Introduction of Dr. Michael Sarr

Hans G. Beger, M.D., F.A.C.S.

As Vice President of The Society for Surgery of the Alimentary Tract, I am honored to introduce Dr. Michael Sarr who will deliver the Presidential Address. The office of President of The Society for Surgery of the Alimentary Tract has always been connected with high qualities of leadership. John P. Kotter, Professor of Leadership at Harvard Business School, defines leadership as "what the future should look like, aligns people with that vision and inspires them to make it happen despite the obstacles."

In the case of the Forty-First President, Michael Gregory Sarr, the road to this position has been straight ahead with no major diversions. Dr. Sarr was born in Butler County, Pennsylvania, which has neither mountains nor hills, but a city called Harmony, founded by the German Society of Harmony. There are two typical characteristics of the people of Pennsylvania. If you study Dr. Sarr's face, you will note that he possesses these traits, which have led to his successful career as a leading surgeon and scientist. He appears friendly, and his eyes are wide open and full of curiosity. Friendliness is one important prerequisite if one is to become a good doctor who is accepted by his patients, and curiosity is another basic quality that is needed to become a successful scientist.

During his childhood Dr. Sarr developed his natural talent as a musician, playing the guitar in his rock and roll band, which was called "The REAS." In fact, I think that Dr. Sarr looks a bit like George Harrison of the Beatles. This blend of a special talent in music combined with an interest in surgery is a common feature of especially gifted surgeons; both of these talents have a highly creative component. One of the most famous surgeons from the past in Europe, Dr. Theodor Billroth, was an enthusiastic pianist, who played together with his best friend, the composer Johannes Brahms. Dr. Billroth not only played the piano, but he wrote several compositions as well.

In 1968 Dr. Sarr began his studies at Colgate University in Hamilton, Ontario. It was during this first period, while formulating his individual goals, that he became particularly fascinated with organic and physical chemistry and biochemistry. He was an excellent student who passed all of his examinations with the highest degree of success. This was also true of his personal life. In 1974 he married his lovely wife Barbara, who at that time was a registered nurse. I suppose that choosing Barbara, who earned a Master of Science degree in nursing, was the best decision of Dr. Sarr's career. Everyone knows that the most important decision in the career of a successful surgeon is the choice of a life partner, and Barbara is the ideal partner for Dr. Sarr. Not only is she involved in medicine, but they share the same hobbies as well. Both Dr. Sarr and his wife are enthusiastic about fishing, and they are both quite skilled at this sport.

Dr. Sarr took his first major step into the world of academic surgery when he entered the Johns Hopkins School of Medicine in Baltimore. As a medical student and later as a physician, his focus changed from chemistry and biochemistry to physiology, and he ended up doing surgical research. During his residency at Johns Hopkins he came to know Professor John Cameron, and I suspect that Dr. Cameron was the force that helped shape him into the precise, straight-talking, and forward-thinking surgeon that he is today. These remain Dr. Sarr's most predominant traits. First he formulates a concept and then asks the appropriate questions, although these are sometimes provocative, and it becomes a simple matter of intuition and creativity to carry out the shortest and most precise experiments in order to obtain the correct answers; that is the basis for his success as a scientist. His first publication in the *American Journal of Physiology* was entitled "Canine Jejunal Absorption and Transit During Interdigestive and Digestive Motor States." In going through his vast collection of original articles up to the present, it is clear that his principal research activities over the past 20 years have remained in the area of gastrointestinal physiology and motility and have included basic research studies, animal experiments, and clinical trials.

Professor of Surgery, University of Ulm, Ulm, Germany.

In 1981 he visited the Department of Surgery at the John Radcliff Hospital in Oxford, England, because of his curiosity about what was going on at other research institutions, particularly in the field of transplant surgery. During his time in Baltimore, in cooperation with Dr. Keith Kelly, Dr. David Nagorney, Dr. Gregory Bulkley, and Dr. George Zuidema, his efforts resulted in a series of significant research activities in the area of gastrointestinal physiology and motility. However, there is no doubt that during this time, guided by Dr. Cameron, he became "obsessed" with the pancreas. If one observes the world of very successful surgeons, it becomes clear that their major surgical achievements are based on research activities.

In 1985 Dr. Sarr joined the staff of the Mayo Clinic in Rochester, and began his clinical duties as a consultant in gastroenterology and general surgery, and he continued his intensive research studies with the group in Baltimore.

As an ever-relaxed surgeon, always ready for a joke, giving generously of his time in discussions with coworkers, or listening to and talking with patients—all with excellence and dignity—he was able to chart a course for the entire staff of the Mayo Medical School with his vision of surgery and inspired them to make things happen. So it came as no surprise when, as a member of the faculty of the Mayo Medical School, in the Department of Surgery, he was awarded the title "Teacher of the Year" in 1986, 1988, and 1997.

In 1992 he assumed chair of the Division of Gastroenterologic and General Surgery at the Mayo Clinic. As a Professor of Surgery at the Mayo Medical School, he was responsible for a large group of fellows who have undergone training in the research and residency programs at the Mayo Clinic in Rochester. He has always taken the time to listen to his staff despite any time constraints such as, for example, trying to make the next flight. He ends each discussion by trying to form a consensus that can be accepted by all participants. I experienced his techniques first hand while writing a book entitled "The Pancreas," which we began together. He composed a short letter to spark renewed interest following a declining level of interest in this vast work, with immediate success. He is never in a hurry, but always works very quickly, except in matters concerning his patients, where he never fails to do whatever is necessary to benefit those who need his help in matters of health care. It comes as no surprise that in 2000 he was named "Outstanding Clinical Educator" at the Mayo School of Health-Related Sciences.

At the Mayo Clinic he continues to achieve success in his surgical research in the area of gastrointestinal physiology and gastrointestinal motility studies. In the field of pancreas research he has shared a special bond with gastroenterologists Dr. Gene DiMagno and Dr. Sidney Phillips. His research is very attractive to a large number of research fellows from abroad, including gastroenterologists from the University of Ulm Medical School. Others who have had a major impact on his clinical and research studies include Dr. van Heerden, Dr. Nagorney, and Dr. Niteki. Dr. Tsiotos was one of Dr. Sarr's closest coworkers who, under his guidance, also became "obsessed" with the pancreas.

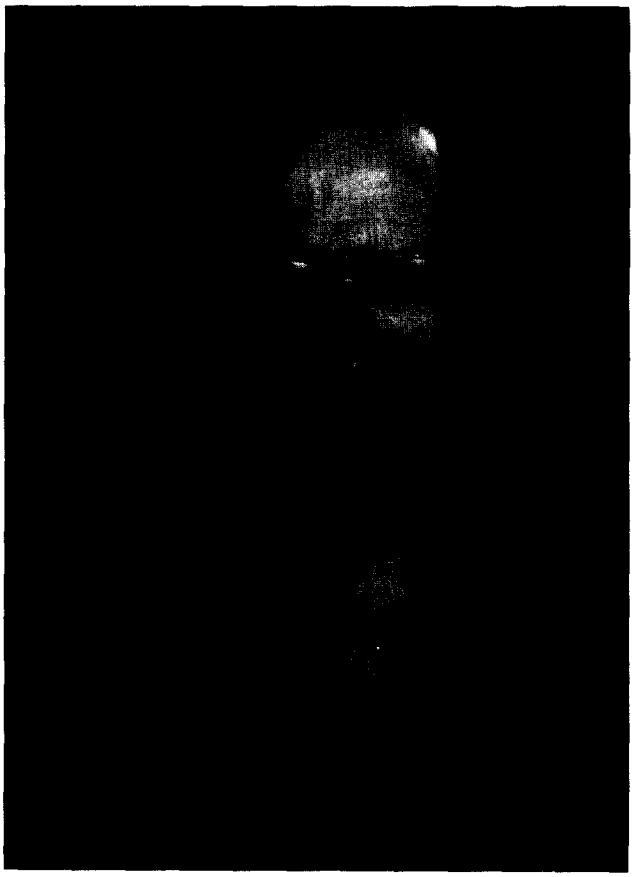
Clearly Dr. Sarr loves his job and enjoys life with his friends, certainly with his co-workers, and last but not least with his family. In 1982 his daughter Lindsay was born, and 2 years later his son Chase was born. Interestingly, during these 2 years Dr. Sarr wrote 12 additional papers, but I suppose it was Barbara who cared for Lindsay and Chase most of the time because Dr. Sarr was quite well aware of the importance of continuing to publish. As Sir Francis Bacon wrote 400 years ago, "Reading makes a full man, conference a ready man, and writing an exact man."

As Chair of the Division of Gastroenterologic and General Surgery at the Mayo Clinic, Dr. Sarr has taken on more than is necessary for any surgeon and chairman. The tremendous burden of these commitments leaves him little time for his hobby of pheasant hunting, a carryover from his Pennsylvania days. In the pleasant surroundings of Rochester, this hobby not only relaxes him, it offers the added advantage of supplying food for his family.

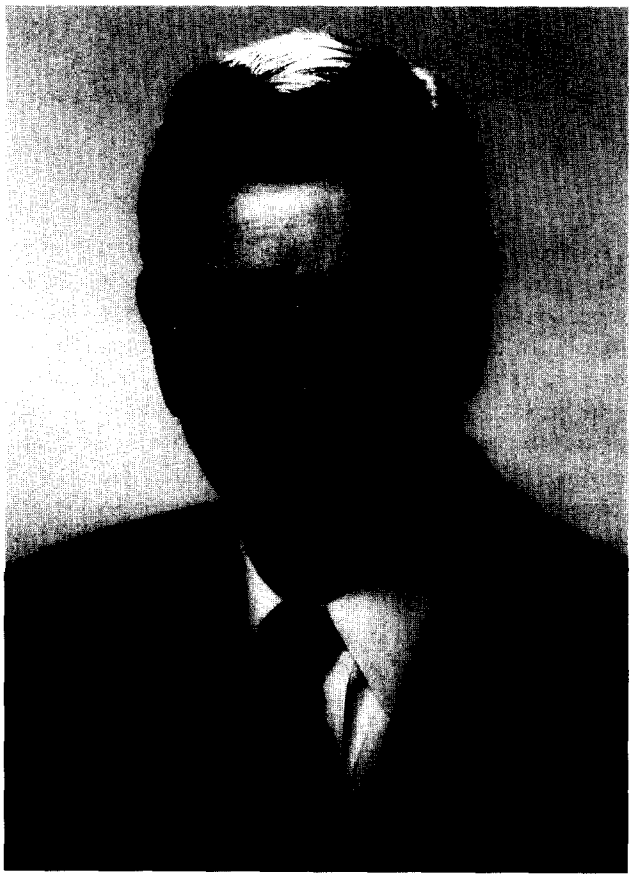
In addition to all his accomplishments, both professional and personal, Dr. Sarr has three main goals. The first is to build and further develop an improved Department of Gastrointestinal Surgery, which he considers the legacy of the Mayo doctors who preceded him. Second is his desire to educate his colleagues in the Department of Gastrointestinal Surgery to the highest possible degree by taking his research and clinical duties to the highest possible level. Third, he wishes to represent the Mayo Clinic with the greatest distinction as a gastrointestinal surgeon in the national and international community of academic surgeons. At this high level of responsibility, he has taken on more than most surgeons and department chairs could shoulder. In addition to serving as President of The Society for Surgery of the Alimentary Tract, he serves as coeditor of one of the world's leading journals of surgery, and is also codirector of the Pancreas Club. His other activities include serving on the Editorial Board of the JOURNAL OF GASTROINTESTINAL SURGERY and being a member of various academic and business committees at the Mayo Foundation.

In conclusion, I would like to state that the Forty-First President of The Society for Surgery of the Alimentary Tract is a very successful surgeon. What makes Dr. Sarr so likeable, however, is his unpretentiousness. As Popper wrote in 1984, "The most important quality of a scientist is intellectual humility." (Popper KR. Logik der Forschung. Tubingen: JCB Moho, 1984.)

Ladies and gentlemen, members of the Society, I present to you the Forty-First President of The Society for Surgery of the Alimentary Tract, Dr. Michael Gregory Sarr.

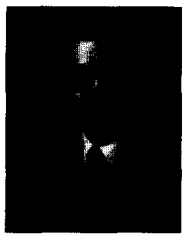


Dr. John L. Cameron



Dr. Keith A. Kelly

The Electronic Environment: How Has It, How Will It, and How Should It Affect Us?



Michael G. Sarr, M.D.

Wow! The electronic environment is everywhere. The net, the Web, the Internet, web page, home page, web-based, virtual this/virtual that, www, http//, aol.com, poop.com (seemingly appropriate for a gastrointestinal surgery audience), .com, .org, .edu; in fact, eEVERYTHING. What does this all mean, how has it, how will it, and how should it affect us? Much of all this eEVERYTHING has occurred over *only* the past 7 years since the World Wide Web became readily available to the public. Interestingly, all this came from the defense department's idea of DARPA (Defense Advanced Research Projects Agency), a communications network that would be functional even if a nuclear attack should destroy several major sites. Amazing! Even scary!

Let me start off by fully acknowledging that I am not an expert on this topic—let me repeat, I am not an expert on this topic; in many ways I represent an eFRAUD in that I (and many of my naive colleagues—myself included) have been swept along by this technology rather than becoming a primary, proactive part of it. All that must change. As J. Goldsmith wrote, "In health care, institutional computing is a different . . . sad story. The health care field as a whole . . . is 25 years behind banking and the airlines in the application of (electronic) technology."[1] How true!

What I am writing today (May 2001) will be outdated next year, next month even, and is already outdated in many medical institutions and practices currently. The following discussion represents my personal viewpoints and thoughts. Although me personally addressing this topic is probably best described as an eFRAUD, because I know very little about this (hardware, software, providers, HTML, etc.), yet I am a believer and I am (slowly) becoming eTRAINED. The current and future generations have been and will be raised and educated in the eWorld and will not require retraining in the electronic world.

The goals of this short treatise are to provide an overview of this eREVOLUTION from the perspectives of a middle-aged surgeon with 25 years of clinical experience, functionally 20 years of research endeavors, and 18 years of a serious dedicated educational interest. I plan to address some of the important aspects of how the electronic environment has impacted our clinical practice, the research world, and our education of medical personnel. I hope to point out some of the problems that arise with integrating a digital electronic world into our analog, paper-and-pencil mentality of the *established medical world*—that is, establishing a true electronic medical record. Finally, I will speculate on the future and the benefits to be reached through the electronic media.

OVERVIEW

Think back over the centuries about methods of communication—smoke signals, carrier pigeons, scouts, pony express, and so forth; how would history have been changed had Caesar and Napoleon known about the movements of their enemies at the exact time the armies embarked on their campaigns? In our generation, the written word has traveled by U.S. Mail/Air Mail (days → weeks), telegram (hours—someone needed to deliver the telegram from the receiving telegraph office), Fax (minutes), to now e-mail

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(nanoseconds), and even "instant message systems" allowing online "talking" or typed dialogue via the Internet (for those of you with college students, it is a lot cheaper than phone calls). This complex system of potentially "universal" instantaneous communication (at least on the planet earth) allows this person-to-person interchange to be typed words (the future will be electronic, voice-activated, computer-generated), pictures, and video images. The electronic medium now allows us this ease of information transfer. For instance, in 1985, a great deal of information existed; it was "out there" in books, journals, theses, and so forth, but the challenge and problems were how to identify or access the appropriate source. Where to *go to get it?* How much time would it take? The problem was categorization and then person-time searching. Now, in May 2001, despite 15 years of additional exponential information growth, the electronic medium allows this information to be much more easily (and rapidly) accessed through net-based databases, search engines, computerized searches—the computer does the search in digital time (nanoseconds) compared to human time (minutes → hours → days). Thus the Web really represents a tool of information technology–information transfer–information cataloging.

This electronic environment has numerous obvious advantages (Table I). The electronic medium provides rapid availability (provided computer access is available) in less space (no record rooms, storage cabinets, physical libraries, etc.), universal retrieval of data (at work, at home, in a plane, in your car, from your wireless personal digital assistant (PDA), or even from your cell phone), virtually instantaneous communication, efficiency, and potentially fewer errors in medicine—witness the interest in the recent report from the Institute of Medicine concerning errors in medicine.[2] However, there are many relative disadvantages and immediate problems to overcome such as computer literacy, "user-friendly" software—a term many of the uninitiated (and some of the "initiates in training" like myself) consider to be an oxymoron, and typing skills. In addition, what happens when the computer is "down"? How about the universality of hardware/software, computer applications, Apple vs. the PC, the typical problems of a free-enterprise market competition, patents, intellectual property, and so forth, and then the problem that will have to be addressed in the very near future—confidentiality!

So how does this electronic environment affect our clinical practice, research endeavors and educational opportunities, and our work?

CLINICAL PRACTICE

What does the electronic environment offer our day-to-day care of patients (Table II)? The paper chart (which is often/usually unorganized—yes, even on occasion at the Mayo Clinic) will be unnecessary. Indeed, the electronic medical record will always be available—think of that—seeing patients with "the chart" always and readily available! X-ray films will be available for review without having to first obtain the hard copies and then sort through multiple chest x-ray, barium enema, or computed tomography (CT) films to get to the ones you really want. Once data are entered, they will be immediately accessible to all parties simultaneously. For instance, at the Mayo Clinic, we now "electronically" list patients who are to have an operation the next day. Once these patients are electronically listed, the operating room staff, preadmission office (for insurance purposes), hospital admissions, my secretary, blood bank, anesthesiology and radiology departments, pharmacy, intensive care unit, chaplain, and, of course, the business office are all made aware of this operation simultaneously, and this knowledge precipitates planning for staffing, beds, and so forth immediately and simultaneously—hours before the paper record sent through tube systems, carried by the patient, or communicated by phone calls or faxes in the past (just 1 year ago) initi-

Table I. Electronic environment—Considerations

Advantages	Disadvantages
Rapid availability	Computer literacy
Less space needed (record rooms, library)	Software not always user friendly
Universal retrieval capabilities	Typing skills needed
Instantaneous communication	Computer is often "Down"
Efficiency	No universality of software/hardware
Fewer errors in medicine	

Table II. Electronic environment—What does it offer our clinical practice?

Paperless record
Immediate access to "the medical record"
Immediate access to radiographic images
Efficiency across
Institution
Physician offices
Business office/billing
Minimizes errors in medicine
Physician handwriting
Drug interactions

ated a similar downstream effect. This electronic medical record promotes efficiency across all aspects of the institution including distant physicians' offices and, of course, for billing by the business office. Moreover, errors in medicine should be minimized—no more problems with reading our universally atrocious handwriting; in addition, the ready possibility/reality of computerized (automatic) review of drug interactions or patient allergies will decrease pharmaceutical errors.

This is all very attractive; but there are, of course, numerous hurdles. Gary Baldwin commented "Physicians often sit in the driver's seat when it comes to successful implementation of information technologies,"[3] but sometimes as physicians we are our own worst enemies (Table III). As physicians, many of us lack computer skills (this is often age related); we cannot type efficiently, many of us are not effective or well-versed web browsers (clickers), and we may refuse to take the time or claim we do not have the time to learn. Of additional concern is the concept of the "electronic signature." When we write (or when some of us type—not me, however) something, we know we have done it, and we do not have to reread it. However, when we dictate it, and someone else (or the computer when voice recognition comes of reliable age) types it, we will have to reread it to ascertain and verify its accuracy (right vs. left, orchiectomy vs. orchiopexy, benign breast cyst vs. malignant breast cyst); similarly, when pathology reports are made "online" without a piece of paper for us to review, we will need to be certain that the gallbladder we removed laparoscopically yesterday did not harbor an occult stage II carcinoma that requires further treatment. Similarly, what we used to do (and bill) as face-to-face "encounters" may now be done largely electronically—how will or how should we be compensated for our time?

Table III. Application of electronic medium—Problems

The Doctor
Many physicians
Lack computer skills (age-related)
Cannot type efficiently
Are not efficient web browsers (clickers)
Refuse to take the time to learn
Concept of the electronic signature
Compensation from electronic (vs. face-to-face) encounters
The Institution
Cost to implement
Hardware—computers, digital x-rays
Customized Internet vs. applications server provider
Hire more stenographers
Educate staff
Transition from paper to electronic transactions

The institutions in which we all work also face many problems. Conversion to an electronic medical record has numerous, often hidden, inherent costs. Hardware must be installed, including computers, networking, conversion of analog radiographic data to digital data, workstations, and so forth. Either the infrastructure (and software) can be homegrown through customized intranets (which assumes the institution has the expertise to do so), or smaller institutions or private practices can use so-called applications server providers, which offer off-site net services just as if you would rent their services or "space," in this case virtual space. Most institutions and practices will require a bit of customizing of the outside applications server providers to fill their individual parochial needs. To become electronic, data (consultations/notes/lab work/reports/etc.) need to be entered digitally, which requires the hiring of more transcriptionists. The staff needs to be educated in the electronic environmental services—entering and accessing information—hardware, software, and networking. Thus the transition from paper to the eTransaction involves many steps—expense, education, and a new mindset for everyone involved—from laboratory worker to nurse to physician to business office. The goal, of course, is to improve efficiency, cut costs, and ultimately improve the delivery of health care within, as well as across, institutions without breaking the bank getting there.

Having been at the Mayo Clinic for 16 years, I have reaped the benefits of one of the best medical records systems in the world, beginning with Henry Plummer's idea in 1909 of a common medical record for *each* patient. At that time, this was one of, if not the first medical group practices. Plummer was the idea man, Harry Harwick was the organizer, and their vision and organizational skills led to the development of one of the most thorough and easily accessible medical records (paper) systems ever known. Indeed, the Mayo Clinic has built a considerable part of its academic and clinical reputation on this readily available and complete medical records system. Currently Mayo Clinic Rochester has more than 16,000 employees. In about 1997, the institution made the commitment to go "paperless" by 2003. We have lived and worked (and continue to do so) through a staged implementation, with a largely customized, "homegrown" intranet in addition to partnering with several outside, non-Mayo electronic vendors that offer preprogrammed software for certain "applications." From now on, my use of the word "application" will apply to individual data retrieval (software) systems, such as retrieval of laboratory data, provision of the ability to view radiographic studies, or a means of providing for service

recognition (billing), and so forth. Each application is different, requires different software, accesses a different database, is unique in itself, and may be offered (sold) by a different company or provider. One immediately appreciates the potential problems of merging, integrating, and "home growing" all of these separate applications into one common package. Currently the Mayo Clinic has numerous applications; some have been integrated into a common system, but many others remain separate, each requiring a separate "log on" with a user name, a confidential password, and finally a patient identification number. It can be extremely frustrating—and may on occasion generate what I refer to as eRAGE, the electronic equivalent of "road rage." At present, we at the Mayo Clinic are in the integration phase with the goal being an intranet, web-based system by the year 2003 for all aspects of outpatient and inpatient encounters.

Overall, a summary of our road to a paperless electronic medical record would best be described as **painful, expensive,** and **disjointed,** but will be worth the effort when it is fully established. I will elaborate on these descriptions. You will note that my interests revolve around the care of and interaction with the patient. The formal business aspects of our esteemed profession (billing, interaction with third-party payers, governmental "reimbursement" for resident education, etc.) are a whole different but related electronic world of which I know little in my current position.

Painful

Imagine going from a *very* well-organized, efficient (for the times) system, where the entire institutional complex was geared to rapid movement of the paper record between the clinic and two hospitals, to a system that requires everyone to relearn a new method of ordering, requesting, or locating the desired information via a computer terminal. For the new (young) employees, this transition has been relatively easy since they have grown up in the electronic age (video games, a computer at home since they were born, and computer training in grade school as well as in high school). Contrast this generation with those who have worked at the Mayo Clinic for 20 or more years, some of whom (yes, it is hard to believe) do not have a computer at home and may or may not be comfortable even with e-mail. Similarly, many surgeons who have organized their practices around the efficiency of the paper record are now being asked (initially politely) to try electronic scheduling, and more recently not being asked but told (or better yet mandated) how it would have to be done! Painful is indeed the appropriate word, and the pain continues. To make matters worse, many of these changes at the Mayo Clinic have taken place under the supervision of internists with little or no input from our surgical audience. Applications were designed for the internist's practice or by well-meaning but sometimes naive groups of administrators with no knowledge of the marked differences in types of practices, documentation, and busy travel between many different floors (within both the outpatient clinic and the hospital) for surgeons at the Mayo Clinic (we go to see patients at the internist's office, the patients do not necessarily come to us—a practice plan somewhat unique to the Mayo Clinic) in contrast to outpatient-oriented internists. Each different location requires finding a computer, logging on at each place, security concerns, and so forth. In addition, each new "improved update" or each new application requires learning the software package—again, beware of the oxymoron "user friendly," which may generate more eRAGE.

Expensive

Although the Mayo Clinic had a large information technology staff, even as early as 1985 (approximately 250 employees), today we have more than 800 persons in the field. The introduction of the electronic medical record as applied to more than 16,000 employees was and still is a daunting task involving the added expenses of hardware, software, upgrades, and electronic maintenance, to say nothing of education of the staff. From a hardware aspect, our "electronic gurus" predicted that approximately 12,000 computer terminals are needed within just the Mayo Clinic Rochester complex; that is, in almost all employees' offices (including physicians), all examining rooms, all patient rooms, all nursing stations and work areas, all operating rooms (two needed, one for anesthesia and one for the operating room personnel), several in the recovery room, in the business office, and so forth. The imaging capabilities needed to be converted from analog to digital data, which requires the following: (1) conversion of analog radiographic machines to digital films, which requires approximately 780 "imaging plates" (total ~ $1,000,000); (2) conversion of 40 analog ultrasonography units to digital data (total ~ $200,000); (3) an easier and less expensive conversion of CT as it was already in digital format; and (4) development of the hardware for electronic movement and the software applications for processing, trafficking, and viewing the images (cost unknown but presumably quite high). Next, the education of the staff is an equally staggering (and expensive) proposition because we have approximately 1400 staff physicians, 1000 residents, 4000 nurses, and roughly 9000 other paramedical staff. Much of the development costs of this electronic medical record

will not be directly recoverable as such; the hopes are, of course, that these development costs will be recovered by improved efficiency in all aspects of health care delivery.

Disjointed

All of this development has been going on simultaneously. Currently, multiple pilot projects are being evaluated in diverse settings throughout the institution. Some locations are fully electronic, some (currently very few) are minimally electronic, and some are partially electronic, often raising the question "is it paper or electronic?" The level of staff education and electronic usage is also very disproportionate. Moreover, to review a patient's medical record, numerous and varied applications are available, yet each requires an individual "log on." For example, the laboratory data, endoscopy notes, and operative notes are in one application, outpatient notes and consultations are in another application, and x-ray films are available (currently just CT scans, ultrasound images, selected chest x-ray films, etc.) in yet another application and only from areas where the radiographic equipment has been converted to digital processors. Although all these applications are in and of themselves marvelous when they work, each application requires a separate log on with a user number, a password (for confidentiality), and a patient identification number. This need to "open" each computer every time we as surgeons move from floor to floor is exasperating (and sometimes generates considerable eRAGE).

But the future looks hopeful. We are currently integrating and merging almost all of these separate applications into one common application provided by an outside consulting firm. This interactive home page will allow centralization of patient information, creation of "clickable" patient lists so it will not be necessary to always type in a patient identification number, correspondence, consultations/notes/clinic visits, laboratory and radiology results, ability to order various tests, diagnostic procedures, and medications, and to capture billing documentation; all at a one-stop "shopping center" (home page). In addition, several functions are available for the individual health care provider by centralizing lists of personal inpatients, consultations, "to do" lists, and so forth, to say nothing of coordinating the patient's schedule. With such a central web-based home page, retrieval and entry of patient data will be markedly facilitated.

The next major step is to progress with the electronic medical record in the hospital inpatient setting. Although almost all of the applications that are used currently in the outpatient setting are also electronically available for inpatients as well (e.g., laboratory data, x-ray films, other reports, etc.); many others, such as inpatient consultations, daily notes, orders, nurses' notes, and so forth, have yet to be addressed at the Mayo Clinic Rochester. In contrast, our two sister Mayo Clinic facilities in Jacksonville, Florida, and in Scottsdale, Arizona, with their new hospitals, have already in large part developed their electronic medical records for inpatients; the size of the mother Mayo institution in Rochester is such that the inpatient electronic medical records will require a prolonged phase-in program.

WILL IT BE WORTH IT?

Once established and once our mindset changes from analog processing to an electronic (digital) mode, I am convinced that the answer is "yes," once the electronic medical record system is in place. Think of the possibilities: (1) direct, easy access to all available data from home, office, clinic, hospital, car, personal digital assistant, cell phone—in essence anywhere in the world with Internet access; (2) x-ray films, CT scans, and so forth *always* available (no films); (3) legible consultations from other physicians (imagine or eMAGINE that); (4) immediate distribution of information institution wide; (5) ready transportability of a patient's medical record via a floppy disk, CT, or web address; and, of course, (6) better revenue recognition and thus better revenue recovery.

IS IT COST-EFFECTIVE?

Early on, of course not! Indeed, the initial startup costs will be borne by the individual institutions or software development companies; the former will not be able to directly recover the costs, whereas undoubtedly the latter will pass the cost on to the users. However, the real questions will be in the future with the use of the electronic medical record—does it improve efficiency? Does it save time? Does it decrease manpower? And does it improve healthcare? These are difficult questions to quantify.

One group in Yakima, Washington, has developed a homegrown, computer-based intranet-enabled electronic medical record (Worthylake T, personal communication). This group of 33 physicians, including family practice physicians, internists, and two gastroenterologists, worked actively with an outside, web-based service provider in an effort spearheaded by one of their physicians (Dr. Victor Sharpe). Their electronic medical record system has been established and integrates their medical practices. They estimate that their monthly payroll has realized a savings of approximately $1000 per physician, while individual physician productivity has increased by about 5%.[4]

These savings are largely secondary to the downsizing of their medical records personnel. Although obviously very nebulous and difficult to quantitate, other ostensibly unrealized but likely financial benefits may relate to revenue recognition, rapid billing, better documentation (if it wasn't documented, it wasn't done), and ultimately improved (but appreciable) financial reimbursement.

There are other more global concerns. Beyond improved revenue, the electronic medical record offers potential improvements in patient safety and improved medical provider communication. Each year, approximately three billion prescriptions are written. One controversial estimate[2] is that 7000 persons per year die as a result of prescription errors. Electronic prescriptions would avoid misreading of the physician's handwriting, allow automatic checking of drug interactions, and review the compatibility of prescription drugs with individual patient allergies. In addition, immediate transfer of medical records data between different practices, from state to state, and even internationally would be possible.

RESEARCH

The electronic environment has virtually revolutionized the research world. Not only have electronic collection and processing of data markedly decreased analysis and computational time (contrast the old smoked-drum recordings with current online digitally reproduced recordings), but also the Internet allows development and ready access to multiple databases (e.g., the Human Genome Project) through many types of search engines. In addition, the new field of "bioinformatics" has been born. As will be discussed (briefly) below, it is now possible to conduct electronic "research" online by "mining" the databases, generating a hypothesis, and then confirming your work experimentally!

As with the organization of our clinical practices, the electronic medium is the ultimate organizer in research. The Internet does not create data—scientists do, but the electronic medium brings all of these data together. The information has always been there, but it was always difficult to access and was often found in foreign or obscure specialty journals to which only the largest libraries could afford to subscribe. It is now incredibly easy to access this enormous amount of integrated data.

For instance, let us address the past experimental literature. Prior to the Internet, literature searches took a long time (i.e., laborious searching by hand through multiple volumes of *Index Medicus*, etc.). Currently it takes minutes or seconds; indeed, calling up a search engine and typing in key words, the author, or the journal takes "much longer" than the actual computer search itself. PubMed, as organized by the National Library of Medicine, Medline, and numerous other public or private (for profit) collections of "the literature," both as journal articles or collections of reference books, are available to the investigator online in his or her office or home or through the institutional library (as at Mayo Clinic)—all one needs is Internet access. Indeed, the need to *go to* a library is becoming archaic. Almost all major journals are (or will soon be) available online, and their recent past articles can be accessed (often for a price) either as a citation, abstract, or full text. Such benefits are phenomenal for the investigator; however, there are potentially serious problems for publishers. For instance, our department of immunology no longer subscribes to the major immunology journals—"Why subscribe? Everyone has direct, immediate access on their PC!" Indeed, the subscriptions are now online, manuscript reviews are sent, done, and received online, and soon all submissions may be allowed or eventually required online as well. Will the paper journals disappear? What will happen to our beautiful libraries? Will they be transformed into virtual libraries and exist in physical space as "beautiful computers?"

From a different perspective, one need only walk through a bench-space laboratory now to see how the electronic age has impacted even bench-type research. My research laboratory has 400 square feet of bench-top space with six computers, seven monitors, three printers, and two scanners; my large-animal wet laboratory has 300 square feet with three monitors, three computers, and one printer (Fig. 1). In addition, my secretary also has her own computer as do I—and, of course, all are networked together and to the Mayo computer intranet! None of this existed 20 years ago, and very little 10 years ago—what a change!

Literature searches and computer-assisted analyses are now accepted, expected, and somewhat passé, but the Internet has created a new field of bioinformatics. The electronic age has enabled this new field of "computational biology" to really come of age as a discipline in and of itself. There are two major aspects of bioinformatics: databases and data mining.

Use of Databases. There are many web-based resources available for bioinformatics research. One such example is the National Center for Biotechnology Information (NCBI or www.ncbi.nlm.nih.gov), a governmental collecting house maintained by the National Library of Medicine and the National Institutes of Health. The NCBI website is a collection of linked databases accessible to the public that includes any sequence of DNA, cDNA, and protein/protein structure published including a working draft of the human genome. For instance, let's say you have identi-

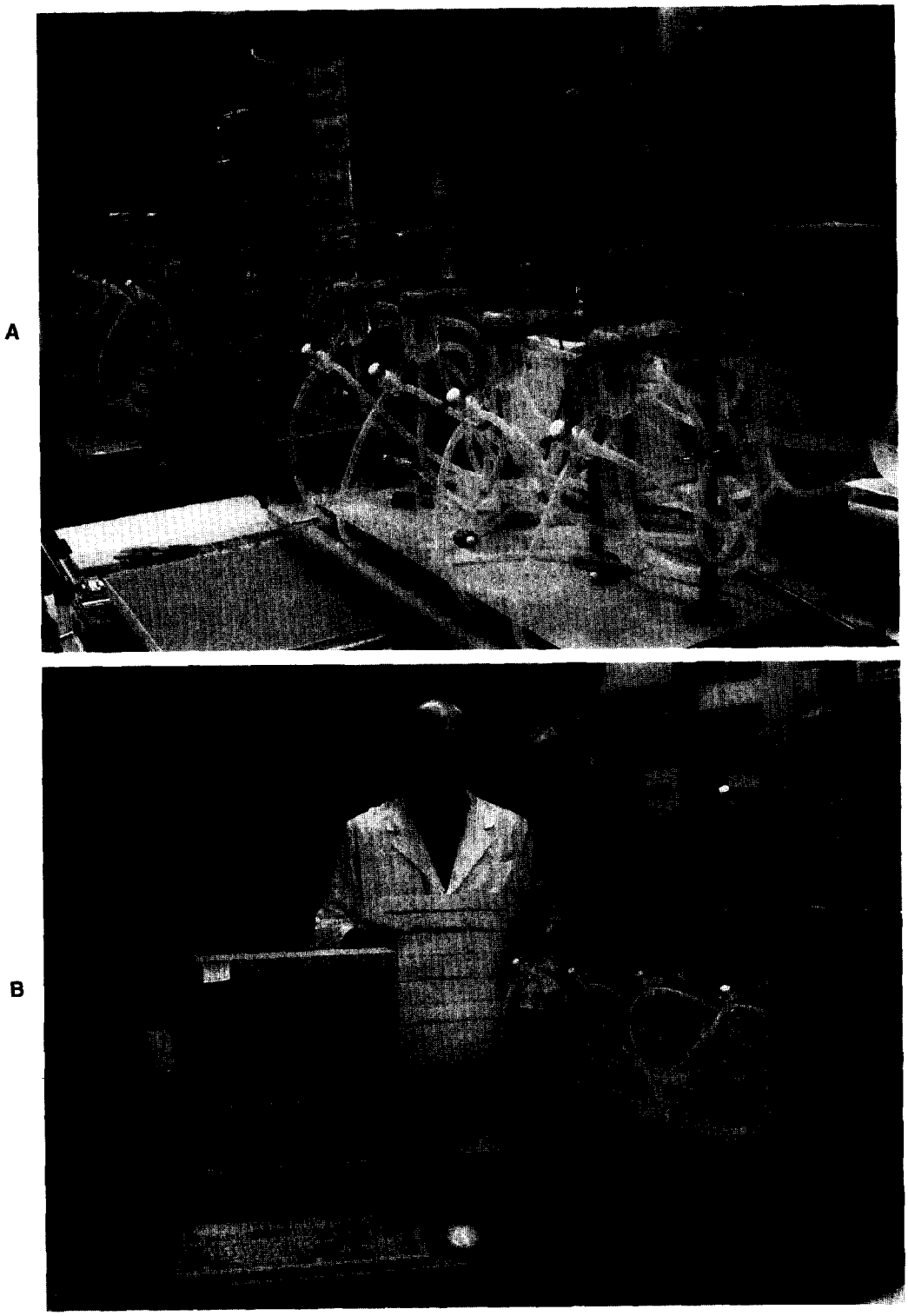
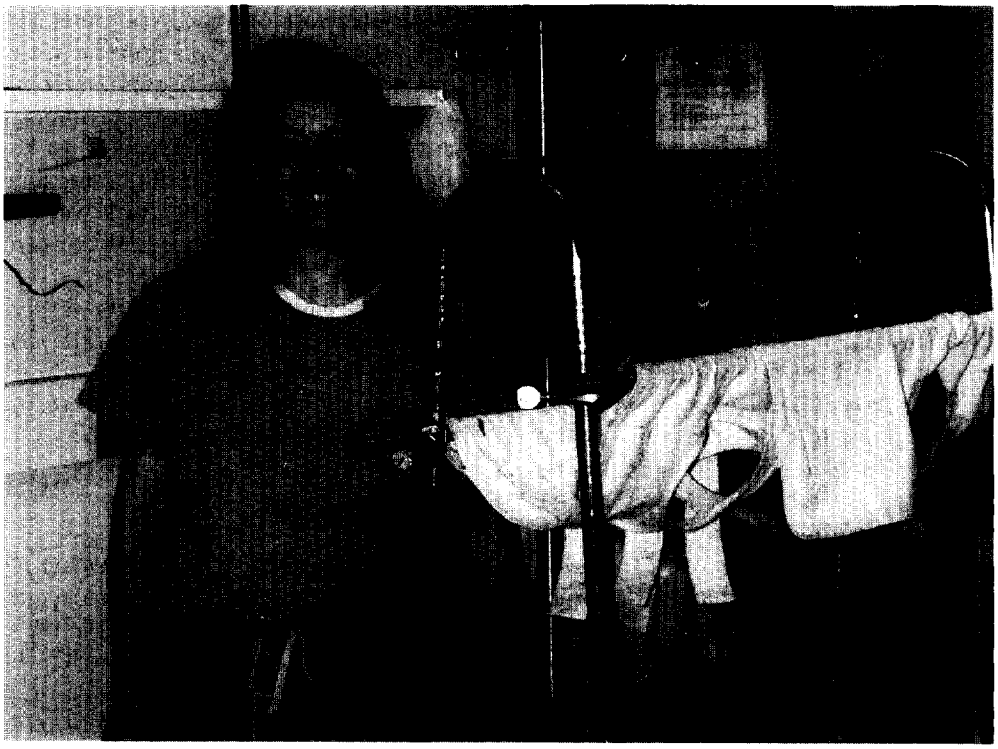


Fig. 1. Research setup. **A,** Old in vitro setup (note tissue chambers and chart recorder). **B,** Current setup (note computer).

C



D

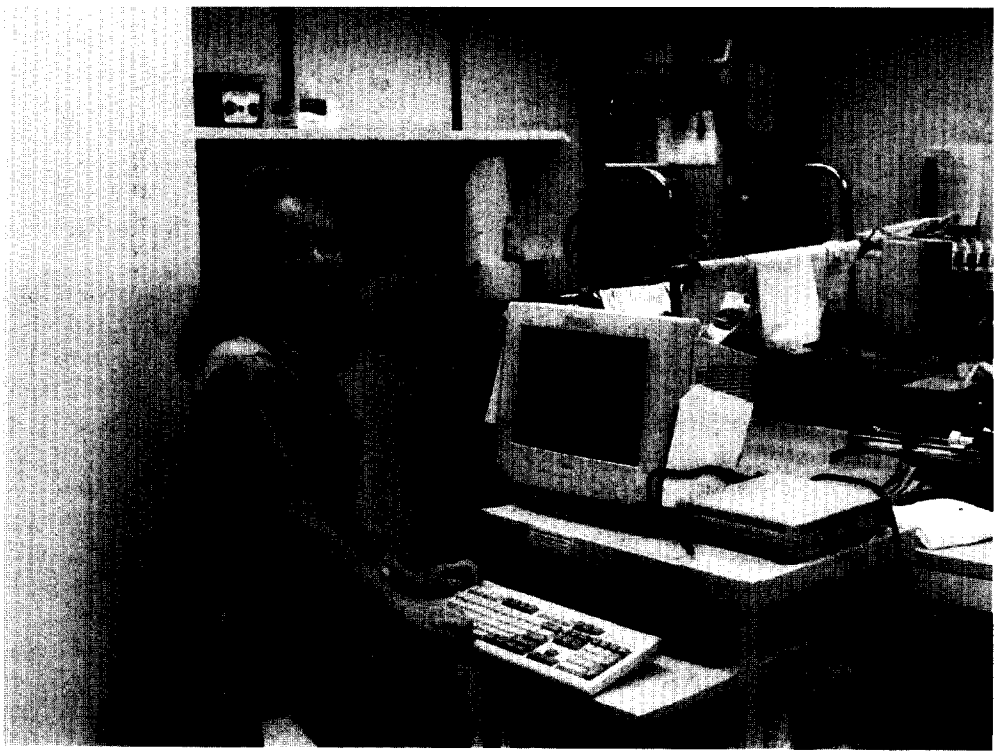


Fig. 1, cont'd. C, Old in vivo dog laboratory. **D,** Current in vivo dog laboratory (note computer).

fied an unknown protein important in your work; you have sequenced it and want to determine if there is any homology with other known proteins in humans or other species. The same approach is possible with a "novel" nucleotide sequence. Software algorithms for sequence alignments provide a very powerful way to compare novel sequences (your protein or nucleotide sequence) with previously reported and characterized proteins or genes. Functional, structural, and evolutionary information can be inferred from well-designed queries and alignments including relevant literature citations. One of the algorithm search engines (BLAST 2.0, Basic Local Alignment Search Tool) provides for rapid searching of protein and nucleotide databases; because this search algorithm detects segmental as well as global alignments, any region(s) of homology or similarity embedded within seemingly unrelated peptides or genes can be detected. These types of searches can identify homology that can provide important clues to function and evolutionary development. Using another search algorithm, one can determine the following: parts of your protein or another homologous gene that have been identified in other species and specifically in the human genome; data (if published) on tissue site expression; the chromosome and region on which the protein is encoded or the gene is located; as well as available probes (either commercially or via NCBI) with which to further characterize your protein or nucleotide. For instance, with a protein, another search algorithm allows you to attempt to identify and characterize your sequence, examine for potential post-translational predictions, explore primary, secondary, or tertiary structural configurations, and determine the presence of known transmembrane domains and nuclear localization sequences. In addition, many of these websites offer links to other major molecular biology servers, such as the European Bioinformatics Institute, Japanese Genome Net, Australian National Genomic Information Service, and many others. This powerful process, accessible to the public, has only become possible through the development, use, and worldwide expansion of the Internet.

Data Mining. The other part of bioinformatics is "data mining" or what I am going to call eRESEARCH. Data mining is really the field of what some have referred to as computational biology. In some respects, it is a new field requiring new expertise. Classically, we have approached scientific research by making an observation (clinically or in the research laboratory), then reviewing the literature, forming a hypothesis based on this observation, and then confirming or disproving the hypothesis by an experiment. By "mining the Internet," the researcher makes a hypothesis, mines/searches the databases for an observation (accrued through an electronic approach) that supports the hypothesis, and *then* designs an experiment. With this approach, the observation is made electronically through searching known sequences for potentially novel proteins, genes, or nucleotides that might be expected to have important functions, and only then conducting an experiment to see if the electronic prediction was correct (i.e., an "electronic assay," which is then confirmed by a "biologic assay").

This discussion of the electronic environment and its uses just breaks the surface of the current uses and potential future uses. The most important concepts of the electronic environment in research are communication and data availability.

EDUCATION

What about eDUCATION? One need not stress one's thought processing much to begin to realize the benefits, market, and challenges of the electronic environment in the educational world. The classic approach of books and lectures is being challenged by the power of an "interactive" website. "Colleges and universities may exist as we currently know them as social *rites* of passage. (But) People don't only go off to college to learn Plato, but rather to come of age."[4] Currently there are "virtual universities" that offer educational courses and degrees fully online or what some have called "distance education." Many of these online electronic courses also offer interactive sessions with other eStudents or the instructors (eProfessors), usually through e-mail.

At the Mayo Clinic, we have developed (of course) a web-based medical student curriculum as well as a website for our residency program. Use of an electronic intranet environment allows many imaginative approaches to material presentation. For instance, our anatomy department uses a web-based interactive program to help teach anatomy to the first-year medical students.[5] Our group maintains that anatomy is a visual science with information conveyed through drawings and via hands-on, three-dimensional anatomic dissections, and prosections. Using animated images projected by computer graphics, computer-assisted manipulations of colors for, as an example, the arteries, veins, and bile ducts of the liver, and interactive input from the viewer at the convenience of a web-based format is very attractive and has received high acclaim from our students. The ability to edit and modify the digitally formatted figures through imaging processing software allows a new form of self-teaching. Similar in principle are many of the virtual teaching modules being used in laparoscopic training curricula popularized and supported by the Society of American Gastrointestinal Endoscopic Surgeons (SAGES). A "virtual liver" has been devel-

oped by Dr. Jonathan Silverstein at the University of Illinois to help visualize the important three-dimensional relationships within the liver (Silverstein J, personal communication).

What about production and availability of educational materials? One might think of the digital format (digitized books, magazines, newspapers, etc.) as providing for readers (and writers today) what Gutenberg's printing press made available in the past. Many versions of texts, handbooks, formularies, and so forth are becoming downloadable onto personal digital assistants. Indeed, the picture of the surgical house officer with a white coat stuffed with a pharmacy formulary pocketbook, a primer of surgical infections, a notebook with the important phone numbers, a surgical journal, "tips for the house officer," and a pager might soon be replaced by a white coat containing only a wireless personal digital assistant or even only a modification of the current cell phone.

Educational benefits accrue for the patient as well. Estimates for the year 2001[6] claim that 76 million Americans are online with 41 million seeking some form of health care information, and fully 22 million seeking information on a specific topic (interestingly, the most common topic is obesity). This has not gone unnoticed by insurance companies; indeed, 1.6 million people e-mailed health insurance providers last year. What this means is that the public is equally involved in the eVOLUTION of healthcare. As of March 2001, there were more than 26,000 health-related websites,[7] and with 54% of American adults online, many will have both the interest and the ready access to visit these websites. Indeed, any surgeon today will attest to the use of the Internet to access data concerning a patient's condition. Internet-based data are readily offered by the patient or family to the surgeon for his or her comment. Be prepared! This is both good and bad. For instance, there are just too many data available—for instance, search "colon cancer" with almost any search engine and you will get more than 290,000 sites! Information, thus, is there, *but* how good is it? Many of these sites are not credentialed, promise "sure cures," offer unproven treatments, or involve chat rooms with unreliable personal opinions. In addition, beware possible fraud. Interpretations of such broad searches require informed choices and insight—commodities that are often difficult for the lay public to find, especially when the searcher has a serious disease (e.g., cancer), and he or she or the family is desperate.

THE FUTURE

Many opportunities, improvements, upgrades, and problems confront us in the future. On the near horizon is voice-recognition software and voice command. Although used by some currently, there is no current consensus on its accuracy in a medical practice. Does it save time if a report needs to be carefully edited by a secretary who might be able to actually type it faster than it can be read and edited as the secretary goes on? Another technologic advance will be wireless systems that avoid the need for much of the hardware networking between computer terminals. In this respect, the communications industry will continue to grow exponentially. Also, the ready access of *all* healthcare providers to the full electronic medical record is immeasurably attractive. No longer should we need to write, fax, or call for the "medical records" to be sent; indeed, the electronic medical record will be presented by the patient to the physician either on a floppy disk, CD, or web address (Fig. 2).

However, as our technologic sophistication improves, several major problems await solutions. For instance, the electronic medium makes all types and complexities of data collection theoretically possible. However, we must never lose sight of the fact that the process of data collection is not the goal; rather *analysis* of appropriate data collection is the final goal. We must be careful to avoid the suggestion by our "bean counters" that data need to be collected just "because we can"—collecting the data is not the end but rather a potential process that aids the analysis. Second, the cost of the electronic coming-of-age needs to be factored into the equation, especially in smaller medical practices, research laboratories, and educational facilities, and by health care providers. Undoubtedly some practices and businesses will fail because they do not have the financial backup to compete—free enterprise!

Probably one of our biggest challenges comes in the field of data privacy and security regulations. The Health Insurance Portability and Accountability Act (HIPPA) will be a major problem for health care providers and their medical institutions. This congressional act requires all Internet-based applications to be HIPPA compliant in the near future. The regulations require these electronic systems to provide for the following: (1) authentication of *both* sender and receiver; (2) authentication of the message (when, where); (3) some form of encryption of message contents; and (4) message integrity verification. This act mandates a *secure* firewall to protect any dial-in entry point to the network to be effective against a professional hacker. Similarly, intrusion detection applications, which alert the system and the inquirer to unauthorized access, need be functional and in place. Although technology attempting to meet HIPPA mandates is available and is in use in most such systems, full implementation will require several novel solutions to be developed. For instance, current encryption technology works only when both the sender and

Fig. 2. Availability of the patients' medical records will be by floppy disk, CD, or a web address.

recipient of a transmission have the same software for encoding and decoding a message.

Other implications of the HIPPA have to do with patient rights. Patients will determine who (and only who) will be allowed to view their medical records. This concept is important for, as an example, a major league baseball player who enters the electronic hospital—many curious employees (or the newspapers, television stations, or even coaches from other competing baseball teams) could in theory review the medical record with very serious consequences. Or, how about a movie star, the mayor, or the CEO of a large company? Thus patients may restrict their information to only a few health care providers, even within a single health care institution. But think of the potential headaches for a consulting physician or a new nurse if they have not been "approved" by the patient.

SUMMARY

Nevertheless, the future is bright and offers vastly improved **communication, organization, speed of search,** and **efficiency** enabled by the concept of digitized data entry and retrieval. The opportunities are virtually boundless and will change (and may even abolish) the paper world as we now know it. We must be prepared, keep an open mind, and constructively control the electronic environment to meet our needs—the computer should be our slave and not vice versa. As Peter Kilbridge, a pediatrician turned information technology consultant stated, "Physician's main obstacle is time . . . for any Internet application to catch on, it has to be easy to use and time efficient."[3] Not all electronic applications or technologies save time, improve efficiency, or save money. The fact that it *can be* done electronically does not always mean that it *should be* done. The challenge will be to control its implementation in a rational, insightful way—for many of us, it will require thinking "outside the box."

I would like to thank Drs. Nicholas Zyromski and Raul Urrutia, Judith Duenes, and Deborah Frank for all their help and advice with this manuscript.

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Pancreatic Cancer: From Genes to Patient Care

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It has been more than a century since Dr. William S. Halsted reported the first successful resection of an ampullary cancer.[1] In the years following Halsted's report, Dr. Kausch, a German surgeon from Berlin, would report in 1912 the first successful two-stage partial pancreaticoduodenectomy, and in the 1930s Dr. Allen Oldfather Whipple would popularize the surgery that now bears his name.[1] During the ensuing decades, the Whipple procedure would fall out of favor at many institutions because of high operative mortality rates; yet it has been resurrected at centers of excellence where it is now routinely performed with low operative morbidity and mortality.[2,3] Today, 5-year survival rates for patients with small, node-negative pancreatic cancer following pancreaticoduodenectomy can approach 40%.[4]

As exciting as these surgical advances are, the sad truth is that the vast majority of patients with pancreatic cancer present too late in the course of their disease to be surgical candidates, and most of the surgically treated patients who live to 5 years eventually die of their disease.[5,6] Clearly, another revolution as radical as Dr. Halsted's first surgery for a periampullary cancer is needed. I believe the genetic revolution of the past decade will provide this advance.

PANCREATIC CANCER IS A GENETIC DISEASE

The past 10 years have witnessed an exponential growth in our understanding of the nature of pancreatic cancer and it is now clear that pancreatic cancer is fundamentally a genetic disease—a disease caused by inherited and acquired mutations in cancer-causing genes. Many of the genes targeted in pancreatic cancer have been identified and they can be divided into three classes—oncogenes, tumor suppressor genes, and genome maintenance genes (Table I).

Table I. Genetic alterations in pancreatic adenocarcinomas

Gene	Chromosome locus	Frequency (%)
Oncogenes		
K-*ras*	12	90
Tumor suppressor genes		
p16	9p	95
p53	17p	50-75
DPC4	18q	55
BRCA2	13q	7
LKB1	17p	4
MKK4	19p	5
ALK4	12q	2
Genome maintenance genes		
hMSH2, hMLH1	2p, 3p	4

Oncogenes

Oncogenes are genes that encode for proteins which, when *activated* by mutation or overexpression, possess transforming (cancer-promoting) properties. The analogy has been made between an activated oncogene and the gas pedal of a car being stuck in the "floored" position. The K-*ras* gene is probably the best example of an oncogene that is activated in pancreatic cancer. The K-*ras* gene is activated in more than 90% of pancreatic cancers by point mutations at codon 12 of the gene.

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Tumor Suppressor Genes

Tumor suppressor genes are genes that encode for proteins that normally function to restrain cell proliferation, and *loss* of their activity may lead to unrestrained cell growth. The analogy has been made between an inactivated tumor suppressor gene and a broken brake pedal on a car. The tumor suppressor genes most often inactivated in pancreatic cancer include the *p16* gene (inactivated in >90%), the *p53* gene (inactivated in 50% to 75%), the *DPC4* gene (inactivated in 55%), and the *BRCA2* gene (inactivated in ~7%).

Genome Maintenance Genes

Genome maintenance genes, also known as "DNA Mismatch Repair Genes," are genes that code for proteins that check the fidelity of DNA replication. When a genome maintenance gene is *inactivated*, errors that normally occur during DNA replication are not corrected. Extending the analogy of the car further, an inactivated genome maintenance gene can be thought of as having a drunk mechanic maintain your car. He is bound to make mistakes and sometimes these mistakes will affect the brake or gas pedal of the car. Genome maintenance genes targeted in pancreatic cancer include the *hMLH1* and *hMSH2* genes and when one of these genes is inactivated, it results in a characteristic alteration in the tumor DNA called "microsatellite instability."

WHY IS THIS IMPORTANT TO ME?

In Sophocles' *Oedipus, The King*, Tiresias says to Oedipus "It is but sorrow to be wise when wisdom profits not." At first glance, one might reasonably argue that Tiresias' comments apply to today's genetic revolution; however, I would argue that our knowledge of the genetics of pancreatic cancer is already having an impact on patient care in the following four areas: familial pancreatic cancer, screening for early pancreatic cancer, tumor classification, and treatment of pancreatic cancer.

Familial Pancreatic Cancer

For years it was assumed that pancreatic cancer was not a familial disease. When the aggregation of pancreatic cancer was noted in a family, it was assumed that it was either due to chance or to shared environmental exposures. Because of advances made in our understanding of the genetics of pancreatic cancer over the past decade, we now are in a position to establish definitely that 5% to 10% of pancreatic cancers arise because of a familial predisposition, and some of the genes responsible for this familial predisposition have been identified (Table II). As a result, selected members of families in which there has been an aggregation of pancreatic cancer can now be tested for inherited genetic abnormalities. Family members found to carry a germline (inherited) genetic abnormality in a gene that predisposes to pancreatic cancer can be carefully screened for early disease, and those found not to carry an inherited genetic abnormality will be relieved of their anxiety. It is important to note that almost all of these genetic syndromes are also associated with an increased risk of other nonpancreatic malignancies, and gene carriers can be screened for early curable forms of these other malignancies.

The genetic abnormalities discovered to date account for only about 10% of the families in which

Table II. Genetic syndromes associated with hereditary pancreatic cancer

Syndrome	Mode of inheritance	Gene	Fold increase in risk of pancreatic cancer	Manifestation
Peutz-Jeghers	AD	*STK11*	140×	Hamartomatous polyps of the gastrointestinal tract; mucocutaneous melanin macules
Hereditary pancreatitis	AD	*PRSS1*	60×	Recurrent episodes of severe pancreatitis starting at a young age
Familial pancreatic cancer	Unknown	Unknown	18×	At least one pair of first-degree relatives with pancreatic cancer
FAMMM	AD	*p16*	20×	Multiple nevi, atypical nevi, melanomas
Familial breast cancer 2	AD	*BRCA2*	10×	Breast, ovarian, and pancreatic cancer
HNPCC	AD	*MSH2* *HLH1*	Unknown	Colonic, endometrial, and gastric cancers; mutator phenotype

AD = autosomal dominant; FAMMM = familial atypical multiple mole melanoma; HNPCC = hereditary nonpolyposis colorectal cancer.

there is an aggregation of pancreatic cancer. The identification of the clustering of pancreatic cancer in the remaining 90% of the kindreds remains a hot area of research.

The National Familial Pancreas Tumor Registry

The National Familial Pancreas Tumor Registry (NFPTR)* was established at Johns Hopkins in 1994 to help elucidate the yet-unidentified causes of familial pancreatic cancer. A total of 636 kindreds were enrolled in this registry as of February 1, 2001. These include 256 kindreds in which at least one pair of first-degree relatives have been diagnosed with pancreatic cancer. Eleven new cases of pancreatic cancer have developed in these kindreds. To quantify this risk, we prospectively followed the first 341 kindreds enrolled in the NFPTR and found that when the family contained at least one pair of first-degree relatives with pancreatic cancer at the time the family enrolled in the NFPTR, then apparently healthy first-degree relatives of the index patient had an 18-fold increased risk of developing pancreatic cancer.[7] Remarkably, if three or more members of the family had pancreatic cancer at the time the family enrolled in the NFPTR, then the *prospective* risk of pancreatic cancer in first-degree relatives of the index patient rose to 56-fold greater then expected.[7] These studies not only quantify the risk of pancreatic cancer in family pancreatic cancer kindreds, but they also highlight the importance of identifying the genes responsible for familial pancreatic cancer. Any physician who has a patient with a strong family history of pancreatic cancer, should consider referring the patient to the NFPTR for registration in ongoing research protocols.

The identification of the genes responsible for the familial aggregation of pancreatic cancer and the demonstration that members of these kindred have an increased risk of developing pancreatic cancer raises the critical question—"How can we screen at-risk patients for early pancreatic cancer?" Here again, the genetic revolution may hold the key.

Screening for Early Pancreatic Cancer

Screening tests for early breast, prostate, cervical, and colorectal cancers have all saved many lives. Unfortunately, sensitive and specific screening tests for pancreatic cancer, the one cancer that cries out most for a screening test, do not exist. Carbohydrate antigen 19.9 (CA 19.9) is often elevated in patients with advanced pancreatic cancer, but the marker lacks the sensitivity and specificity needed to screen the general population.

A major focus of our research efforts is, therefore, to develop novel screening tests for early pancreatic cancer. Three advances make these efforts promising.

First, although many of the genetic alterations that occur in pancreatic cancers are losses of genetic material and therefore would be difficult to detect in screening tests, Ueki et al.[8] have recently demonstrated that specific genes are selectively hypermethylated (the addition of a carbon group to the DNA) in pancreatic cancer. Each of these sites of hypermethylation provides a potential target for screening. Indeed, Palmisano et al.[9] have already shown that DNA hypermethylation can be used to detect early lung cancers.

Second, techniques have recently been developed to analyze the gene expression patterns in tumors, not one gene at a time, but instead thousands. These exciting techniques include "serial analysis of gene expression" (SAGE) and the "gene chips" that have been popularized in the news. To demonstrate the power of these technologies, we performed SAGE on tumors and normal tissues and identified more than 100 genes that appear to be selectively overexpressed in pancreatic cancer.[10] Each of these overexpressed genes is a potential new marker for pancreatic cancer. For example, tissue inhibitor of metaloproteinase-1 (TIMP-1) was found by SAGE to be overexpressed in pancreatic cancer, and Zhou et al.[11] have shown that TIMP-1 levels are indeed elevated in the serum of patients with pancreatic cancer.

The third area that has advanced our search for an early detection test is the field of "proteomics." Genes code for proteins and protein expression patterns in pancreatic cancer and in pancreatic secretions have been studied using recently developed protein chip technologies. Using this approach, the levels of hundreds of proteins can be examined simultaneously in a large number of samples, allowing one to compare directly the proteins present in samples from patients with and without pancreatic cancer. The goal of this approach is to identify proteins selectively elevated in the cancer samples. Once identified, each of these proteins is a potential new target for the development of a screening test for pancreatic cancer.

Although none of these approaches is currently clinically available, all three have provided strong leads for the future development of screening tests for early pancreatic cancer.

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Tumor Classification

Because I am a pathologist, it is only natural that one of my interests is improving tumor classification, and the genetic revolution has led to great advances here too. Let me give an example. For years, pathologists noted intraductal proliferations adjacent to infiltrating cancers of the pancreas, and they hypothesized, but had no way to prove, that these proliferations were the precursors to the infiltrating carcinomas. Our group has extensively studied these genetic alterations in these intraductal lesions (called "Pancreatic Intraepithelial Neoplasms [PanINs]) and, quite remarkably, PanINs harbor many of the same fundamental genetic alterations as infiltrating adenocarcinomas of the pancreas.[12] These similarities establish that PanINs are true neoplasms and that they are the precursors to infiltrating cancer. Establishing a noninvasive precursor to invasive pancreatic cancer provides critical support to efforts to screen for early pancreatic cancer. Just as screening for benign adenomas of the colon can reduce deaths from invasive colon cancer, so screening for PanINs could save lives that would otherwise have been lost to invasive pancreatic cancer.

Treatment of Pancreatic Cancer

Even if an effective screening test can be developed, new therapies will still be needed to treat established invasive disease. The genetic revolution has provided critical advances here too. For example, Jaffee et al.[13] have developed a novel vaccine therapy against invasive pancreatic cancer. They have recently reported that it is safe and that it induces an intensive antitumor immune response. This allogeneic vaccine was developed by transfecting cultured human pancreatic cancer cell lines with the gene that codes for granulocyte-monocyte colony–stimulating factor (GM-CSF). Transfected cultured cells expressing GM-CSF are treated with low-dose radiation so that they remain alive but can no longer divide. These cells are then injected into the dermis of patients with pancreatic cancer. Once in the dermis, the GM-CSF–secreting vaccine cells attract antigen-presenting cells, which in turn activate cytotoxic T-lymphocytes. These activated lymphocytes circulate and selectively and effectively kill large numbers of tumor cells throughout the body. This vaccine is now being evaluated in phase II trials at Johns Hopkins. It demonstrates the power of genetics to harness the sensitivity and specificity of the immune system.

These are just a few examples, but they demonstrate the importance that our understanding of the fundamental genetic nature of pancreatic cancer can have.

WHAT DOES IT MEAN FOR THE AVERAGE SURGEON?

As our understanding of the fundamental genetic nature of pancreatic cancer grows, surgeons will be in a unique position to translate these advances to patient care. Surgeons with an understanding of the genetic basis for the familial aggregation of pancreatic cancer will be able to counsel patients and their families on genetic testing and, in selected individuals, may even recommend prophylactic surgery. Surgeons with an understanding of the power of new gene-based screening technologies will be the first to harness these new technologies to detect early, and perhaps even preinvasive, pancreatic neoplasms. Finally, surgeons with an understanding of the genetics of pancreatic cancer will be the first to apply rational gene-based therapies to treat patients with invasive disease.

By contrast, surgeons unwilling to accept these advances will miss the opportunity to treat their patients at a stage when their patients would benefit most from surgery—early, before the disease has already spread to other organs.

WHAT WOULD HALSTED THINK?

What would William Halsted think of all of this if he were alive today? He would love it. Dr. Halsted was a true pioneer of the scientific method. He was able to make remarkable advances in the operating room because he was able to combine experimental work in physiology and pathology with innovative surgical techniques. For example, he developed the submucosal intestinal suture after carefully examining a variety of anastomotic methods in dogs in the laboratory. This advance proved critical in the development of alimentary tract surgery. Similarly, he spent many months in the laboratory before he formed a method of overlapping tissues and other refinements that resulted in a firm closure of hernias. Clearly, if Dr. Halsted were alive today, he would be the first to grasp the genetics revolution and apply it to surgery.

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Duodenal Carcinoid Tumors: How Aggressive Should We Be?

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Duodenal carcinoid tumors are uncommon. It is not known whether they behave more like carcinoid tumors in the appendix (indolent course) or those in the ileum (often virulent)—crucial information for determining the need for radical resection. A retrospective review at our tertiary referral center (from 1976 to 1999) identified 27 patients with primary duodenal carcinoid lesions, excluding functional islet cell tumors. Endoscopic biopsy provided the diagnosis in 78% of patients. Treatment was by endoscopic excision (n = 11), transduodenal excision (n = 8), pancreaticoduodenectomy (n = 3), segmental distal duodenectomy (n = 2), or palliative operation (n = 2). One patient did not undergo operation because of comorbidity. Eighteen of 19 patients with tumors smaller than 2 cm remained disease free after local (endoscopic or transduodenal) excision. The exception was a patient with a small periampullary carcinoid lesion. In contrast, all four patients with carcinoid tumors 2 cm or larger who were resected for cure developed a recurrence (2 to 9 years postoperatively). We conclude that duodenal carcinoid tumors smaller than 2 cm may be excised locally; to ensure complete resection we recommend open transduodenal excision for tumors between 1 and 2 cm. Endoscopic follow-up is indicated. It is unclear whether patients with larger tumors benefit from more aggressive locoregional resection. Ampullary/periampullary carcinoid tumors should be considered separately, as their behavior is unpredictable. (J GASTROINTEST SURG 2001;5:588-593.)

KEY WORDS: Carcinoid tumor, duodenum, neuroendocrine tumor, endoscopy

Carcinoid tumors are of neuroendocrine origin; they arise from enterochromafin cells and are found most commonly in the gastrointestinal tract. Primary duodenal carcinoid lesions account for less than 5% of all gastrointestinal carcinoid tumors,[1-3] making it difficult to accrue a large enough experience to identify prognostic factors specific for the duodenum. Broader experience with carcinoid tumors in the remainder of the gastrointestinal tract has allowed more complete study and relatively clear therapeutic recommendations. Small appendiceal and rectal carcinoid tumors have an indolent course; tumors smaller than 2 cm in these locations without evidence of local invasion can be safely treated by conservative excision without major concern about metastatic disease.[4-7] Carcinoid tumors of the distal small bowel, on the other hand, are more aggressive. Even small tumors (especially in the ileum) are associated with mesenteric lymphatic metastases and distant metastases.[6,8] Indeed, most ileal carcinoid tumors have already metastasized to regional lymph nodes by the time of diagnosis. Segmental resection with wide mesenteric excision to include the draining lymph nodes has thus been considered minimal therapy for these tumors, regardless of size. In addition, some investigators have recently suggested that carcinoid tumors of the foregut may portend a worse prognosis than those arising elsewhere in the gastrointestinal tract, although

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these series included only a few duodenal carcinoid lesions.[6,9]

It remains unclear whether duodenal carcinoid tumors behave more like appendiceal and rectal tumors, with a more indolent course, or like ileal carcinoid lesions, with a greater likelihood of locoregional or distant hematogenous metastases—crucial information for determining the necessity of radical resection in this anatomic region where a formal segmental resection including regional lymph nodes may require a pancreaticoduodenectomy. This study was therefore undertaken to evaluate treatment and outcomes of patients with duodenal carcinoid tumors from our large tertiary referral practice.

METHODS

All consecutive patients evaluated for treatment of duodenal carcinoid tumors at the Mayo Clinic in Rochester, Minnesota, from January 1976 to December 1999, were identified by a retrospective chart review. We have defined "carcinoid tumor" according to the World Health Organization *International Histological Classification of Tumours*.[10] Those patients with neuroendocrine tumors that were functional at the time of diagnosis (e.g., gastrinoma, somatostatinoma), as well as those with tumors arising from structures surrounding the duodenum (pancreas, common bile duct), were specifically excluded from this study. Patient demographics, clinical features, diagnostic procedures, details of the operation, and pathologic findings were recorded. Because carcinoid tumors in other regions of the gut have been associated with multicentricity (up to 30%) and with other primary malignancies (up to 40%), we evaluated these parameters as well. Complete follow-up to date was obtained from patient charts, tumor registry, or by contacting patients or their primary physician by telephone or letter. The study protocol was approved by the Mayo Clinic Institutional Review Board.

RESULTS

Patient Characteristics

Twenty-seven patients (15 men, 12 women) had duodenal carcinoid tumors. Median age at the time of diagnosis was 66 years (range 43 to 86 years). The most common presenting symptom was abdominal pain (48%). Other symptoms included vomiting, weight loss, gastrointestinal blood loss, pancreatitis, abdominal mass, and other nonspecific gastrointestinal complaints (Table I). Four patients were asymptomatic and diagnosed at the time of workup for an unrelated gastrointestinal condition. Diagnosis was secured in the vast majority of patients (78%) by endoscopic biopsy.

Table I. Symptoms of duodenal carcinoid tumors

Symptoms	No. of patients (%)
Abdominal pain	13 (48)
Vomiting	4 (15)
Weight loss	4 (15)
Gastrointestinal blood loss	3 (11)
Pancreatitis	3 (11)
Mass	1 (4)
Nonspecific gastrointestinal complaints*	11 (41)
Asymptomatic	4 (15)

*Dyspepsia, nausea, bloating, heartburn, diarrhea.

Duodenal carcinoid tumors were found incidentally at operation for another unrelated condition in two patients. In two others, diagnosis was made by computed tomography–guided fine-needle aspiration.

Pathologic Findings

The majority of these tumors (16 of 27) were located in the first portion of the duodenum. Nine others were in the second portion, including two periampullary tumors, and only two were located in the distal duodenum.

One patient with a periampullary tumor was noted to have several small (<0.4 cm) localized tumors within the pathologic specimen; no other patient in this series had multicentric gastrointestinal carcinoid lesions. Nine patients (33%) had one or more other primary malignancies, including three patients each with adenocarcinoma of the colon and prostate, and one patient each with jejunal adenocarcinoma, lymphoma, small cell carcinoma of the lung, and renal cell carcinoma. Twelve tumors in this series underwent evaluation with special immunohistochemical stains. The majority (10 of 12) stained positive for the nonspecific neuroendocrine marker chromogranin. In addition, five tumors were positive for synaptophysin, two each for neuron-specific enolase and cytokeratin, and one each for gastrin, somatostatin, and the neuroendocrine marker nkh1.

Treatment

Endoscopic Excision. Eleven patients were treated by endoscopic excision, all with tumors 1.0 cm or smaller. All patients are alive and tumor free at a median follow-up of 4.2 years (range 1.5 to 8 years). One patient had an early local recurrence (noted at 3 months) treated successfully by endoscopic reexcision; he is alive with no further recurrence 7 years later.

Table II. Characteristics of patients undergoing open operation for duodenal carcinoid tumors (sorted by outcome)

Outcome	Age (yr)	Sex	Location	Size (cm)	Depth of invasion	Metastasis	Operation	Time until recurrence (yr)	Follow-up (yr)
Died of disease	43	M	D2	4.0	Through serosa	Liver	Gastrojejunostomy, hepatic artery ligation	—	2.5
	60	F	D2	2.0	Through serosa	1 node	Pancreaticoduodenectomy	2.0	6.5
	62	F	D2	3.5	Through serosa	No	Pancreaticoduodenectomy	5.0	12.0
Alive with disease	36	F	D2	3.0	Through serosa	Liver, 3 nodes	Pancreaticoduodenectomy	—	6.8
	67	F	D3, D4	8.0	Into muscularis	No	Segmental excision	2.2	2.3
	69	F	Periampullary	1.0	Mucosal	No	Pancreaticoduodenectomy	3.0	5.5
	58	F	D4	4.0	Through serosa	No	Segmental excision	9.0	22.5
Died of other causes	66	M	Periampullary	0.4	Mucosal	No	Transduodenal excision	—	2.7
Alive	77	M	D1	1.1	Mucosal	No	Transduodenal excision	—	2.5
Disease free	53	M	D1	1.1	Mucosal	No	Transduodenal excision	—	4.5
	60	M	D2	1.4	Into muscularis	No	Transduodenal excision	—	1.5
	64	M	D1	0.5	Mucosal	No	Transduodenal excision	—	4.3
	67	M	D1	1.2	Mucosal	No	Transduodenal excision	—	2.5
	67	F	D1	0.8	Mucosal	No	Transduodenal excision	—	4.25
	49	M	D1	1.7	Mucosal	No	Transduodenal excision	—	12.8

Open Excision. Fifteen patients were managed operatively (Table II). Operative approaches included transduodenal local excision (n = 8), grossly curative pancreaticoduodenectomy (n = 3), grossly curative segmental excision of the third and fourth portions of the duodenum (n = 2), and palliative operation (n = 2; gastrojejunostomy with hepatic artery ligation in one and palliative pancreaticoduodenectomy in one). Thirteen of these patients thus underwent resection with curative intent (median follow-up 4.3 years, range 1.5 to 23 years).

Five of the 13 patients resected for cure developed recurrent carcinoid tumor. The mean size of the primary tumor in patients with a recurrence was 3.6 cm (range 1 to 8 cm); the size of the primary tumor was greater than 2 cm in all patients except one who had a 1 cm periampullary carcinoid lesion. Four of the five patients with a recurrence had a primary tumor invading into the muscularis or through the serosa at the time of the initial operation (the exception being the one patient with a submucosal periampullary tumor). Only one patient with recurrent carcinoid tumor had metastatic lymph node involvement at the time of the initial operation. Two patients with recurrent tumor died of disease 7 and 12 years postoperatively, and three are alive with progressive disease 2, 6, and 22 years postoperatively.

Seven patients resected for cure are alive and tumor free (median follow-up 4.3 years, range 1.5 to 12.8 years), and one died of unrelated causes with no evidence of recurrence. All of these patients had primary carcinoid tumors smaller than 2 cm (mean size 1 cm, range 0.4 to 1.7 cm). The primary tumor was confined to the mucosa in seven of these patients and invaded the muscularis in one.

The one patient managed by observation alone because of advanced age and significant comorbidity is alive 2.5 years after the initial diagnosis.

Carcinoid Syndrome

Three patients in this series (all with advanced recurrent disease) developed symptoms of carcinoid syndrome (sweating, flushing, and diarrhea) after grossly curative resection. None of these patients had symptoms at the time of the initial diagnosis; all symptoms of carcinoid syndrome become evident after recurrence with multiple bulky hepatic metastases. The 24-hour urine 5-hydroxyindoleacetic acid (5′-HIAA) level was normal in one of these patients, and measurements were not performed in the other two. Another patient who developed metastatic disease with elevated urinary 5-HIAA levels did not have symptoms of carcinoid syndrome. No patient had elevated levels of 5′-HIAA at the time of initial presentation.

DISCUSSION

In 1907 Oberndorfer[11] coined the term "karzinoide" to describe a carcinoma-like tumor with less aggressive behavior, a term that remains appropriate today. Although carcinoid tumors are generally considered more indolent, especially when compared to other adenocarcinomas of the gastrointestinal tract, it is important to emphasize that the natural history of carcinoid tumors is variable depending on their location within the gastrointestinal tract. Small carcinoid tumors (<2 cm) arising in the appendix or rectum have a very low incidence of metastasis and are adequately treated by local excision (appendectomy or local excision with fulguration, respectively). In contrast, carcinoid tumors arising in the distal small bowel are more aggressive; even small tumors (<1 cm) here have a much greater propensity to metastasize.[4,6,12,13] Because of this aggressive behavior, segmental excision with wide local mesenteric excision is recommended for treatment of distal small bowel carcinoid tumors.

Finding a carcinoid tumor in the duodenum presents a unique clinical dilemma because, unlike carcinoid lesions of the distal small bowel, appendix, or rectum, prognostic factors specific for carcinoid tumors arising in the duodenum are poorly characterized as a result of the rarity of this neoplasm. Proposed treatment of duodenal carcinoid tumors has spanned a broad spectrum including aggressive resection by pancreaticoduodenectomy, transduodenal local excision, full-thickness laparoscopic excision, and endoscopic excision by snare or strip biopsy.[6,14-16] Optimal therapy for duodenal carcinoid tumors, however, remains unclear. The need to understand the specific natural history of carcinoid tumors in the duodenum is important for several reasons. First, radical duodenal resection is a more complex undertaking than wide segmental excision of the distal small bowel. Although major duodenal and pancreatic resections are being performed more commonly and safely in the current surgical era,[17,18] it is not clear whether a major resection provides any added therapeutic benefit over local excision for patients with duodenal carcinoid tumors. Second, the morbidity and mortality of carcinoid tumors are related to metastatic disease (liver replacement from metastases and carcinoid syndrome) and usually not from the primary tumor. A third important consideration is that duodenal carcinoid tumors will be identified more frequently with the increased use of upper gastrointestinal endoscopy. Indeed, almost 60% of patients in our series were diagnosed within the past 5 years, mostly by endoscopy.

Most previous reports of treatment of duodenal carcinoid tumors have involved anecdotal experience, collective reviews of multiple small experiences, or reports of unique technical approaches. There has been only one other large study of 99 duodenal carcinoid tumors, compiled by Burke et al.[12] at the Armed Forces Institute of Pathology. These pathologists identified size greater than 2.0 cm, presence of mitotic figures, and invasion of the muscularis as independent risk factors for metastasis. No patient in their series with a tumor smaller than 1.0 cm developed metastatic disease at minimum follow-up of 24 months.

In our single-institution experience, although several different operative treatments were used, patients seem to be differentiated into two groups based on the size of the primary tumor and the presence of invasion of the muscularis, similar to the series from the Armed Forces Institute of Pathology.[12] Nineteen patients in our series had duodenal carcinoid tumors smaller than 2 cm; in only one of these patients had the tumor invaded past the submucosa into the muscularis. Of these 19 patients, 18 remain disease free after local resection (endoscopic excision in 11 and transduodenal excision in 7) with a mean follow-up of 4.7 years (range 1.5 to 13 years). The one exception was a patient with a 1 cm submucosal periampullary carcinoid lesion. Despite aggressive resection by pancreaticoduodenectomy, the patient had a recurrence 3 years postoperatively. She is alive with liver metastases 2.5 years after recurrence. This patient highlights the unpredictable behavior of even small ampullary/periampullary carcinoid tumors, which should probably stand alone when considering therapeutic options. The distinction between "ampullary" and "periampullary" carcinoid tumors in the literature is difficult to make; these tumors are quite rare, with only 89 cases reported. They do, however, appear to have a more aggressive course, with development of early metastatic disease despite very small size.[19,20] One other patient in our series had a periampullary carcinoid tumor. He underwent local excision of a 0.4 cm lesion and died of unrelated causes with no evidence of recurrence 3 years postoperatively.

One of the 11 patients treated by endoscopic resection was found to have a local "recurrence" at 3-month endoscopic follow-up, which was most likely due to incomplete initial resection. This patient underwent endoscopic reexcision and remains free of disease 7 years later; this patient underscores the need for at least one endoscopic reexamination several months after any form of local excision (endoscopic or open).

In contrast to patients with tumors smaller than 2 cm, all four patients in our series with duodenal carcinoid tumors 2 cm or larger who underwent curative resection (pancreaticoduodenectomy in 2 and segmental distal duodenectomy in 2) developed recurrent disease 2 to 9 years later. In all four patients the primary tumor invaded through the submucosa into

the muscularis. It is also notable that only one of these patients had lymphatic nodal metastases at the time of the initial operation. Interestingly, two of these four patients remain alive 2 and 23 years postoperatively, and two have died 6 and 12 years postoperatively (4.5 and 7 years after metastatic recurrence). These latter patients further reinforce the less aggressive natural history of gastrointestinal carcinoid tumors, particularly when compared to gastrointestinal adenocarcinomas.

From our single-institution experience and that of others,[12] we suggest that duodenal carcinoid tumors smaller than 2 cm be excised locally. Endoscopic excision is appropriate for patients with tumors smaller than 1 cm, with at least one follow-up endoscopy 2 to 3 months later to ensure completeness of resection and rule out local recurrence. Tumors between 1 and 2 cm may best be treated by open transduodenal local excision, as it may be difficult to ensure complete endoscopic excision of these larger lesions.

Appropriate management of duodenal carcinoid lesions 2 cm or larger is more problematic. All of our patients with tumors 2 cm or larger who were resected for cure developed recurrent disease despite segmental resection or pancreaticoduodenectomy. Only one of these patients had nodal metastases within the resected specimen. This experience questions the added benefit of an extended oncologic-type resection. Although a segmental resection may be needed to completely excise the primary tumor for local control, whether a more extended resection (pancreaticoduodenectomy) designed to include the draining nodal basin is of any added benefit remains unknown. Endoscopic ultrasonography may be helpful in determining the depth of invasion of the primary tumor; it is unclear whether an extended resection will benefit patients with small (<2 cm) tumors that invade into the muscularis.

Although the collective experience with ampullary/periampullary carcinoid tumors is small, and it is difficult to draw conclusions from only two patients in this series, based on our collective experience and findings in the literature, these tumors appear to behave unpredictably and should probably be viewed as a distinct category of carcinoid tumor when considering treatment options. Even very small (<1 cm) ampullary/periampullary carcinoid tumors display a distinctly different aggressive behavior and may metastasize early.[19,20]

Carcinoid tumors of the gastrointestinal tract have been associated with high rates of multicentricity (up to 30%)[5,21,22] as well as with a 12% to 40% association with other primary malignancies.[6,23] Although no patient in our series had distant multicentric carcinoid tumors, nine patients had an associated second primary malignancy (either synchronous or metachronous), an important consideration during the workup or long-term management of patients with duodenal carcinoid tumors.

In summary, our review of 27 patients with duodenal carcinoid lesions suggests that tumors smaller than 2 cm may be safely excised locally (endoscopically for tumors smaller than 1 cm or by open transduodenal local excision for tumors 1 to 2 cm). Close endoscopic follow-up of these patients is clearly warranted. Size greater than 2 cm, invasion of the tumor through the submucosa, or a periampullary location may predict a poorer prognosis. It is unclear whether patients with larger tumors will benefit from more aggressive locoregional resection; however, even patients with metastatic disease may enjoy a reasonably long survival. Ampullary and periampullary carcinoid tumors should be considered separately, as their behavior is more unpredictable.

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Analysis of Tumor Characteristics and Survival in Liver Transplant Recipients With Incidentally Diagnosed Hepatocellular Carcinoma

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The use of orthotopic liver transplantation (OLTX) for the treatment of hepatocellular carcinoma (HCC) has generally become restricted to carefully selected cases of small oligocentric tumors. However, it is not uncommon to find previously undetected HCC within recipient cirrhotic livers at the time of hepatectomy. The impact of unsuspected HCC on patient outcomes remains unclear. A retrospective analysis of our institutional experience with adult primary OLTX was performed comparing recipients with incidental HCC (group 1), recipients with known or suspected HCC (group 2), and recipients confirmed by pathologic examination to be tumor free (group 3). Between 1984 and 2000, 27 patients in group 1, 12 patients in group 2, and 612 patients in group 3 underwent primary OLTX. Tumors were smaller ($P = 0.0172$) in group 1 than in group 2; however, the number of tumors and the histologic findings were similar in the groups. Incidence of bilobar involvement, vascular invasion, portal vein tumor thrombus, lymphatic involvement, and distant metastasis at the time of OLTX did not differ significantly between these groups. Four-year patient survival appeared to be lower in group 1 (70.0%) than in group 3 (79.0%) ($P = 0.0546$); 4-year patient survival was significantly worse in group 2 (31.0%) compared to group 3 ($P = 0.0106$). Thus, in our experience, incidentally diagnosed cases of HCC possess many of the same features of malignancy as preoperatively diagnosed HCC. Indeed, patient survival after OLTX appears to be adversely affected by the presence of incidental HCC. (J GASTROINTEST SURG 2001;5: 594-602.)

KEY WORDS: Liver transplantation, hepatocellular carcinoma, incidental

Since its early development, liver transplantation has been employed as a treatment strategy for primary hepatic tumors. In fact, several of the earliest human liver transplantations were performed in patients with unresectable hepatic malignancies.[1-3] Dismal rates of tumor recurrence and postoperative liver failure after surgical resection for hepatocellular carcinoma (HCC) led to an initial enthusiasm for using orthotopic liver transplantation (OLTX) as a definitive form of therapy for HCC, particularly in cases of unresectable lesions arising in the preneoplastic setting of cirrhosis. However, an unacceptably high rate of treatment failure after liver transplantation, combined with the universal limitation of available donor organs, quickly tempered this early enthusiasm.[4-8] More recently, selective use of OLTX has begun to demonstrate an oncologic advantage over resection in carefully selected cases of early, small, oligocentric HCC.[9-24]

For the most part, the issue of OLTX performed in recipients with incidentally diagnosed cases of HCC has been only indirectly examined. Several single-center series have reported slightly more favorable survival results for incidentally diagnosed HCC as compared to preoperatively diagnosed cases.[13,25-28] In contrast, however, other single-center series and a long-term analysis of tumor registry data have suggested that the incidental diagnosis of HCC in OLTX recipients portends an unfavorable prognosis that

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Table I. Patient characteristics

	Group 1	Group 2	Group 3	*P* value
Mean age (yr)	55.4	46	49	0.0112
Sex (% male)	85.2	75	60.1	0.015
HBV/HCV etiology (%)	55.6	41.7	25.5	0.00178
Child-Pugh score				
(% A)	3.7	50	N/A	
(% B)	40.7	41.7	N/A	
(% C)	55.6	8.3	N/A	0.00103

HBV/HCV = hepatitis B/C virus.
N/A = not available.

cannot be statistically segregated from that seen in transplant recipients with known tumors.[24,29-33] To date, no studies have directly compared outcomes of patients with HCC diagnosed incidentally after transplantation with patients confirmed to be free of tumor. To examine this issue more closely, we retrospectively analyzed our institutional experience with OLTX among recipients whose native, explanted cirrhotic livers were incidentally diagnosed with previously unsuspected HCC at the time of pathologic examination. This population was compared with patients undergoing OLTX for known HCC, and OLTX patients found by pathologic examination to be free of HCC.

METHODS

By means of chart and liver transplant database review, adult patients undergoing primary OLTX for end-stage cirrhotic liver disease at the University of Wisconsin Hospital and Clinics between 1984 and 2000 were identified. Patients awaiting liver transplantation were followed with yearly abdominopelvic computed tomography (CT) scans and serum alpha-fetoprotein levels every 6 months. Cirrhotic patients with previously unsuspected HCC diagnosed at the time of pathologic examination of the recipient liver were considered to have incidental HCC (group 1). Cirrhotic patients with any preoperative suspicion or diagnosis of HCC by radiography, biopsy, and/or alpha-fetoprotein serology confirmed by pathologic examination were considered to have known HCC (group 2). These groups were compared with patients undergoing OLTX for cirrhotic liver disease whose native livers were confirmed by pathologic examination to be free of HCC or other neoplasms (group 3). Of note, fibrolamellar variants of HCC were excluded from these analyses. No patients in this series underwent neoadjuvant therapy with transarterial chemoembolization or tumor ablation prior to OLTX. Patient and tumor characteristics were statistically compared using the two-tailed Fisher's exact test and analysis of variance. Patient survival was compared using Kaplan-Meyer methodology, and clinical factors associated with patient survival were determined by means of the log-rank test and Cox proportional hazards model.

RESULTS

Patient Characteristics

In a review of all cases of adult primary OLTX performed for cirrhotic liver disease between 1984 and 2000, 27 patients in group 1, 12 patients in group 2, and 612 patients in group 3 were identified. These numbers indicated a 4.2% prevalence of incidental HCC among cirrhotic patients with no previously diagnosed tumors who had undergone liver transplantation. OLTX among patients in both groups 1 and 2 took place between 1987 and 2000; OLTX among patients in group 3 took place between 1984 and 2000. Patient characteristics in the three groups are shown in Table I. Patients in group 1 (mean age 55.4 years) were older than those in group 2 (mean age 46.0 years) ($P = 0.018$) and group 3 (mean age 49.0 years) ($P = 0.0047$). There was a stronger male predominance in groups 1 (85.2%) and 2 (75.0%) compared to group 3 (60.1%) ($P = 0.0150$). Prevalence of hepatitis B or C virus as an etiology of cirrhosis was higher in group 1 (55.6%) and group 2 (41.7%) than in group 3 (25.5%) ($P = 0.00178$). Prevalence of hepatitis C alone was also higher in group 1 (37.0%) and group 2 (33.3%) than in group 3 (16.3%) ($P = 0.007$). In addition, severity of cirrhosis as determined by Child-Pugh classification was significantly greater in group 1 than in group 2 ($P = 0.00103$).

Tumor Characteristics

Tumor characteristics in groups 1 and 2 are shown in Table II. The size of the largest tumor identified in each liver as determined by direct pathologic measurement was significantly smaller in group 1 (median size 2.0 cm) than in group 2 (median size 9.5 cm) ($P =$

Table II. Tumor characteristics

	Group 1	Group 2	*P* value
Median size (cm)	2	9.5	0.0172
Large (>5 cm) tumors (%)	0	50	0.000283
Number of tumors			
(% with 1)	55.6	50	
(% with 2)	22.2	16.7	
(% with 3)	3.7	0	
(% with 4)	3.7	8.3	
(% >4)	14.8	25	0.838
Differentiation			
(% well)	77.8	50	
(% moderate)	18.5	33.3	
(% poor)	3.7	16.7	0.153
Bilobar involvement (%)	22.2	41.7	0.50
Vascular invasion (%)	25.9	16.7	0.693
Portal tumor thrombus (%)	22.2	16.7	1
Lymph node invasion (%)	0	0	N/A
Distant metastasis (%)	0	0	N/A

N/A = not applicable.

0.0172). Similarly, classification of the largest tumor as small (≤5 cm) or large (>5 cm) identified fewer tumors that were larger than 5 cm in group 1 than in group 2 ($P = 0.00028$). The number of tumors did not differ significantly between group 1 (44.4% with ≥2 lesions) and group 2 (50.0% with ≥2 lesions) ($P = 0.838$). Histologic assessment of tumor differentiation in group 1 tumors was statistically similar to that seen in group 2 tumors ($P = 0.153$). There were no differences in bilobar involvement between group 1 (22.2%) and group 2 (41.7%) ($P = 0.496$). Similarly, incidences of vascular invasion (25.9% in group 1 vs. 16.7% in group 2), portal vein tumor thrombus (22.2% in group 1 vs. 16.7% in group 2), lymph node metastases (0% for both groups), and distant metastases (0% for both groups) were not significantly different between the groups ($P = 0.693$ and 1.000, not applicable for both lymph node and distant metastases).

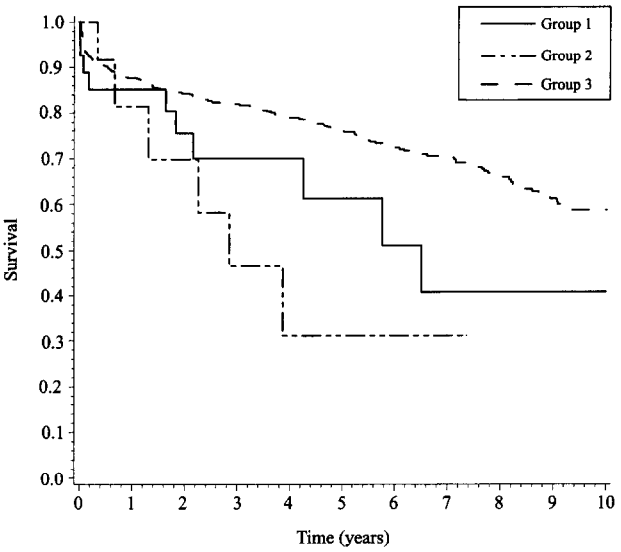

Fig. 1. Overall survival probability curves. Group 1 vs. group 2, $P = 0.0546$; Group 2 vs. group 3, $P = 0.0106$.

Survival

Mean lengths of follow-up for groups 1, 2, and 3 were 35.3, 28.8, and 59.2 months, respectively. As represented in Fig. 1, one-year overall patient survival was 85.2%, 91.7%, and 90.2%, respectively, for groups 1, 2, and 3. Three-year overall patient survival was 70.0%, 46.6%, and 81.9%, respectively, for groups 1, 2, and 3. Five-year overall patient survival was 61.3%, 31.0%, and 76.0%, respectively, for groups 1, 2, and 3. Differences in overall patient survival were nearly statistically significant between group 1 and group 3 ($P = 0.0546$). Patient survival was significantly worse for group 2 than for group 3 ($P = 0.0106$).

Recurrence-free survival (not censored for patient death) in group 1 was 95.8% at 1 year, 78.8% at 3 years, and 68.9% at 5 years. Uncensored recurrence-free survival for group 2 was 81.5% at 1 year, 46.6% at 3 years, and 31.0% at 5 years. Recurrence-free survival (censored for patient death) for group 1 was 100% at 1 year, 76.2% at 3 years, and 76.2% at 5 years. Censored recurrence-free survival for group 2 was 60.8% at 1, 3, and 5 years. Of the nine deaths in group 1, four (44.4%) were a result of recurrent tumor. In group 2, all six of the deaths were due to recurrent tumor.

Table III. Clinical factors associated with survival

Factor	Overall survival (*P* value)	Recurrence-free survival (*P* value)
Sex	0.74	0.739
Age	0.319	0.155
Child-Pugh score	0.0656	0.111
HBV/HCV etiology	0.423	0.164
Size (>5 cm)	0.0016	0.0003
Number	<0.0001	<0.0001
Differentiation	0.0084	<0.0001
Bilobar involvement	0.197	0.0169
Vascular invasion	0.0436	0.0042
Portal tumor thrombus	0.0272	0.0009

Table IV. Transfusions associated with survival

	Overall survival		Recurrence-free survival	
Type of transfusion	RR	*P* value	RR	*P* value
Red blood cell	1.021	0.0769	0.956	0.241
Cell-saver	1.05	0.0145	0.858	0.568
Fresh-frozen plasma	0.996	0.719	0.971	0.145
Cryoprecipitate	1.048	0.0218	1.007	0.88

RR = risk ratio per unit transfused.

Clinical Factors Associated With Survival

Associations of various patient and tumor characteristics with overall survival and recurrence-free survival (not censored for patient death) in groups 1 and 2 are shown in Table III. Clinical factors associated with worse overall survival after OLTX were tumor size ($P = 0.0016$), number of tumors ($P < 0.0001$), less differentiated tumor histology ($P = 0.0084$), vascular invasion ($P = 0.0436$), and portal vein tumor thrombus ($P = 0.0272$). Clinical factors associated with worse recurrence-free survival after OLTX were similar: tumor size ($P = 0.0003$), number of tumors ($P < 0.0001$), tumor histology ($P < 0.0001$), bilobar involvement ($P = 0.0169$), vascular invasion ($P = 0.0042$), and portal vein tumor thrombus ($P = 0.0009$).

Transfusion Requirements Associated With Survival

The amounts of various blood products transfused intraoperatively were compared with post-transplantation survival outcomes (Table IV). The number of packed red blood cell and fresh-frozen plasma transfusions did not appear to influence overall survival (risk ratios 1.021 and 0.996, respectively; $P = 0.0769$ and 0.719, respectively). In contrast, cell-saver autotransfusion, and cryoprecipitate transfusion demonstrated significant risk ratios with respect to overall survival (risk ratios 1.050 and 1.048, respectively; $P = 0.0145$ and 0.0218, respectively). However, when analyzed with respect to recurrence-free survival, packed red blood cell, fresh-frozen plasma, cell-saver, and cryoprecipitate transfusions were not associated with statistically significant risk ratios.

DISCUSSION

An accumulating body of evidence has strengthened the contention that OLTX is an effective form of therapy for certain presentations of HCC.[9-24] Specifically, small oligocentric tumors have been shown to be particularly responsive to transplantation, with transplant recipients demonstrating superior overall or disease-free survival compared to similar patients treated with conventional surgical resection. Given the apparent efficacy of OLTX for early-stage HCC, it would be reasonable to speculate that OLTX for previously undiagnosed HCC would also be associated with particularly favorable patient outcomes. However, the few analyses that have been performed regarding OLTX recipients treated for known or incidental HCC have been varied in their conclusions.[13,24-33]

In the present study we observed a 4.2% prevalence of incidental HCC among adult patients undergoing primary OLTX for end-stage cirrhotic liver disease. Although our value is slightly higher than the range of 1.3% to 2.8% reported elsewhere,[3,8,25-27] this

is likely due to the fact that the denominator for our calculation was restricted to those adult patients with end-stage cirrhosis, which would be the patient population most likely to harbor incidental HCC. When this calculation is performed based on the entire number of OLTX procedures performed at our institution during the study period, as has been done by others, the prevalence is reduced to 2.9%. In actuality, the true frequency of incidental HCC is probably much higher; one careful pathologic analysis of 80 consecutive explanted cirrhotic livers yielded a prevalence of 17.5%.[34] This discrepancy is most likely due to the fact that these livers were examined in 2 to 3 mm sections, which is likely to be far more sensitive than the pathologic examinations typically performed on native hepatectomy specimens.

Patient age in group 1 was higher than in group 2 and group 3. The relatively young age observed in group 2 may reflect a selection bias arising from attempts to optimize clinical characteristics among patients undergoing OLTX for the generally unfavorable indication of known HCC. The higher age of patients in group 1 as compared to group 3 may be a reflection of the increased incidence of HCC that is observed among patients with cirrhosis as a function of time.[35-37] Previous data have estimated a yearly incidence of new-onset HCC of 3% among patients with cirrhosis.[16] The higher number of men in the two HCC groups is consistent with previous data describing a higher frequency of HCC among male patients.[35-37] Similarly, the well-established association between chronic hepatitis B and C with HCC likely explains the higher percentage of viral hepatitis as an underlying etiology of liver disease in groups 1 and 2 compared to group 3.[35-37]

In terms of size, the tumors found incidentally in group 1 tended to be more favorable from an oncologic perspective than those tumors diagnosed preoperatively in group 2. However, group 1 tumors were not statistically less multifocal or histologically better differentiated than group 2 tumors. These findings suggest that incidentally diagnosed HCC tumors may tend to be earlier in development but are potentially no less aggressive in nature than known HCC tumors. Furthermore, the absence of any detectable differences in other tumor parameters, such as vascular invasion or portal venous tumor thrombus, indicate that incidental HCC can certainly express some of the same malignant characteristics commonly associated with preoperatively diagnosed HCC. These findings are in slight contrast to those reported by Klintmalm[30] in a report of tumor registry data. In this multi-institution voluntary database, incidental tumors were noted to be statistically smaller (90.8% were <5 cm in size), as well as less likely to be multifocal (48.3%), bilobar (27.0%), poorly differentiated (6.6%), or with vascular invasion (16.1%) than nonincidental tumors. However, the actual percentages of incidental tumors meeting these various characteristics are similar to the percentages observed in our experience. It is possible that the sharper differences noted between incidental and nonincidental tumors in the registry data may be due to the fact that the nonincidental tumors in the registry database were oncologically more advanced than the nonincidental tumors treated with OLTX at our institution.

It is interesting to note that a few of the patients in group 1 were found to have tumors up to 4 or 5 cm in size. One might reasonably expect such tumors to be readily detectable prior to transplantation by standard computed tomography or ultrasonography. However, despite considerable improvements in technology, contemporary radiographic modalities continue to demonstrate suboptimal sensitivities of 35% to 71% for HCC in the setting of chronic liver injury.[38-45] Furthermore, pretransplant evaluations in this population of patients with end-stage cirrhosis not infrequently reveal radiographic inhomogeneity in the liver that is often due to benign causes such as regenerating nodules, adenomas, and so forth, making the preoperative diagnosis of HCC no less challenging than in the past. Indeed, 15 of the 27 patients in group 1 underwent OLTX between 1998 and 2000, suggesting that recent advances in preoperative imaging techniques have not significantly reduced the incidence of previously undetected HCC found after OLTX.

Among the HCC patients treated with OLTX at our institution, tumor size, number of tumors, histologic findings, vascular invasion, and portal vein tumor thrombus were identified as significant negative prognostic factors for overall survival. When analyzed with respect to recurrence-free survival, the same tumor parameters were again identified as significant negative prognostic factors, along with bilobar involvement. These observations are in general agreement with most analyses of HCC tumors treated with OLTX.* There is some controversy over the independent impact of tumor histology on post-transplantation outcomes, with transplant tumor registry data and recent single-center retrospective analyses also identifying degree of tumor differentiation as a significant negative prognostic element.[24,30,46] However, our analysis is limited in that no multivariate

*References 9, 12, 14, 15, 20, 22, 24, 30, 46.

analyses were performed given the small sample sizes involved.

An attempt was made to examine the influence of blood autotransfusion on outcomes among transplant recipients with HCC. The presence of tumor cells in blood shed intraoperatively during surgical oncologic procedures is well documented.[47,48] Several authors have observed that HCC tumor cells can be identified using sensitive polymerase chain reaction techniques in the peripheral blood circulation during operative resection or transplantation procedures.[49-51] In fact, this occult hematogenous dissemination has been implicated as a potential mechanism for the curious observation that the majority of HCC recurrences after OLTX occur in the new, noncirrhotic liver.[30,52] With this in mind, we tested whether extensive use of autotransfusion could be associated with poor patient survival and tumor recurrence. Indeed, we measured a significant risk ratio of 1.050 for cell-saver autotransfusion with respect to overall patient survival. However, we also observed a similarly significant risk ratio of 1.048 for cryoprecipitate transfusion; no significant association was observed with the use of packed red cell or fresh-frozen plasma transfusions. The correlation between cell-saver or cryoprecipitate transfusion and lower survival could simply be a reflection of higher transfusion requirements during operative cases with extensive blood loss or coagulopathy. Indeed, when analyzed with respect to tumor recurrence, none of the transfusion types were associated with significant risk ratios; therefore no adverse oncologic outcome could be associated with the use of autotransfusion.

As would be expected, patients in group 2 exhibited significantly worse overall post-transplantation survival outcomes than did patients in group 3. However, it should be noted that most of the OLTX procedures performed for known HCC at our institution took place prior to the establishment of guidelines of tumor size and number of tumors that are often used as indications for transplantation today.[12,53] Seven of the 12 patients in group 2 underwent OLTX for bulky, unresectable tumors that would typically be considered too large for transplantation by current standards. Indeed, these seven patients who possessed tumor burdens exceeding modern United Network of Organ Sharing (UNOS) status 2B criteria for HCC underwent OLTX prior to 1996; these patients would not be considered candidates for transplantation by our program today. Furthermore, until recently our institution has not aggressively utilized OLTX as a treatment modality for known HCC, so the number of patients in group 2 is quite small. It is quite possible that OLTX outcomes for patients with HCC meeting strict tumor inclusion criteria of size and number may match the outcomes we have observed among incidental HCC. Indeed, five of the patients in group 2 exhibited tumor burdens that would be considered stage I or II (and therefore within the criteria for status IIB candidacy) by current UNOS staging criteria; all five of these patients were still alive without tumor at the time of follow-up.

It is important to note that patients in group 1 also demonstrated inferior survival outcomes when compared with patients in group 3, although not to the sharp extent seen in group 2. The finding of lower overall survival suggests that even the incidental diagnosis of HCC after OLTX could portend a negative prognosis with respect to post-transplantation survival. Given the fact that incidental tumors and preoperatively diagnosed tumors differed only in size but not in number, bilobar involvement, histologic grade, vascular invasion, and portal venous tumor thrombus formation, it is perhaps not surprising to find that these incidental tumors retained sufficient malignant potential to adversely affect patient survival. In fact, the survival data we observed for the group 1 population of tumors are very similar to survival results one would expect after transplanting preoperatively diagnosed tumors of similar nature. Mazzaferro et al.[12] demonstrated that 4-year overall patient survival was 85% among patients undergoing transplantation for HCC pathologically proven not to exceed specific tumor criteria (one lesion $\leq$5 cm or up to three lesions $\leq$3 cm each). In our study, 21 (77.8%) of 27 of the tumors found in group 1 did not exceed these same pathologic criteria, yielding a similar 4-year overall survival of 78.8%.

CONCLUSION

To our knowledge, no analyses to date have directly compared outcomes between OLTX patients with incidental HCC and OLTX patients with no HCC. In the present analysis, incidentally diagnosed HCC tumors were smaller, but otherwise not significantly different in terms of other oncologically relevant tumor characteristics from preoperatively diagnosed HCC tumors. Patients with incidentally diagnosed HCC demonstrated worse survival after transplantation compared with those recipients confirmed to be tumor free, although not to the extent observed among transplant recipients with known HCC. These observations suggest that even the incidental finding of HCC in native livers after OLTX portends a negative impact on ultimate patient survival. As such, this population of recipients may deserve special attention during follow-up for tumor recurrence. Fortunately the relatively

small tumors found in these incidentally diagnosed cases tended to be treated rather effectively with OLTX, and the decrement in patient survival as compared to tumor-free cohorts was small. Perhaps future adjuvant therapies that are found to confer survival benefit for transplant recipients with known HCC may be applicable for this subset of patients as well.

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Discussion

Dr. W.S. Helton (Chicago, Ill.). This is a very nicely presented study. Your results are in contrast to a very large body of literature that already exists on transplantation. We know that the currently used UNOS criteria for transplantation for known tumors, if they are adhered to, indicate that survival is no different from that in patients without tumor. We also know from the literature on the explanted liver that patients found to have "incidental" tumors have no difference in their survival compared to patients with known tumors prior to transplant. So it becomes a question of how you define incidental tumors. Can you tell us what the cutoff is for sensitivity at your institution using either CT or MRI detecting known tumors preoperatively, because maybe there is a big difference between what is found at your institution and what has been reported by others.

Dr. C.S. Cho. Those are obviously very important questions. First, with respect to sensitivity of pretransplantation imaging, I do not know what our particular numbers are, but I can tell you that there is a good body of evidence to suggest that, as good as CT scans, MRIs, and ultrasound images are, the sensitivity for picking up and accurately diagnosing HCC in the setting of cirrhosis or long-standing hepatic fibrosis is fairly poor.

There are some data, I agree, supporting the notion that if the established UNOS or so-called "Milan" criteria are strictly adhered to, the outcomes can be comparable to what would be seen in other cirrhotic tumor-free patients. I think the best example in the literature on that particular point is from Figueras et al.[22] in Barcelona, Spain. My reading of it is that if you actually look at their overall survival for the tumor-free cohort of patients, the rate is lower than what has been observed at other institutions, including our own, and so perhaps that may account for the difference we are able to see in the patients with incidental tumor.

Dr. S.M. Strasberg (St. Louis, Mo.). Congratulations on a well-presented study. My question is purely statistical. You are drawing comparisons between incidental and known HCCs. As an example, you showed in one table that

in the "known" group 44% of patients had tumor on both sides of the liver but in the "incidental" group the tumor was bilateral in only 22% of patients. It would seem that with such results one would expect to find a statistically significant difference and that the failure to find a statistical difference may be due to the small numbers in each group. Therefore, under such circumstances, one must draw conclusions with caution. What kind of power does this study have to make those kinds of discriminations?

Dr. Cho. I too was surprised that the results were not statistically significant, and I agree that that is likely attributable to the fact that there really was not sufficient power for that particular analysis. However, what makes me feel a little bit better about the observations that we made defining incidental tumors is the fact that the actual percentages, for instance, with respect to bilobar involvement or histologic grade, and so forth, are almost identical to the percentages from a very large registry reported by Klintmalm[30] in Dallas, Texas. So, based on the fact that our numbers are very similar to findings in that study, which were drawn from hundreds of patients, I think that our incidental tumors are very characteristic of what other investigators have observed.

PI-3′ Kinase and NF-κB Cross-Signaling in Human Pancreatic Cancer Cells

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Because tumor necrosis factor-alpha (TNF-α) and some chemotherapeutic agents activate both apoptosis and NF-κB–dependent antiapoptotic genes, they may neutralize their own antitumor effects. The cell-signaling mechanisms for such chemoresistance are not clear but may involve phosphotidylinositol-3′ kinase (PI3K). To clarify this we examined whether cross-signaling between PI3K and NF-κB enhances the antitumor effect of TNF-α in human pancreatic cancer cells. Quiescent pancreatic cancer cells (Panc-1, MiaPaCa-2) with TNF-α, Ly294002 (PI3K inhibitor), alone or combined, were restimulated with mitogen (10% fetal calf serum [FCS] to induce cell cycle entry). Proliferation (monotetrazolium), cell cycle progression (ApoBrDU and fluorescence-activated cell sorter analysis), and apoptosis (PARP cleavage; caspase-3 activation) were measured. Akt activation (Akt kinase assay) and IκBα degradation were determined by Western blot analysis. Translocation of NF-κB into the nucleus was examined by EMSA, whereas an NF-κB/luciferase reporter gene was used to quantify NF-κB–dependent gene expression. Statistical analysis was carried out by means of two-tailed *t* test ($P < 0.05$). PI3K inhibition significantly enhanced the antiproliferative and proapoptotic effects of TNF-α in both cell lines. Ly294002 also blocked TNF-α–induced Akt activation but failed to alter cytoplasmic IκBα degradation or subsequent NF-κB nuclear translocation. NF-κB–dependent gene expression, however, was ultimately suppressed by Ly294002, suggesting that PI3k-dependent activation of NF-κB is IκBα independent. PI3K inhibition can block NF-κB–dependent gene expression regardless of cytoplasmic IκBα/NF-κB activation. Because it also regulates the antitumor effects of TNF-α, PI3K may in part determine NF-κB–induced chemoresistance in human pancreatic cancer. (J GASTROINTEST SURG 2001;5:603-613.)

KEY WORDS: Akt, pancreatic cancer, PARP, apoptosis, IκBα, caspase-3

Tumor resistance to chemotherapy occurs commonly in human cancer. Such acquired or inducible chemoresistance usually develops by unknown mechanisms. Because most anticancer chemotherapeutic agents are discovered empirically, our understanding of the cellular mechanisms of their effect is often lacking.[1] Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine whose role in oncologic therapy remains unclear. TNF-α, a proinflammatory cytokine that mediates numerous host responses, stimulates cell signaling pathways that converge with activation of the transcription factor NF-κB and regulate apoptosis.[2-4] TNF-α has an antiproliferative effect on pancreatic cancer cells, but whether this is due primarily to apoptosis remains unknown. On activation, NF-κB is responsible for the transcription of antiapoptotic genes, which function to suppress cell death.

NF-κB refers to a group of binary complexes of proteins, most notably a p65/p50 heterodimer.[5] NF-κB is kept sequestered in the cytosol by its inhibitor, IκBα. On activation by TNF-α, IκBα undergoes phosphorylation, ubiquination, and subsequent degradation, allowing NF-κB to enter the nucleus.[2,6] Regulation of NF-κB, similar to other transcription factors, is controlled by signaling mechanisms that control its nuclear translocation (IκB) and through mechanisms that are responsible for upregulating the

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transactivation function of NF-κB. Activation of NF-κB by TNF-α is accompanied by increased phosphorylation of the p65/Rel A subunit.[7]

Phosphotidylinositol-3′ kinase (PI3K) has been implicated in signal transduction events regulating transcription factor activation.[8,9] It also regulates cell survival signals in response to growth factors, cytokines, and tumor oncogenes such as *Ras*.[10,11] PI3K consists of a catalytic subunit (p110) associated with a regulatory polypeptide (p85).[12] PI3K produces second messengers as 3′ phosphorylated lipid products, activating downstream kinases such as Akt and certain isoforms of protein kinase C.[13]

The induction of NF-κB and its antiapoptotic machinery may factor in pancreatic cancer chemoresistance. We have previously shown that TNF-α–induced apoptosis is significantly enhanced when pancreatic cancer cells are pretreated with proteasome inhibitors, which prevent the degradation of IκBα in the cytoplasm.[2,14] In addition to IκB, phosphorylation of NF-κB (relA/p65) has also been reported.[15] This phosphorylation appears to be critical for NF-κB function, but the kinases responsible and its overall significance have not been fully elucidated. Recent studies of interleukin-1 and TNF-α signaling pathways suggest that PI3K is central to the activation of NF-κB.[16-18] Today it remains unclear whether PI3K and its downstream effectors feed into a signal transduction cascade that leads to the activation of NF-κB in human pancreatic cancer cells (Fig. 1).

Our results indicate that PI3K factors in the activation of NF-κB in human pancreatic cancer stimulated by TNF-α. TNF-α stimulates the PI3K cascade in rapid fashion and its downstream target Akt. PI3K then activates a pathway that parallels but is separate from IκBα degradation. Specific PI3K inhibition with Ly294002 inhibits PI3K activation and NF-κB–dependent gene expression but has no effect on IκBα degradation, the nuclear translocation of NF-κB, or the ability of NF-κB to bind to DNA. PI3K plays some role in the antiapoptotic activities of NF-κB in response to TNF-α. Ly294002 strongly augments the proapoptotic and antiproliferative effects of TNF-α in human pancreatic cancer cells. Because it also regulates the antitumor effects of TNF-α, PI3K may in part determine NF-κB–induced chemoresistance in human pancreatic cancer.

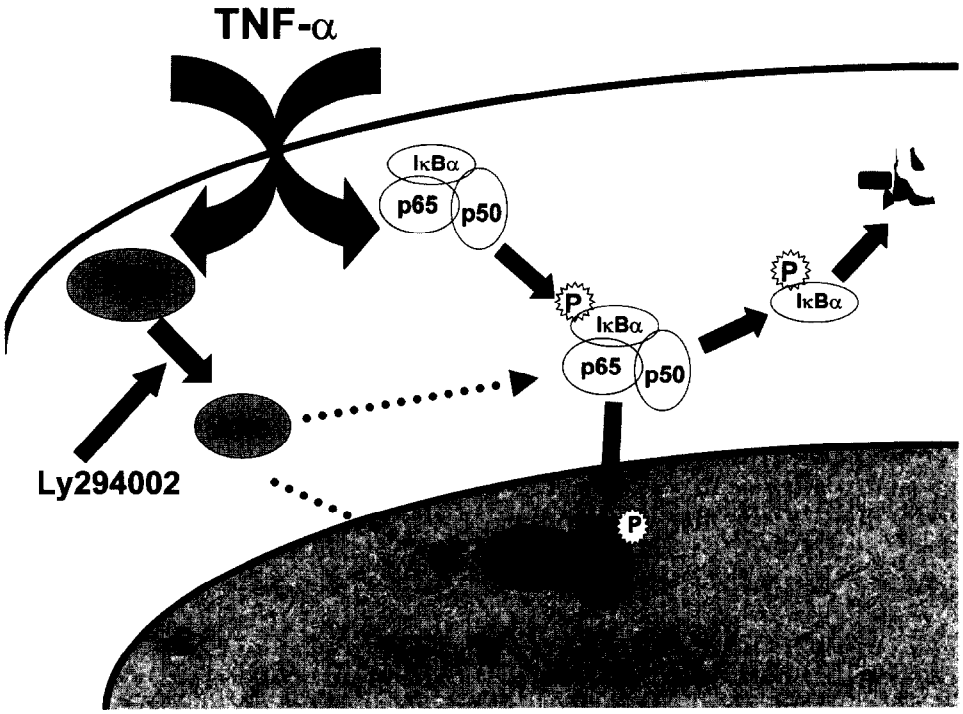

Fig. 1. TNF-α activates PI3K and NF-κB signal transduction pathways. A proposed schematic of the dual activation of PI3K and NF-κB in response to TNF-α. PI3K activates NF-κB in response to TNF-α, but the site and mechanism of action remain unclear. The downstream kinase Akt either factors in IκBα degradation or directly phosphorylates the p65 subunit of NF-κB.

MATERIAL AND METHODS

Cell Culture and Treatments

The MiaPaCa-2 and Panc-1 human pancreatic adenocarcinoma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, Va.). Cells were grown and propagated in Dulbecco modified Eagle medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum (FCS), 1% penicillin and streptomycin, and maintained at 37° C and 5% CO_2 atmosphere. Rapamycin and Ly294002 (Sigma, St. Louis, Mo.) were reconstituted in dimethylsulfoxide (Sigma) and used at concentrations of 20 ng/ml and 40 nmol/L, respectively. TNF-α (Promega, Madison, Wis.) was reconstituted in phosphate-buffered saline (PBS) with 5% FCS and a standard concentration of 20 ng/ml was employed. Following serum starvation for 60 hours to induce quiescence, cells were stimulated to proliferate by resupplementation with medium containing mitogen (10% FCS) alone or in combination with Ly294002 and TNF-α.

Proliferation Assay

Pancreatic cancer cell proliferation was determined by monotetrazolium (MTT) assay. Protocols followed similar previous MTT protocols used in our laboratory.[14]

Immunoblotting. Western immunoblot analysis was performed as previously described.[14] Immunoblots were analyzed using primary antibodies for phospho-Akt, Akt, IκBα, caspase-3 (New England BioLabs, Beverly, Mass.), and PARP (Upstate Biotechnology, Lake Placid, N.Y.).

Cell Cycle Progression and Apoptosis (ApoBrDU)

1×10^6 cells were plated in parallel in 25 cm^2 culture flasks. After serum starvation for 60 hours, quiescent cells were repleted with 10% FCS alone or in combination with Ly294002 and TNF-α. Cells were harvested by trypsinization, with care to include all floating cells, and then resuspended in PBS at a concentration of 2×10^6 cells/ml. The cell suspension was then placed in 1% (weight/volume) paraformaldehyde in PBS and placed on ice for 15 minutes. After washing two times with PBS, cells were then fixed with 70% ethanol and stored at −20° C for 24 hours. Cells were then stained with fluorescein–PRB-1 and treated with propidium iodide Rnase A according to the manufacturer's protocol (ApoBrDU, Phoenix Flow Systems, San Diego, Calif.). DNA histograms were obtained by fluorescence-activated cell sorting (FACS). Cell cycle analysis was performed using the Modfit program (Verity, Maine). Apoptosis was measured using a 488 mm argon laser as a light source, with propidium iodide (total cellular DNA) and fluorescin (apoptotic cells) as the two dyes. Flow cytometer data acquisition creates a gating display in which nonclumped cells and a second gated dual-parameter display is generated. By using the dual-parameter display method, apoptotic cells are resolved and their location in the cell cycle is also determined.

Akt Kinase Assay

1×10^6 cells were plated on a six-well plate and cells were treated under experimental conditions as described earlier. After rinsing cells with PBS, cells were lysed with cell lysis buffer (Cell Signaling Technology, Beverly, Mass.) and incubated on ice for 10 minutes. Cells were then centrifuged, the supernate was extracted, and Lowry assay (Bio-Rad Laboratories, Inc., Hercules, Calif.) was performed for equal loading. A monoclonal phosphoantibody to Akt was used to immunoprecipitate selectively phosphorylated Akt from cell lysates. The resulting immunoprecipitate was incubated with GSK-3α/β (Ser 21/9) fusion protein in the presence of adenosine triphosphate and kinase buffer. Active Akt, if present, will then phosphorylate GSK-3 at Ser21 (α) or Ser9 (β), major phosphorylation sites used by GSK-3 to confer GSK-3–dependent transcriptional activity. Phosphorylation of GSK-3 was then measured by immunoblotting and activity of Akt was determined.

Electromobility Shift Assay of NF-κB

Gel shift assays were performed as previously described.[14]

Transfection and Luciferase Reporter Assays

Subconfluent Panc-1 and MiaPaCa-2 cells were transiently transfected using Superfect reagent (Qiagen, Valencia, Calif.). Plasmid constructs for luciferase with NF-κB and other cDNA (2 μg of total DNA) as promoters (Promega, Madison, Wis.) were diluted in serum-free medium and mixed with Superfect reagent. Fifteen minutes was allotted to allow complexes to form, and then medium containing 1% FCS was added to the mixture. After washing cells with PBS, the Superfect-DNA complexes were added to the cells and incubated for 3 hours at 37° C and 5% CO_2. Cells were then washed with PBS, and fresh medium containing 1% FCS was added. Twenty-four hours later, cells were washed with PBS and lysed in

reporter lysis buffer (Promega, Madison, Wis.) for 20 minutes. Luciferase assays were performed on equal amounts of protein, using D-luciferin as a substrate. Relative light units were measured using an Analytical Luminescence Laboratories (ALL) 2001 technique.

Statistical Analysis

All experiments were performed in triplicate. Statistical significance was determined by two-tailed Student's t test ($P < 0.05$) or analysis of variance ($P < 0.05$) when indicated.

RESULTS

PI3K Inhibition Augments the Antiproliferative Effects of TNF-α in Human Pancreatic Cancer Cells

Cells were serum starved for 60 hours to induce quiescence (G_0-G_1 arrest) and then stimulated with 10% FCS, with or without TNF-α or Ly294002 or both. In the presence of 10% FCS alone at 24 hours, cells proliferate so that there are approximately 1.5-fold more cells in both cell lines. With escalating doses of Ly294002 in the presence of TNF-α, there is an associated decrease in proliferation (Fig. 2, *A*). Forty μmol/L of Ly294002 has the greatest synergistic effect with TNF-α, without inducing necrosis. Studies with Ly294002 alone have shown 40 μmol/L to be the therapeutic dose in blocking proliferation, but this effect required more than 40 hours.[19]

Forty μmol/L of Ly294002 enhanced the antiproliferative effects of TNF-α in both Panc-1 and MiaPaCa-2 cell lines by 24 hours. When Panc-1 cells were exposed to TNF-α alone, an 11% reduction in proliferation was seen compared to 10% FCS alone (Fig. 2, *B*). Pretreatment with Ly294002 and then exposure to TNF-α/Ly294002 in combination induced a 56% reduction in proliferation ($P < 0.05$). In MiaPaCa-2 cells, TNF-α/Ly294002 combination inhibited proliferation by 66% compared to 14% with TNF-α alone ($P < 0.05$). This effect was seen by 24 hours and continued for up to 72 hours. Similar experiments with vehicle control did not affect proliferation in either cell line.

To determine if Akt may be the kinase responsible for enhancing TNF-α cytotoxity in pancreatic cancer cells, parallel experiments were performed with rapamycin. Rapamycin is a selective inhibitor of FRAP/p70s6K, a downstream target of Akt and

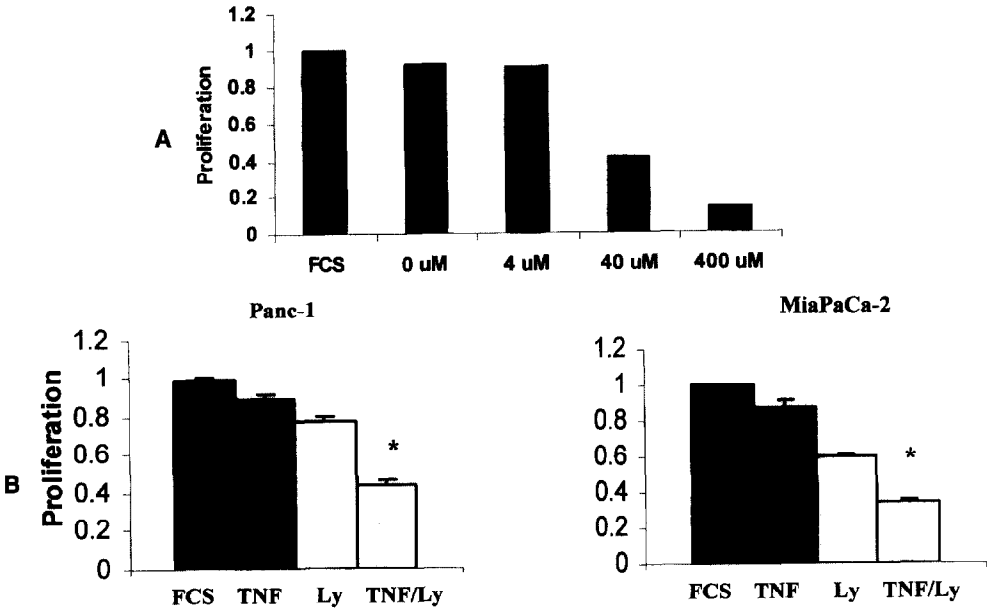

Fig. 2. PI3K inhibition enhances the antiproliferative effects of NF-κB. **A,** 2×10^6 human pancreatic cancer cells (Panc-1 shown) were serum starved for 60 hours to induce quiescence and then restimulated with 10% FCS, 20 ng/ml TNF-α, and varying doses of Ly294002. Twenty-four hours later, MTT assay was performed. 0 μmol/L and 4 μmol/L did not augment the antiproliferative effects of TNF-α, but 40 μmol/L Ly294002 had the greatest synergistic reaction with TNF-α without inducing necrosis. **B,** Quiescent cells allowed to proliferate with the addition of 10% FCS and TNF-α, 40 μmol/L Ly294002, or both for 24 hours. In both cell lines, Ly294002 enhanced the antiproliferative effects of TNF-α (Panc-1, 45%; MiaPaCa-2, 41%). Results are representative of three parallel experiments ($P < 0.05$; two-tailed t test).

PI3K.[20] Rapamycin did not augment the antiproliferative effect of TNF-α in either cell line (data not shown).

Ly294002 Augments the Apoptotic Effect of TNF-α

Treatment of human pancreatic cancer cells with Ly294002 in addition to TNF-α significantly enhanced apoptosis in a time-dependent fashion compared to either agent alone. Two early protein markers of apoptosis, PARP and caspase-3, were evaluated by Western immunoblot analysis. PARP cleavage (115kDa fragment ->87kDa fragment) was evident by 4 hours only when cells were treated with combination TNF-α/Ly294002 therapy (Fig. 3, *A*). TNF-α or Ly294002 alone did not induce cleavage by 4 hours. Caspase-3 cleavage (17kDa fragment) was also significantly enhanced only when Ly294004 was added to TNF-α (Fig. 3, *B*). PI3K inhibition appears to enhance the apoptotic effects of TNF-α in a synergistic manner in human pancreatic cancer cells.

ApoBrDU is a novel method of analyzing cell cycle changes during apoptosis. We used this test to determine if cells became arrested with PI3K inhibition before the induction of apoptosis. Cells were serum starved for 60 hours to induce quiescence, and then restimulated with 10% FCS to induce cell cycle entry with or without TNF-α, Ly29004, or both. Serum starvation caused an arrest of cellular proliferation, with a majority of cells in G_0-G_1 phase, as we have previously described.[14,19] Exposure to 10% FCS for 24 hours results in progression from G_1 to S phase (Fig. 4, *A*). When Ly294002 was added in addition to 10% FCS, this G_1 to S phase progression was significantly inhibited with most cells arrested in G_1 (Fig. 4, *B*). TNF-α alone did not halt cell cycle progression, but when Ly294002 was added cells tended toward G_1 arrest (Fig. 4, *C* and *D*). These findings confirm that PI3K inhibi-

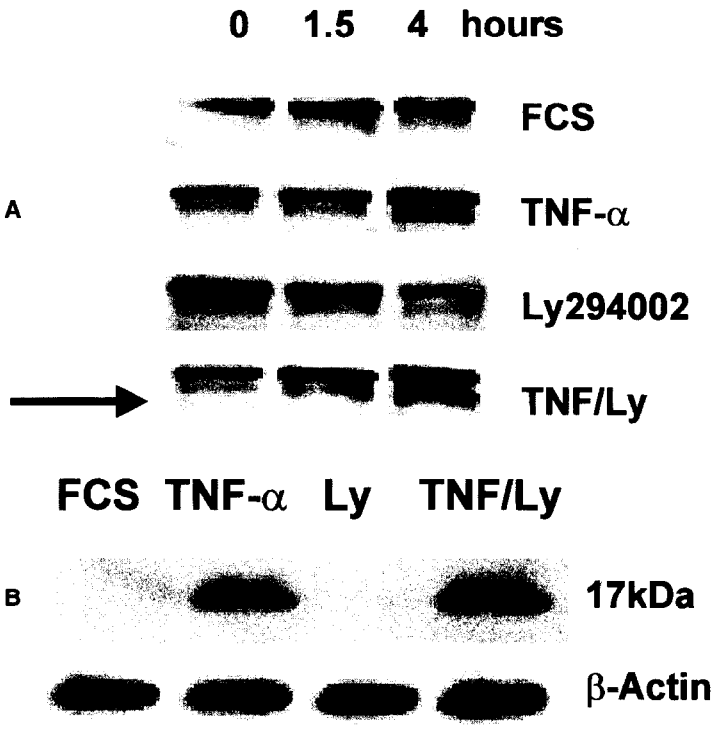

Fig. 3. Ly294002 augments TNF-α–induced apoptosis in human pancreatic cancer cells. 1×10^6 quiescent Panc-1 cells were restimulated with 10% FCS and TNF-α, Ly294002, or both for varying time points shown, cells were lysed, and Western blot analysis was performed. **A,** Representative Western blot of PARP cleavage. On induction of apoptosis, the 115 kDa fragment of PARP undergoes cleavage to an 87 kDa fragment *(arrows)*. TNF-α induces apoptosis only when cells are pretreated with Ly294002. Either treatment alone results in minimal or no evidence of the 87kDa fragment. **B,** Caspase-3 cleavage of the 17 kDa fragment is shown here after 4 hours of exposure to the various agents. The intensity of the 17 kDa band was greatly enhanced when cells were pretreated with Ly294002 and then exposed to TNF-α/Ly294002 in combination. Ly294002 alone did not induce apoptosis. Western blots are representative of three experiments. β-Actin is shown as a control and another measure of equal protein loading.

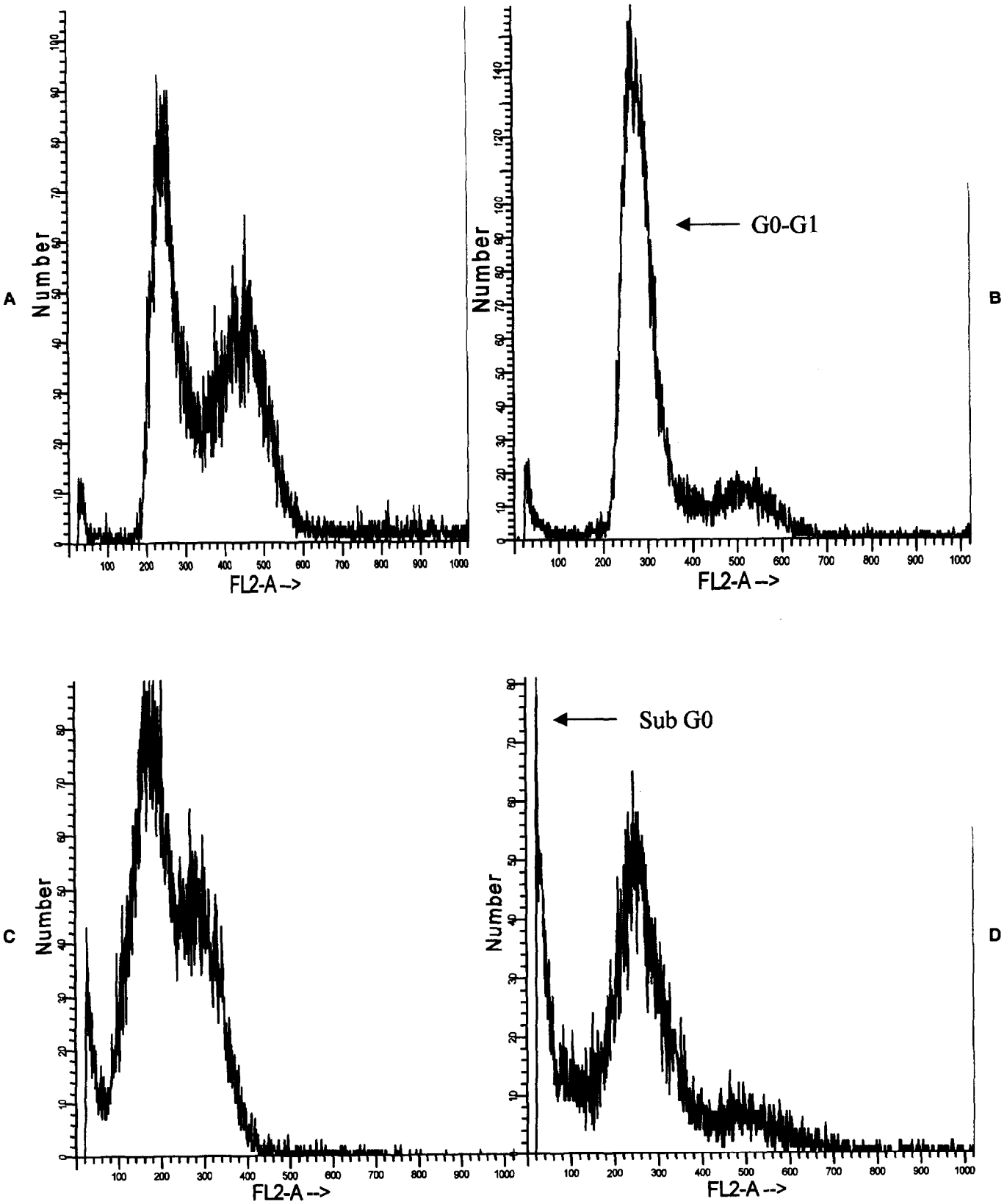

Fig. 4. Ly294002 induces a G_1 cell cycle arrest. ApoBrDU cell cycle analysis of the pancreatic cancer cells after various treatments is shown. Quiescent cells were restimulated with 10% FCS and either TNF-α, Ly294002, or both for 24 hours. **A,** Cells exposed to 10% FCS alone progress from G_0-G_1 to S phase. **B,** Stimulation with 10% FCS and Ly294002 induced a marked G_1 arrest, evident by the early peak. **C,** TNF-α did not appear to arrest cells, but **(D)** pretreatment with Ly294002 blocked cell entry into S phase.

tion induces G_1 arrest in human pancreatic cancer cells, when administered alone or in combination with TNF-α.

Propidium iodide and fluorescin analysis was used to analyze apoptotic changes in relation to cell cycle progression. Ly294002 arrested cells in G_1, before apoptosis induction. TNF-α or Ly294002 alone was not able to significantly induce apoptosis, but in combination cells were apoptotic when arrested at G_1.

TNF-α Stimulates PI3K and Akt Activity

We used the downstream target, Akt to assess PI3K activity by examining Akt phosphorylation and kinase activity with Western immunoblotting. We have previously shown that Akt is not constitutively phosphorylated in human pancreatic cancer cells.[14] TNF-α induced rapid phosphorylation of Akt by 15 minutes (Fig. 5). This effect was blocked with Ly294002 indicating specific PI3K pathway inhibition.

We then used an Akt kinase assay to confirm that changes in phosphorylation correlated with activity (data not shown). When cells were exposed to 10% FCS alone, there was minimal Akt activity. TNF-α induced significant activation of Akt, but this was blocked with Ly294002. To assess the specificity of Ly294002, we used rapamycin in combination with TNF-α to compare changes in activity. Rapamycin, a specific inhibitor of downstream FRAP-p70s6K, had no effect on Akt activity. This implies that Akt may be involved with cross-signaling between PI3K and NF-κB signal transduction pathways.

PI3K Enhances TNF-α–Induced NF-κB Function and Activity

TNF-α induced IκBα degradation by 5 minutes (Fig. 6). The addition of Ly294002 did not affect cytoplasmic degradation of IκBα. Ly294002 alone had no effect on degradation.

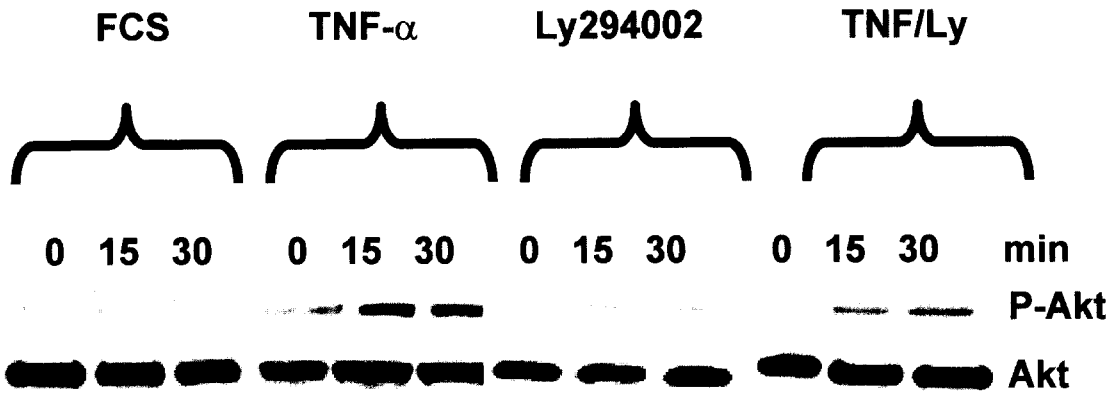

Fig. 5. TNF-α induces Akt phosphorylation. Quiescent 1×10^6 human pancreatic cancer cells were treated with 10% FCS with or without TNF-α, Ly294002, or both for the 30-minute time course and then harvested for Western immunoblot analysis. TNF-α stimulated Akt phosphorylation by 15 minutes, but this was blocked with Ly294002 pretreatment. Ly294002 alone had no effect on Akt phosphorylation confirming specificity for Akt. Representative Western blot of three parallel experiments.

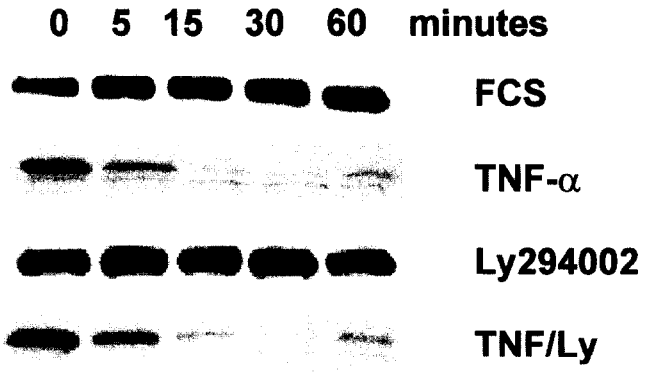

Fig. 6. PI3K does not affect TNF-α–induced IκBα degradation. TNF-α induced rapid degradation of IκBα in the cytoplasm, regardless of Ly294002 pretreatment. Ly294002 alone had no effect on IκBα degradation. Western blot is representative of three different experiments in both cell lines.

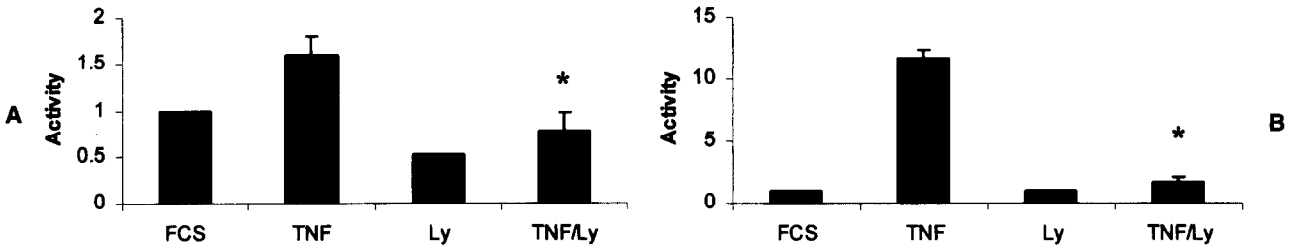

Fig. 7. NF-κB–dependent gene expression is stimulated by TNF-α and inhibited by Ly294002 in human pancreatic cancer cells. 1×10^6 human pancreatic cancer cells were plated and then serum starved for 24 hours. Cells were then transfected with NF-κB cDNA using the Superfect reagent. Cells were appropriately treated and 24 hours later cells were harvested and analyzed using the luciferase reporter system. Data are expressed as fold induction, the ratio between the expression level in the experimental group and the level in the untreated control cells. Statistical significance was determined by means of two-tailed *t* test ($P < 0.05$). **A,** In Panc-1 cells, TNF-α stimulated NF-κB gene activity 1.6-fold compared to 10% FCS alone. Pretreatment with Ly294002 blocked TNF-α–induced stimulation by 50%. Treatment with Ly294002 did not induce NF-κB–dependent gene activity. **B,** TNF-α augmented NF-κB–dependent gene activity 11.7-fold compared to 10% FCS in MiaPaCa-2 cells. Pretreatment with PI3K inhibition blocked TNF-α stimulation back to basal levels (86% reduction). Results are representative of three different experiments.

To identify the role of PI3K in TNF-α signaling, we tested the effect of Ly294002 on the DNA-binding activity of NF-κB. The ability of TNF-α to rapidly induce the degradation of cytoplasmic inhibitor IκBα is a critical step before the transcription factor can be translocated to the nucleus. TNF-α increases the DNA binding of NF-κB in the nucleus by EMSA with peak stimulation at 2 hours (data not shown). PI3K inhibition had no effect on this DNA-binding activity. This implies PI3K is not involved in the signaling pathway leading to the degradation of IκBα and subsequent activation of NF-κB but must activate some parallel pathway.

IκBα alone does not fully account for the functional activation of NF-κB–dependent genes.[15] An NF-κB luciferase reporter gene was used to examine the NF-κB–dependent gene transcription. Cells were treated with TNF-α, Ly294002, or both for 4 hours, and then gene activity was measured with the luciferase assay system. Ly294002 blocked TNF-α–mediated induction of NF-κB reporter gene in both cell lines (Fig. 7). In Panc-1 cells, Ly294002 blocked 50% of TNF-α–induced gene activity, but in MiaPaCa-2 cells the effect was much more profound, with an 86% reduction in NF-κB reporter gene activation. This effect of PI3K inhibition suggests that NF-κB is regulated at different steps through parallel signaling pathways in pancreatic cancer cells, as has been previously described in HepG2 hepatoma cells.[16] One pathway leads to the degradation of IκBα and the other may lead to an increase in the transactivation potential of NF-κB.

DISCUSSION

We have examined the involvement of the PI3K signal transduction pathway in NF-κB–induced chemoresistance in human pancreatic cancer. TNF-α stimulates both the PI3K and NF-κB signaling cascades. Inhibition of PI3K enhances the antiproliferative and proapoptotic effects of TNF-α in human pancreatic cancer cells. Specific PI3K inhibition with Ly294002 strongly inhibited both PI3K activation and NF-κB dependent gene expression but had no effect on TNF-α–induced degradation of IκBα in the cytoplasm or the subsequent nuclear translocation of NF-κB.

Apoptosis is a natural mechanism for cell death and plays a major role in regulating tumor growth. Enhancing apoptosis can lead to tumor regression. Ionizing radiation, certain chemotherapeutic agents, and TNF-α can induce apoptosis, but their clinical efficacy is variable and side effects are significant.[21] These agents also simultaneously activate an NF-κB salvage pathway, which limits apoptosis. Wang et al.[3] reported that NF-κB activation by TNF-α, radiation, and daunorubicin inhibited apoptosis otherwise induced by these stimuli in fibrosarcoma cells. A cell survival response occurs through the induction of antiapoptotic genes such as IAP-1 and IAP-2.[4] Such resistance to apoptosis is a principle mechanism by which cancer cells escape death and grow unregulated.[22]

Cell survival signals stimulated by growth factors, cytokines, and oncoproteins are initiated by PI3K- and Akt-dependent signal transduction pathways. Finco and Baldwin[23] found that oncogenic *Ras* stimu-

lates NF-κB–dependent transcription and that NF-κB is required for *Ras*-mediated transformation.[24] Madrid et al.[25] demonstrated that oncogenic H-Ras (V12) stimulated NF-κB–dependent transcription in a PI3K- and Akt-dependent manner.[25] PI3K has been reported to be involved in the activation of NF-κB in various cell types in response to TNF-α, interleukin-1β, pervanadate, and platelet-derived growth factor (PDGF) signaling.[16-18,26-29]

The specific signaling mechanisms implicating PI3K in NF-κB activation remain unclear. Some studies have shown that both PDGF- and TNF-mediated NF-κB activation involve Akt and its interaction with IκBs.[28,29] Ozes et al.[28] reported that the PI3K inhibitor wortmannin, a dominant-negative p85 PI3K mutant, and a kinase-deficient Akt kinase are all able to abolish TNF-induced activation of NF-κB. Reddy et al.[17] postulated that PI3K may be involved in a step or a series of steps that regulate the transactivation potential of NF-κB but not its DNA binding. These investigators found that although PI3K participated in modulating increases in the transactivation potential of NF-κB, it appeared not to be involved in TNF-α–induced IκB-α degradation in HepG2 cells. Sizemore et al.[18] reported PI3K inhibition had no effect on interleukin-1–stimulated IκB-α degradation, nuclear translocation of NF-κB, or DNA binding of NF-κB in HepG2 cells. Finally, Bergmann et al.[30] found that inhibitors of phosphatidylcholine-specific phospholipase C and protein kinase C blocked interleukin-1– and TNF-α–induced NF-κB gene expression without affecting cytokine-induced IκB degradation or nuclear translocation or DNA binding of NF-κB.

In our studies, inhibition of TNF-α–stimulated PI3K activity by pretreatment with Ly294002 caused a dramatic reduction in NF-κB–dependent gene expression without affecting IκBα degradation or the nuclear translocation of NF-κB in human pancreatic cancer cells. These findings are consistent with results realized in other cancer models.[17,18] Differences in the luciferase reporter assay among cell lines may be due to transfection efficiency. The ultimate role of IκB in PI3K–NF-κB signaling may center on individual cell-type characteristics (malignant vs. nonmalignant), PKCζ,[31] MEKK1,[32] and ubiquitin proteasome degradation. We are currently exploring some of these pathways to determine their significance.

Our findings also show that inhibition of PI3K enhanced the apoptotic and antiproliferative effects of TNF-α. By examining protein markers of apoptosis and fluorescin staining of cells, we confirmed that decreases in proliferation are due to apoptosis. This implies that PI3K signaling may stimulate NF-κB to generate antiapoptotic genes as a mechanism of inducible chemoresistance. We are now examining mRNA levels of some antiapoptotic genes to determine if their upregulation correlates with changes in proliferation. Ly294002 is the most specific PI3K inhibitor but may also affect other kinases. More specific experiments involving Akt transfection will further clarify the role of PI3K in NF-κB activation. We used wortmannin, a nonspecific inhibitor of PI3K, and found similar results in both cell lines.

These studies provide further insight into the role of PI3K in the activation of NF-κB in human pancreatic cancer cells. We provide the first report of cross talk between these pathways in regard to chemoresistance in human pancreatic cancer. Identifying mechanisms of NF-κB–induced chemoresistance will provide future targets for drug therapy in curing this lethal malignancy.

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Discussion

Dr. K. Behrns (Chapel Hill, N.C.). This work continues the focus of your laboratory studies determining the mechanisms of chemoresistance in pancreatic cancer; this is very important for development of drugs to treat this cancer, which has been fairly resistant to surgery and other types of treatment. My first question for you is, did you determine the PTEN status of these cell lines? PTEN is a tumor suppressor, it is a known inhibitor of Akt, and this might further dissect the PI-3 kinase pathway and allow you to determine whether it is the Akt arm or another downstream arm of the PI-3 kinase pathway.

Dr. S. Shah. Our main studies with PTEN have involved protein analysis in the Panc-1 cells, and we found that PTEN has been expressed just at the protein level, even with exposure to some of these agents done in parallel. The protein expression of PTEN probably is not a valid way to determine what its activity is in these cell lines, and probably stable transfections with knockouts of PTEN would be a better way to determine whether Akt is indeed responsible or if it is upstream or downstream. We have performed parallel experiments with rapamycin, which is an inhibitor of FRAP/mTOR, which is downstream from Akt. I did not present these data in this report, but there was no effect on proliferation, apoptosis, or any of the cell-signaling pathways shown here.

Dr. Behrns. My second question relates to the mechanism of cell death. It may be primary apoptosis or it could be secondary apoptosis. Your early PARP cleavage at 4 hours along with the caspase-3 cleavage at 4 hours would suggest that you have primary apoptosis, but the G_1 cell cycle arrest at 24 hours suggests that you may have cell cycle arrest followed by apoptosis. Could you clarify that?

Dr. Shah. I agree with you; it is probably parallel signaling pathways. The goal of this study was mainly to evaluate the cell-signaling mechanisms versus what was going on in terms of the cell cycle inhibitors and the cyclins. A

really good way to analyze what is occurring with cell cycle changes would be to analyze the protein level of cyclins and the cyclin-dependent kinase inhibitors and see whether they are expressed or not, and we have not yet done that.

Dr. Behrns. My final question relates to the use of a chemotherapeutic agent. Have you performed these studies with the addition of a chemotherapeutic agent, because what we really want to know is how do these cells and these signaling pathways respond when a chemotherapeutic agent is added? We know that PI-3 kinase has multiple effects; it can induce the proapoptotic protein Bad, and it can also inhibit caspase-9. Yesterday a paper was presented on upregulation of insulin-like growth factor 1 receptor. So there are multiple pathways that could be greatly influenced by chemotherapeutic treatment. Have you performed any of these studies with the addition of a chemotherapeutic agent?

Dr. Shah. That is the obvious next step. We have previously shown that CPT-11 activates NF-κB through the degradation of IκB-alpha. So we need to perform these experiments and see if CPT-11 would be a suitable candidate that would activate PI-3 kinase and, therefore, if we could knock out both arms. That would be a good avenue, but we have not fully investigated an entire chemotherapeutic line to see which agents activate both pathways. We know that certain agents, gemcitabine, CPT-11, and adriamycin, in particular, activate NF-κB, but we do not fully know which chemotherapeutic agents activate PI-3 kinase. If you could knock out both arms at this level, you might achieve greater cell death.

Dr. V. Fink, M.D. (Chicago, Ill.). Regarding your compounds, do you have inhibitors to these compounds? Have they effected an increase in apoptosis, and did you have any evidence from your work, in animals or such, that it in any way decreased the incidence and the development of pancreatic cancer or that it really has changed the sensitivity of the cancer to chemotherapy?

Dr. Shah. The one problem with Ly294002 and wortmannin, both of which are PI-3 kinase inhibitors, is that they are not soluble or really bioavailable in the body. In terms of animal studies, we need to develop better pharmacologic agents that will work in vivo. Wortmannin degrades in 24 hours and Ly actually is not soluble unless it is dissolved in dimethylsulfoxide. So those compounds would not necessarily be adequate right now for use in animal studies. The only known agent that we have that could work in the system would be farnesyltransferase inhibitors, which affect *Ras*, which is far upstream from PI-3 kinase, but that is the closest thing we have right now.

In terms of inhibitors to these agents, essentially the next step in our laboratory will be to perform knockout studies of Akt and PI-3 kinase and see if there is any effect. In terms of inhibitors to the inhibitors, I do not know of any.

Incidence of *Helicobacter pylori* in Operatively Managed Acute Nonvariceal Upper Gastrointestinal Bleeding

Christopher S. Callicutt, M.D., Stephen W. Behrman, M.D.

Helicobacter pylori is a known contributor to ulcerogenesis and nonvariceal acute upper gastrointestinal hemorrhage. Its incidence in operatively managed patients with upper gastrointestinal hemorrhage is ill defined. Patients undergoing surgery for upper gastrointestinal hemorrhage secondary to gastroduodenal ulceration between 1993 and 1998 at the University of Tennessee were retrospectively reviewed. Factors examined included age, nonsteroidal drug use, endoscopic intervention, urgency of operation, and *H. pylori* status confirmed by histologic examination. Forty-two patients had surgery with three excluded because of a lack of histologic evaluation. The site of bleeding was gastric in 23 and duodenal in 14. *H. pylori* infection was present in nine (39.1%) gastric and 11 (68.7%) duodenal ulcers. The incidence of *H. pylori* infection was reduced in those over 60 years of age (28.6%). Endoscopy was performed in all patients, but only two had biopsies for assessment of *H. pylori*. Operative morbidity was 17.9% and mortality was 5.1%. No patient had rebleeding following surgery. The incidence of *H. pylori* in this population is less than that reported in uncomplicated ulcer disease. Those older than 60 tended to be *H. pylori* negative. Endoscopic assessment for *H. pylori* was infrequent. Traditional indications for surgical intervention in ulcer hemorrhage should not be altered based on *H. pylori* status. (J GASTROINTEST SURG 2001;5:614-619.)

KEY WORDS: Peptic ulcer, hemorrhage, *Helicobacter pylori*, surgery

The incidence of gastroduodenal bleeding secondary to peptic ulcer disease has not changed dramatically in the past two decades and the number of hospital admissions for hemorrhage has remained stable.[1-3] Further, despite the introduction of antacid therapy and therapeutic endoscopy, the incidence of operations for peptic ulcer bleeding has remained constant ranging from 10% to 20% of all patients hospitalized for upper gastrointestinal hemorrhage.[4,5]

Helicobacter pylori is a known cause of ulcer disease and nonvariceal acute upper gastrointestinal hemorrhage.[6] Despite the improved diagnostic and treatment protocols for *H. pylori*, its prevalence in operatively managed patients with upper gastrointestinal hemorrhage is ill defined. Data regarding its incidence in those with massive upper gastrointestinal bleeding are sparse. Prior reports in those with only modest hemorrhage demonstrated a reduction in recurrent bleeding if *H. pylori* was eradicated.[7,8] Traditionally, surgery has been indicated for those with massive bleeding (>6 units), especially if stigmata of rebleeding are present on upper endoscopy. Aggressive early assessment for *H. pylori* and treatment directed at this pathogen could potentially alleviate the need for emergency surgery if it is present in the majority. However, the magnitude of bleeding may not allow expectant management, and if *H. pylori* is not present, perhaps early surgical referral should be recommended especially in those at high risk for rebleeding.

The purpose of this study was to define the incidence of *H. pylori* in a cohort of patients operated on for massive nonvariceal upper gastrointestinal hemorrhage secondary to gastroduodenal ulcer disease. Its role in the overall treatment algorithm of these patients, as well as surgical outcome, was assessed in hopes of further defining management of this critically ill patient population.

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METHODS

Records of all patients undergoing surgery for upper gastrointestinal hemorrhage secondary to peptic ulcer disease from 1993 to 1998 were retrospectively reviewed. Demographics, risk factors for peptic ulcer disease, ulcer location, and prior ulcer treatment including therapy for *H. pylori* were recorded. Endoscopic findings, assessment for the presence or absence of *H. pylori*, and the need for therapeutic intervention were noted. Perioperative transfusion requirements, surgical procedure, and outcome were examined. The urgency of surgical intervention was defined as follows: emergent, semiurgent (within 24 to 48 hours of admission following stabilization), and elective (>48 hours after admission).

H. pylori infection was documented by Steiner stain from either intraoperative biopsy in those without resection or, in the majority, pathologic analysis of antrectomy specimens. Patient data were accumulated from the University of Tennessee, Memphis, affiliated hospitals. As such, information was obtained from three distinct patient populations: indigent, Veterans Affairs, and two large private institutions. Where appropriate, data were compared using chi-square analysis with significance assessed at the ninety-fifth percentile.

Table I. Distribution of ulcer location, age, and *H. pylori* status

	HP+ (%)	HP−
All patients (n = 39)	20 (51.2)	19
Gastric ulcer (n = 23)	9 (39.1)	14
Duodenal ulcer (n = 16)	11 (68.7)	5
Age >60 years (n = 14)	4 (28.6)	10
Gastric (n = 8)	1 (12.5)	7
Duodenal (n = 6)	3 (50)	3

HP = *Helicobacter pylori*.

RESULTS

During the 5-year period, 42 patients underwent surgery for nonvariceal upper gastrointestinal hemorrhage. Three patients undergoing vagotomy and pyloroplasty had neither preoperative, intraoperative, nor postoperative biopsy for *H. pylori* and were excluded from analysis. Of the 39 evaluable patients, 30 were men and nine were women; mean age was 55 years. Twenty-three had gastric ulcers and 16 had duodenal ulcers. All gastric ulcers were prepyloric. Overall, *H. pylori* was present in only 51.2% (20 of 39 patients). Nine (39.1%) of twenty-three gastric and 11 (68.7%) of 16 duodenal ulcers were *H. pylori* positive. Fourteen patients were over the age of 60—eight with gastric and six with duodenal ulcers. *H. pylori* was present in only four (28.6%)—one (12.5%) of eight with gastric and three (50%) of six with duodenal ulcers (Table I).

Risk factors for upper gastrointestinal hemorrhage are shown in Fig. 1. There were no significant differences between those who were *H. pylori* positive vs. negative with respect to the variables analyzed except for the use of ethanol, which was more common in those with *H. pylori*. Only 17 patients (44%) had a history of nonsteroidal drug use prior to hemorrhage. Nonsteroidal drug use was no more common in those who were *H. pylori* negative than in those who were *H. pylori* positive regardless of whether they were

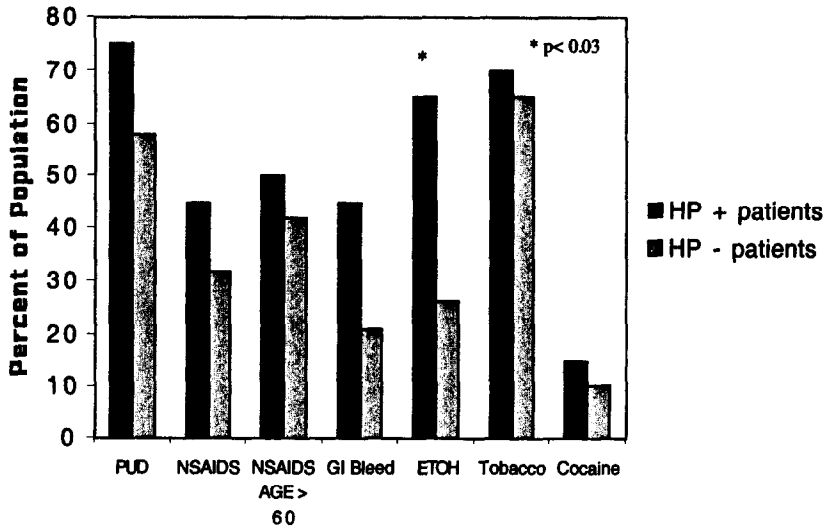

Fig. 1. Risk factors for upper gastrointestinal hemorrhage.

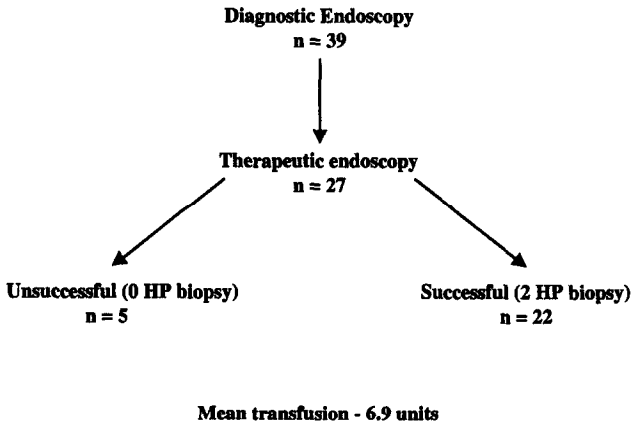

Fig. 2. Endoscopic management and transfusion requirements.

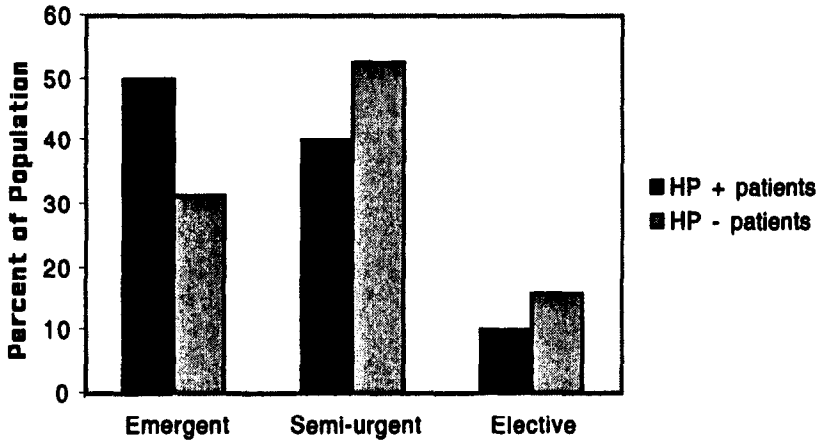

Fig. 3. Urgency of operation and *H. pylori* status.

older or younger than 60. Stated another way, nonsteroidal drug use was not proportionately higher in those who did not have *H. pylori* infection. Thirty (77%) had a history of ulcer disease and 11 (28%) had a history of gastrointestinal hemorrhage. Although a significant number of patients had a history of peptic ulcer disease, only four patients had been taking antacids long term at the time of presentation. No patient had previously been treated for *H. pylori*.

All patients underwent diagnostic endoscopy at presentation (Fig. 2). Diagnostic and therapeutic endoscopy was performed by the medical gastroenterology service. Twenty-seven (69.2%) of 39 patients were treated therapeutically with epinephrine injection and/or heater probe application. Forty-eight percent (13 of 27 patients) were *H. pylori* positive. Therapeutic endoscopy was unsuccessful in stopping the bleeding in five patients—three who were *H. pylori* negative and two who were *H. pylori* positive (20% failure rate). Of all patients undergoing emergent endoscopy, only two (7.4%) had biopsies performed to assess for the presence of *H. pylori*. Both patients were *H. pylori* positive. Only one received treatment but ultimately had surgery for early rebleeding. The second patient was taken directly to surgery before biopsy results were obtained because of large-volume transfusion requirements (6 units). For the group as a whole, the mean packed red cell transfusion requirement was 6.9 units indicating the magnitude of hemorrhage.

Nineteen patients required emergency surgery, whereas 18 had semiurgent surgery and two had elective surgery. Except for the two elective cases, the decision to operate on those with controlled bleeding was based on a combination of large-volume transfusion requirements, age, underlying comorbid disease processes, and endoscopic stigmata indicating a high risk of rebleeding. A similar percentage of patients had surgery emergently or semiurgently regardless of *H. pylori* status, indicating that even if active *H. pylori* infection had been identified at the index endoscopy, the clinical course of these patients necessitated an operation rather than potential medical management (Fig. 3). Vagotomy

Table II. Surgical procedures (N = 39)

	Total	Gastric	Duodenal
Vagotomy/antrectomy Billroth I	24	15	9
Vagotomy/antrectomy Billroth II	9	6	3
Vagotomy/pyloroplasty	4	1	3
Highly selective vagotomy (oversew ulcer)	2	1	1

Table III. Morbidity and mortality

Morbidity	7 (17.9%)
Delayed gastric emptying	2
Pneumonia	2
Cerebral vascular accident	2
Anastomotic leak	1
Mortality	2 (5.1%)

and antrectomy were performed in the majority (Table II). No patient had rebleeding following surgery. Overall operative morbidity was 17.9% and mortality was 5.1% (Table III). One patient (73 years of age) died as a result of prolonged ventilatory management and progressive organ failure. A second patient suffered a loss of airway in the immediate postoperative period and died of hypoxic encephalopathy.

DISCUSSION

Bleeding remains the most common indication for hospitalization and surgery in those with peptic ulcer disease. Although hospital admissions for bleeding duodenal ulcers have remained stable, rates for gastric ulceration have risen dramatically.[2] Despite improvements in the medical and surgical care of these patients, mortality rates have not changed and remain at approximately 10%. This is likely due to an increasing proportion of patients over the age of 60, many with concurrent comorbid disease processes.[9] The need for surgical intervention in this patient cohort remains important. In one large prospective national survey by the American Society for Gastrointestinal Endoscopists, 347 (15.6%) of 2225 patients with bleeding gastroduodenal ulcers required surgical intervention with approximately 70% of these being emergency procedures.[10] Thus surgery is most frequently necessary in the acute setting, usually within 48 hours of initial bleeding. *H. pylori* has been strongly associated with and implicated in the etiology of uncomplicated ulcers and those with mild degrees of bleeding. Treatment of active *H. pylori* infection in these populations prevents recurrent bleeding, markedly improves ulcer healing, reduces long-term antacid use, and avoids the need for surgical intervention. However, the incidence of *H. pylori* in a surgical cohort undergoing surgery for massive bleeding has yet to be ascertained. In addition, the potential role of *H. pylori* treatment in this population and its impact in avoiding the need for surgery is not known.

H. pylori infection is common in those with uncomplicated duodenal, and to a lesser extent gastric, ulceration. Graham et al.,[6] in a randomized prospective study, demonstrated that eradication of *H. pylori* in those with nonbleeding, healed gastroduodenal ulcers resulted in significantly reduced ulcer recurrence rates.[6] Active *H. pylori* infection was documented by ^{13}C-urea breath analysis, anti-*Helicobacter* antibody titers, culture, and histologic evaluation. With the exception of breath analysis, which is not universally available, documentation of active *H. pylori* infection is not readily accomplished. Given that surgical intervention is most often necessary on an emergent basis, before *H. pylori* analysis is possible, the diagnostic and potential therapeutic eradication of *H. pylori* in this cohort remains speculative. Only two studies have examined the therapeutic eradication of *H. pylori* in those with modest gastrointestinal hemorrhage. Both Rokkas et al.[7] and Jaspersen et al.[8] noted a statistically significant reduction in ulcer recurrence following treatment of *H. pylori* versus a group receiving antacids alone. In both studies it is not known what percentage of patients presenting with gastrointestinal hemorrhage were *H. pylori* positive. Only 5 of 51 patients in the study by Jaspersen et al.[8] required blood transfusion, and those with actively bleeding ulcers were excluded from analysis. Transfusion requirements averaged only two units in the study by Rokkas et al.,[7] and it is unclear whether any required therapeutic endoscopy. In contrast, fully 70% of our patients required therapeutic endoscopy at initial surveillance and our cohort required a mean of nearly seven units of blood indicating the magnitude of bleeding. Thus this represents a markedly different patient population from those previously described.

Our data suggest that in those undergoing urgent operations for significant bleeding, *H. pylori* infection is less common than in other populations and infectivity diminishes with age. This is in contrast to most epidemiologic studies, which demonstrates that *H. pylori* infection is more common with increasing age.[11] Excessive use of nonsteroidal anti-inflammatory drugs

was not seen in this group, which suggests that a different pathophysiology accounted for the advanced magnitude of bleeding. Similar observations were noted by Fischer et al.[12] in 28 patients with a mean age of 59 years and giant duodenal ulceration—a population that frequently requires surgical intervention. Twenty-three of these patients had analyses for *H. pylori* infection (rapid urease [CLO] test in 20; serum in 3) for an overall infection rate of 39.1%. This is far lower than that reported for uncomplicated ulcer disease. Tokunaga et al.[13] noted an infection rate of only 55% in 44 patients having an operation for gastroduodenal hemorrhagic ulceration—a rate nearly identical to our findings.[13] Surveillance for *H. pylori* was rarely undertaken in our cohort with only 5% of our study population (2 of 39) having a biopsy performed. In most cases, blood in the stomach, the urgent need for control of active bleeding, and an unstable patient seemed to be factors that precluded *H. pylori* analysis. In the present study, results of both biopsies for *H. pylori* were positive. However, both patients underwent surgery before treatment was instituted—one patient emergently and the other semiurgently for early rebleeding. Thus it is unlikely that in the face of significant bleeding, therapy for *H. pylori* would reduce the need for surgical intervention. Combining data from this study, as well as those from Fischer et al.,[12] and Tokunaga et al.[13] which suggest that *H. pylori* infectivity is less common in high-risk patients, delay in surgical therapy for potential *H. pylori* analysis and treatment cannot be recommended for those with massive bleeding.

The operative procedures used were at the discretion of the attending surgeon and consisted of vagotomy and antrectomy in most patients. Other options include vagotomy, pyloroplasty, and oversewing of the ulcer, vessel ligation with proximal gastric vagotomy, antrectomy only for gastric ulceration, or vessel ligation alone. Vagotomy and pyloroplasty in particular are often performed as they avoid the need for a resection and anastomosis and can be quickly accomplished. However, vagotomy alone carries a known higher rate of ulcer recurrence when compared with concurrent antrectomy. Ohmann et al.,[1] using data extrapolated from uncomplicated ulcers, argued that a less radical operative procedure is indicated because of the presumed high incidence of *H. pylori*, which could easily be treated postoperatively with medication. Given the relatively low incidence of *H. pylori* in this study and others, such an approach would leave nearly one half or more of all patients at risk for rebleeding. Nearly half of our patients required emergency surgery for uncontrolled bleeding. Eighteen patients had semiurgent surgery because of a combination of factors including large-volume transfusion, high-risk stigmata for early rebleeding by upper endoscopy, and underlying comorbid illness. Prior data have demonstrated a reduction in mortality rates with this approach.[14,15] Only two had "elective" surgery. An equal percentage of patients in each group were *H. pylori* positive, suggesting that this variable played a minor role with respect to urgency of operation. In nearly all instances, clinical factors mandated early operation. Morbidity and mortality rates were quite acceptable following surgery, and no patient had rebleeding following operative intervention.

The present study represents further analysis of *H. pylori* infection in a population of patients undergoing surgery for massive bleeding secondary to gastroduodenal ulcers. Inclusive of data from prior work, the incidence of *H. pylori* infection in a surgical population for gastrointestinal bleeding is much less common than in those with uncomplicated disease. We agree with Fischer et al.[12] that the etiology of ulcer disease in this population represents a different pathophysiology. It may, in fact, be based on acid hypersecretion inasmuch as the use of nonsteroidal anti-inflammatory drugs was not found to be extraordinarily high. Most often, surgical intervention is required on an emergency basis before analysis for *H. pylori* infection is possible. Even if the diagnosis of active infection is readily available, delay of surgery for potential medical therapy would be difficult to justify in these patients with significant transfusion requirements. Given the relatively low incidence of *H. pylori* in this patient population, a more aggressive antiulcer procedure should be considered. On the basis of current data, traditional methods of management and indications for surgical intervention in upper gastrointestinal hemorrhage for gastroduodenal ulceration should continue to be used.

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Esophagectomy for Adenocarcinoma in Patients 45 Years of Age and Younger

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Esophageal adenocarcinoma in patients 45 years of age or younger is uncommon. We reviewed our experience with the surgical management of these patients to determine their clinical characteristics, pathologic findings, and treatment results. Thirty-two patients were identified through our surgical pathology database, and their medical records were reviewed to determine clinical characteristics, treatment, treatment-associated mortality, tumor staging, presence of Barrett's mucosa, and survival. In our series, patients were white (100%) males (96.9%) with a history of reflux (56.3%), cigarette smoking (40.6%), and alcohol consumption (59.4%), who presented with progressive solid food dysphagia (78.1%). A prior diagnosis of Barrett's mucosa or use of antireflux medications was noted in five patients each (15.6%). There were no operative deaths. Actuarial survival was 81.1% (95% confidence interval [CI] 66.1 to 96.2) at 12 months, 68.5% (95% CI 49.5 to 87.5) at 24 months, and 56.9% (95% CI 34.6 to 79.1) at 60 months. Our findings show that patients with esophageal adenocarcinoma 45 years of age or younger have similar clinical findings to those reported in other large series where the median age is in the sixth or seventh decade of life, supporting a uniform theory of tumor pathogenesis. Esophagectomy may be performed with low mortality, and survival is reasonable for early-stage disease. Young patients with Barrett's esophagus are not immune from the development of adenocarcinoma and need to be screened accordingly. (J GASTROINTEST SURG 2001;5:620-625.)

KEY WORDS: Esophageal neoplasm, Barrett's, adenocarcinoma

Esophageal adenocarcinoma is currently the most common type of esophageal cancer diagnosed in the United States.[1] Both clinical and laboratory data support the belief that adenocarcinoma arises from Barrett's mucosa by progressing from Barrett's mucosa, to mucosal dysplasia, to invasive adenocarcinoma.[2-9] The median age of patients with adenocarcinoma treated by esophagectomy is 60 to 63 years.[10-13] Esophagectomy for adenocarcinoma in young patients is uncommon. Since 1986 we have performed esophagectomy in 32 patients with adenocarcinoma aged 45 years or younger. The purpose of this study was to define the clinical characteristics of this group of patients and to compare the clinical characteristics of these young patients with the reported demographics of all patients with adenocarcinoma.

METHODS

All patients 45 years of age or younger who had undergone esophagectomy at the Johns Hopkins Hospital for esophageal adenocarcinoma from 1986 through 1999 were identified through the Johns Hopkins Hospital surgical pathology database. The hospital medical records of these patients were retrospectively reviewed to determine demographics (age, sex, and race), reflux and swallowing symptoms (dysphagia, epigastric pain, hiatal hernia, weight loss), social history (smoking and alcohol use), and family history of cancer (esophageal, gastrointestinal, or any other malignancy). The medical records were also reviewed to determine the esophagectomy technique, whether the patient received neoadjuvant chemoradiation therapy, operative mortality, and postresection

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pathologic findings (tumor staging, presence and length of Barrett's mucosa). For the purposes of this study, weight loss was defined as a loss of more than 10 pounds. Operative mortality was defined as postoperative death within 90 days. Medical antireflux therapy included the use of either histamine H_2 blockers or proton pump inhibitors. Tumor staging was carried out using the American Joint Committee on Cancer staging method.[14]

Postesophagectomy survival data were obtained for all but one patient. To determine whether there were any differences in results across the overall age range (19 to 45 years of age), the study group was divided into three age groups—19 to 25 years, 26 to 39 years, and 40 to 45 years—and the results were tabulated both for the subgroups and in total. Survival results were displayed for three groups—patients 40 to 45 years of age, patients under age 40, and the total group. It was thought that inclusion of the group aged 19 to 25 years would not result in meaningful survival results because of the small number of patients in that group.

Clinical characteristics were compared in the three groups of patients with adenocarcinoma—those under 45 years of age from our series, those over 45 years of age from our institution, and patients of all ages compiled from the literature. The composite data from the literature do not contain data from patients treated at our institution in order to avoid data overlap. To determine the clinical characteristics of patients over 45 years of age from our institution, the database from an earlier series[10] was used. This allowed us to collate the patient characteristics for 53 consecutive patients with adenocarcinoma over 45 years of age who underwent esophagectomy at our institution between 1987 and 1996. The following clinical characteristics were compared: age, sex, race, presence of gastroesophageal reflux, dysphagia, weight loss, abdominal pain, smoking, and alcohol consumption. Five-year survival figures were also compared.

Survival statistics were derived using the Kaplan-Meier method, and 95% confidence intervals were determined at each point on the curve using the Peto equation[15] for determination of standard error. Kaplan-Meier curves with 95% confidence intervals that overlapped at a given point were determined to not be significantly different at that point.

RESULTS

Thirty-two patients were identified and comprised the study group. Patient characteristics are summarized in Table I. Four patients were under 25 years of age, 11 were 26 to 39 years of age, and 17 patients were 40 to 45 years of age. All patients were white, and all but one were male. The presenting symptom of dysphagia was noted in 78% of patients. It appeared to be more common in the younger age groups. Epigastric pain was present in 59% of patients and was more common in older patients. A weight loss of more than 10 pounds was present in 16% of

Table I. Patient characteristics

Patient characteristics	Age			All patients
	19-25 yr (n = 4)	26-39 yr (n = 11)	40-45 yr (n = 17)	
White	4 (100%)	11 (100%)	17 (100%)	32 (100%)
Male	4 (100%)	11 (100%)	16 (94%)	31 (97%)
Presenting with dysphagia	4 (100%)	9 (82%)	12 (71%)	25 (78%)
Presenting with epigastric pain	1 (25%)	5 (46%)	13 (76%)	19 (59%)
Presenting with weight loss*	0	3 (27%)	2 (12%)	5 (16%)
History of gastroesophageal reflux	2 (50%)	6 (54%)	10 (59%)	18 (56%)
History of hiatal hernia	0	2 (18%)	5 (29%)	7 (22%)
History of Barrett's esophagus	1 (25%)	0	4 (24%)	5 (16%)
Average length of observation (mo)	18	N/A	36	32
History of medical antireflux therapy	1 (25%)	1 (9%)	3 (18%)	5 (16%)
History of smoking	1 (25%)	5 (46%)	7 (41%)	13 (41%)
History of alcohol use†	1 (25%)	7 (64%)	11 (65%)	19 (59%)
Family history of esophageal cancer	0	0	0	0
Family history of any gastrointestinal malignancy	1 (25%)	2 (18%)	2 (12%)	5 (16%)
Family history of any cancer	1 (25%)	7 (64%)	7 (41%)	15 (47%)

N/A = not available.
*More than 10 pounds within 2 months prior to presentation.
†Defined as consuming an average of three or more alcoholic drinks per day.

Table II. Surgical treatment and pathologic findings

Operation	Age 19-25 yr (n = 4)	Age 26-39 yr (n = 11)	Age 40-45 yr (n = 17)	All patients
Transhiatal esophagectomy	3 (75%)	10 (90.9%)	16 (94.1%)	29 (90.6%)
Ivor-Lewis operation	1 (25%)	0	0	1 (3.1%)
Three-incision esophagectomy	0	1 (9.1%)	0	1 (3.1%)
Thoracoabdominal esophagectomy	0	0	1 (5.9%)	1 (3.1%)
Perioperative mortality	0	0	0	0
Received neoadjuvant chemoradiation	2 (50%)	6 (54.5%)	9 (52.9%)	17 (53.1%)
Tumor stage				
I	1 (25%)	0	7 (41.2%)	8 (25%)
IIB	0	1 (9.1%)	2 (11.8%)	3 (9.4%)
III	3 (75%)	7 (63.6%)	6 (35.3%)	16 (50%)
IV	0	2 (18.2%)	0	2 (6.3%)
Pathologic complete remission†	0	1 (9.1%)	2 (11.8%)	3 (18%)
Barrett's mucosa identified in surgical specimen	1 (25%)	4 (36.4%)	10 (58.8%)	15 (46.9%)
Average length (if reported)	8.0 cm	4.8 cm	7.4 cm	6.9 cm

*Defined as death occurring less than 3 months following operation.
†No other pretreatment staging information available.

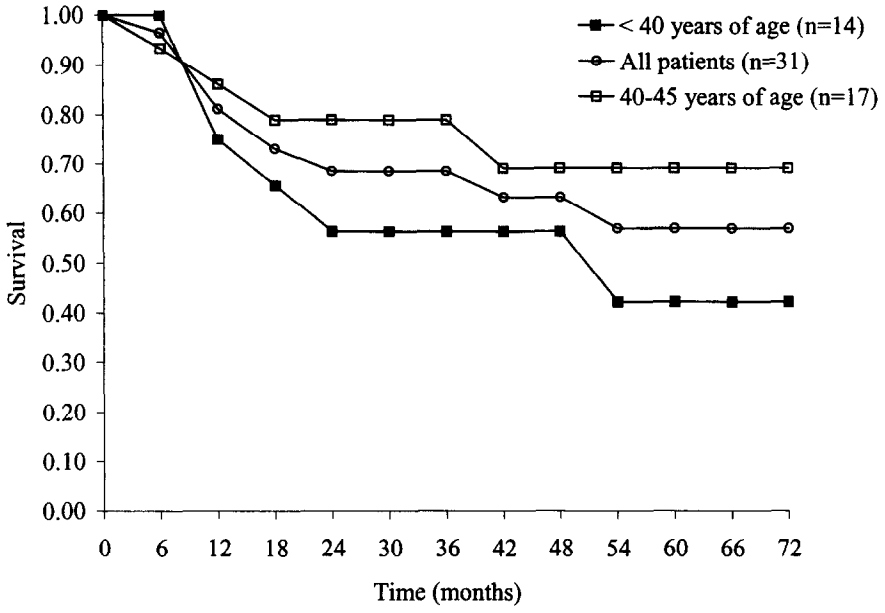
Table III. Comparison of clinical characteristics in patients with adenocarcinoma

Patient characteristics	Present series (n = 32)	Johns Hopkins over age 45 series (n = 53)	Composite data[11,20,22] (n = 1193*)
Average age (yr)	37	65	Range 57-66
Male:female ratio	31:1	12:1	Range 3.3-3.9:1
% White	100	98	Range 97-100
Gastroesophageal reflux (%)	56	60	8
Dysphagia (%)	78	N/A	69-73
Weight loss (%)	16	N/A	45
Abdominal pain (%)	59	N/A	12-15
Smoking (%)	41	81	N/A
Alcohol consumption (%)	59	51	N/A
5-year survival (%)	57	12	30

N/A = not available.

patients. Gastroesophageal reflux and hiatal hernia were noted in 56% and 22%, respectively. A diagnosis of Barrett's esophagus that predated the diagnosis of adenocarcinoma was present in only 16% of patients. For those patients with a prior history of Barrett's mucosa, the average length of preoperative follow-up was 32 months (range 18 to 36 months). Only 16% of patients were receiving medical antireflux therapy before being diagnosed with adenocarcinoma. A history of smoking and alcohol use was noted in 41%, and 59%, respectively. No patient had a positive family history of esophageal cancer. There was a family history of gastrointestinal malignancy or any type of cancer in 16%, and 47%, respectively. Aside from the esophageal neoplasm, the medical history was negative in 62% of patients.

Surgical treatment and pathologic findings are summarized in Table II. Twenty-nine patients (91%) underwent transhiatal esophagectomy, and one patient each (3%) underwent Ivor-Lewis, three-incision, or left thoracoabdominal esophagectomy. There were no operative deaths. Approximately half of the patients (53%) received preoperative therapy consisting of concurrent cis-platinum, 5-fluorouracil, and external beam radiation therapy.[16] Pathologic staging of the resected tumors yielded the following results: stage I in eight patients (25%), stage IIB in three patients (9.4%), stage III in 16 patients (50%), and stage



# at risk:													
< 40 years of age:	14	13	9	7	6	6	5	5	4	3	3	2	2
All patients:	31	30	23	19	17	15	13	13	11	9	9	8	8
40-45 years of age:	17	17	14	12	11	9	8	8	7	6	6	6	6

Fig. 1. Kaplan-Meier survival curves for all patients (open circles), patients 40 to 45 years of age (open squares), and patients under 40 years of age (solid squares).

IV in two patients (6.3%); no residual viable tumor was noted in three patients (9.4%) following preoperative chemoradiation therapy. The pathologic complete response rate was 18%. Barrett's mucosa was identified in 15 patients (46.9%), and the average length of Barrett's mucosa was 6.9 cm.

Table III compares the clinical characteristics of three groups of patients with adenocarcinoma—those under 45 years of age from our series, those over 45 years of age from our institution, and patients of all ages compiled from the literature. Patients under 45 years of age were younger and had a higher male: female ratio, more abdominal pain, and less weight loss than the comparison groups. The incidence of gastroesophageal reflux was similar in patients under (56%) and over (60%) 45 years of age from our institution, and was much higher than that reported in the literature (8%).

Postesophagectomy survival is shown in Fig. 1. Follow-up information was available for 31 of the 32 patients; the remaining patient was lost to follow-up. Ten patients have died as a result of recurrent adenocarcinoma. The average postesophagectomy survival time for these 10 patients was 19.9 months. The remaining 21 patients are alive and well with no recurrence of their disease. One patient has experienced a localized recurrence of tumor at the anastomotic site, which was treated successfully with radiation therapy. The average length of follow-up among the survivors was 50.8 months. The 40- to 45-year-old age group displayed a trend toward increased survival, but the difference was not statistically significant. The actuarial survival for all patients was 81.1% (95% confidence interval [CI] 66.1 to 96.2) at 12 months, 68.5% (95% CI 49.5 to 87.5) at 24 months, 63.2% (95% CI 41.5 to 84.9) at 48 months, and 56.9% (95% CI 34.6 to 79.1) at 60 months.

DISCUSSION

Esophageal adenocarcinoma in young patients is uncommon. Hassall et al.[17] reported the successful resection of an adenocarcinoma arising from Barrett's esophagus in a 17-year-old boy. Prior to Hassall's report, there had been only nine reported cases of esophageal adenocarcinoma in patients 25 years of age or younger. All of these patients presented with advanced-stage disease and died as a result of their cancer.[18] It is well documented that adenocarcinoma is most commonly diagnosed in patients in their sixth and seventh decades of life. Do younger patients with adenocarcinoma have a different disease with unique

clinical features and pathogenesis, or is adenocarcinoma a single disease with a "bell-shaped curve" age distribution? Since 1986 we have performed esophagectomy in 32 patients aged 45 years or younger with adenocarcinoma. The purpose of this study was twofold. The first objective was to define the clinical characteristics of this group of patients. The second objective was to compare the clinical characteristics of adenocarcinoma patients aged 45 years or younger with the reported demographics of all patients with adenocarcinoma to look for similarities, which would support a uniform theory of tumor pathogenesis, or differences, which would support divergent pathways for tumor pathogenesis.

Since 1978 there has been a dramatic increase in the prevalence of esophageal adenocarcinoma, and currently adenocarcinoma is the most common cell type in esophageal cancer diagnosed in the United States.[1] Both clinical and molecular genetic evidence support a unified theory of adenocarcinoma pathogenesis. This theory holds that adenocarcinoma arises from Barrett's mucosa through an intermediate step involving mucosal dysplasia in a process mediated or initiated by gastroesophageal reflux.[2-9] How much time it takes to develop Barrett's esophagus or for the subsequent development of adenocarcinoma is not known with certainty. Cameron and Lomboy[19] documented that the prevalence of Barrett's esophagus increased with age until the seventh decade of life, at which time it reached a plateau. In their review, the median age of patients with Barrett's esophagus was 40 years; however, the mean age at diagnosis was 63 years. Notably, these investigators found that the length of Barrett's mucosa seemed to develop in its entirety at the onset and not change with time; they also found that long delays (>20 years) between the onset of Barrett's mucosa and its diagnosis were possible. Previous studies in which patients with Barrett's esophagus were followed prospectively have shown that only a few patients developed dysplastic mucosa, and this change took a mean of 3.6 to 5.2 years.[2,3,8] In similar clinical studies, patients with Barrett's esophagus and dysplasia were found to progress to adenocarcinoma within 2.6 to 5.0 years.[2,3,8] Current data, therefore, support the possibility of adenocarcinoma developing from Barrett's esophagus in young patients. Cameron and Lomboy[19] documented Barrett's mucosa in patients over a wide age range including under 10 years of age. Given that it takes approximately 6 to 10 years to progress from Barrett's mucosa to adenocarcinoma, this is consistent with the observation that the youngest reported patients with adenocarcinoma have been in their late teens.

Assuming a uniform pathogenesis, patients with adenocarcinoma, regardless of age, should share similar clinical features. In addition, patient characteristics of adenocarcinoma and Barrett's esophagus, which is the precursor for these tumors, should be similar. In our series (see Table I), patients were white (100%) males (97%) with a history of gastroesophageal reflux (56%), hiatal hernia (22%), cigarette smoking (41%), alcohol consumption (59%), who presented with progressive solid food dysplasia (78%). Only five patients (16%) had a prior diagnosis of Barrett's esophagus and only five patients (16%) were taking any antireflux medications. A family history of esophageal cancer, any gastrointestinal cancer, or any cancer regardless of site was noted in 0%, 16%, and 47% of patients, respectively. There were no significant differences in clinical characteristics between the three study groups (ages 19 to 25 years, 26 to 39 years, and 40 to 45 years). Table III compares the clinical characteristics of three groups of patients with adenocarcinoma—those under 45 years of age from our series, those over 45 years of age from our institution,[10] and patients of all ages compiled from the literature.[11,20,21] This comparison shows that patients with adenocarcinoma, regardless of age, are predominantly white males with a history of gastroesophageal reflux and dysphagia. The clinical association between gastroesophageal reflux and adenocarcinoma is well documented. Lagergren et al.[22] have documented a direct, dose-dependent relationship between reflux and the probability of developing adenocarcinoma. Patients 45 years of age or younger have a higher male:female ratio, less weight loss, and a greater frequency of abdominal pain. In our series adenocarcinoma was associated with a history of smoking and alcohol consumption in 41% and 59% of patients, respectively. Adenocarcinoma patients over 45 years of age from our institution had a history of smoking and alcohol consumption of 81% and 51%, respectively. Five-year survival rates are not comparable because the treatments were not the same in the two groups.

Cameron and Lomboy[19] demonstrated that Barrett's esophagus was twice as common in males as in females, and most commonly was diagnosed in the sixth decade of life. In their review of Barrett's esophagus, Sarr et al.[20] noted a mean age of 56 years, with 52% males, 48% females, 91% white, and 9% black. The frequency of heartburn, regurgitation, and hiatal hernia was 30%, 25%, and 70%, respectively. Therefore, when compared to patients with Barrett's esophagus alone, patients with adenocarcinoma aged 45 years or younger were younger with a higher male: female ratio.

Sabel et al.[21] demonstrated that the clinical characteristics of adenocarcinoma patients with and without associated Barrett's esophagus were the same except that there were fewer reflux symptoms in the pa-

tients with no Barrett's esophagus. Sarr et al.[20] compared the patient characteristics of patients with Barrett's esophagus with and without associated adenocarcinoma. The mean age of the two groups was similar; however, those patients with adenocarcinoma were more likely to be white males, with fewer reflux symptoms but a higher frequency of dysphagia.

There were no treatment-related deaths. Postesophagectomy survival data are presented in Fig. 1. It is difficult to generalize from these survival data given the fact that they include all stages and more than half of the patients (53%) received preoperative chemoradiation therapy.[15] Actuarial survival for all patients was 81.1% (95% CI 66.1 to 96.2) at 12 months, 68.5% (95% CI 49.5 to 87.5) at 24 months, 63.2% (95% CI 41.5 to 84.9) at 48 months, and 56.9% (95% CI 34.6 to 79.1) at 60 months. There is a trend toward improved postesophagectomy survival in patients 40 to 45 years of age compared with patients under the age of 40. This finding can be explained by postresection tumor staging. The majority of patients under 40 years of age had stage III tumors, whereas more than 50% of patients 40 to 45 years of age had stage I or IIB tumors.

Patients with adenocarcinoma who were 45 years of age and younger have similar clinical findings to those reported for patients regardless of age, supporting a uniform theory of tumor pathogenesis. We believe that the data regarding pathogenesis of adenocarcinoma also explain the wide age range demonstrated by this disease. Patients may develop Barrett's esophagus early, and then progress to mucosal dysplasia and adenocarcinoma at different times depending on the individual. Exactly what factors initiate this neoplastic process and lead to this observed variability is not known. Esophagectomy may be performed with low mortality, and survival is reasonable for early-stage disease. Current recommendations regarding indications for endoscopy in patients with symptomatic reflux have focused on patients over 50 years of age.[23] Young patients with Barrett's esophagus are not immune to the development of adenocarcinoma and need to be screened accordingly.

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Staging of Pancreatic Cancer Before and After Neoadjuvant Chemoradiation

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Neoadjuvant chemoradiation therapy is used at many institutions for treatment of localized adenocarcinoma of the pancreas. Accurate staging before neoadjuvant therapy identifies patients with distant metastatic disease, and restaging after neoadjuvant therapy selects patients for laparotomy and attempted resection. The aims of this study were to (1) determine the utility of staging laparoscopy in candidates for neoadjuvant therapy and (2) evaluate the accuracy of restaging CT following chemoradiation. Staging laparoscopy was performed in 98 patients with radiographically potentially resectable (no evidence of arterial abutment or venous occlusion) or locally advanced (arterial abutment or venous occlusion) adenocarcinoma of the pancreas. Unsuspected distant metastasis was identified in 8 (18%) of 45 patients with potentially resectable tumors and 13 (24%) of 55 patients with locally advanced tumors by CT. Neoadjuvant chemoradiation therapy and restaging CT were completed in a total of 103 patients. Thirty-three patients with potentially resectable tumors by restaging CT underwent surgical exploration and resections were performed in 27 (82%). Eleven (22%) of 49 patients with locally advanced tumors by restaging CT were resected, with negative margins in 55%; the tumors in these 11 patients had been considered locally advanced because of arterial involvement on restaging CT. Staging laparoscopy is useful for the exclusion of patients with unsuspected metastatic disease from aggressive neoadjuvant chemoradiation protocols. Following neoadjuvant chemoradiation, restaging CT guides the selection of patients for laparotomy but may overestimate unresectability to a greater extent than does prechemoradiation CT. (J GASTROINTEST SURG 2001;5:626-633.)

KEY WORDS: Pancreatic cancer, neoadjuvant, chemoradiation, staging laparoscopy, computed tomography

Accurate staging of pancreatic adenocarcinoma is necessary for the selection of appropriate therapy. Fewer than 10% of patients present with tumors that are amenable to curative resection.[1] More than 40% of patients have distant metastatic disease at the time of diagnosis. For these patients, surgical resection is not indicated because survival rarely exceeds 6 months. The remaining patients (approximately 50%) are considered unresectable because of local tumor invasion. If identified preoperatively, most patients with metastatic and unresectable disease can avoid laparotomy, since endoscopic and laparoscopic approaches can generally provide adequate palliation.

Thin-collimation contrast-enhanced spiral CT is the most widely used modality for the staging of pancreatic cancer. However, despite improvements in the technology, CT remains limited in its ability to detect and characterize small metastatic foci, both within the liver and on the peritoneal surfaces. Staging laparoscopy has therefore been advocated as a means of avoiding nontherapeutic laparotomy in patients with radiographically localized disease. Several studies of staging laparoscopy prior to attempted resection have demonstrated that approximately 25% of patients with localized disease on CT will harbor small-volume liver or peritoneal metastases.[2-5] However, the

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inclusion criteria for these series varied considerably, and controversy still exists over the yield of laparoscopy for the subset of patients with small, radiographically resectable tumors.

In addition to those patients with unsuspected distant metastasis, many patients who appear resectable based on CT criteria will be found to be unresectable at surgical exploration because of local invasion. In 1990 Warshaw et al.[6] published a series in which CT correctly predicted unresectability in more than 90% of cases but failed to identify vascular invasion in 26%. Several subsequent studies have demonstrated that the accuracy of CT has improved,[4,7-9] and other modalities such as angiography, endoscopic ultrasonography, and laparoscopic ultrasonography have been successfully used as adjuncts to CT for the detection of vascular invasion. However, our ability to correctly identify patients preoperatively who are unresectable remains superior to our ability to identify patients who are resectable.

Even in patients who have tumors that are technically resectable, high rates of positive margins, nodal metastases, and local recurrence underscore the locally aggressive nature of this disease.[10] Neoadjuvant (preoperative) chemoradiation therapy (CRT) has therefore been employed at many institutions with the ultimate goals of downstaging locally advanced tumors and improving local recurrence and long-term survival rates in patients with resectable tumors.[11-14] At our institution, all patients with histologically confirmed, localized adenocarcinoma of the pancreas are offered neoadjuvant CRT, whereas the standard treatment for patients with metastatic disease is palliative chemotherapy. The role of staging laparoscopy in this setting is to identify and spare patients with unsuspected metastatic disease the cost and morbidity of aggressive CRT. Following CRT, restaging CT is used for assessment of response to treatment and selection of patients for surgical exploration.

The aim of this study was thus twofold. First, we attempted to determine the utility of staging laparoscopy in candidates for neoadjuvant CRT. Second, we sought to evaluate the accuracy of restaging CT after CRT in predicting resectability and unresectability.

MATERIAL AND METHODS

Between August 1994 and November 2000, 98 patients with histologically confirmed pancreatic ductal adenocarcinoma underwent staging laparoscopy prior to initiation of neoadjuvant CRT. The characteristics of these patients are summarized in Table I. An additional 35 patients who received neoadjuvant CRT without prior staging laparoscopy were included only in the analyses of post-CRT restaging CT. Twelve patients who underwent exploratory laparotomy for diagnosis or palliation prior to CRT, as well as 13 patients who underwent resection without neoadjuvant CRT, were excluded from this study. All patients underwent thin-section, contrast-enhanced dynamic CT. CT scanning was performed with an incremental or spiral scanner, collimation through the pancreas was not more than 5 mm, and images were acquired during the portal predominant phase of enhancement. Many patients underwent CT scanning at referring institutions; CT scans deemed adequate for the purpose of excluding metastatic disease were not repeated. CT evidence of distant metastatic disease included solid focal liver lesions not satisfying the criteria for simple cyst or hemangioma, multiple noncalcified pulmonary nodules, and evidence of peritoneal spread of tumor including fluid, peritoneal thickening and/or nodularity, and mesenteric/omental implants. CT scans were reviewed by one of three radiologists (E.P., K.F., or M.K.). Localized tumors were categorized as potentially resectable if there was no evidence of direct invasion of the superior mesenteric artery (SMA) and celiac axis, and the superior mesenteric vein (SMV) and portal vein were patent. Tumors were categorized as locally advanced in the presence of soft tissue abutting or encircling the SMA or celiac axis or occlusion of the SMV or portal vein.

Table I. Characteristics of patients undergoing staging laparoscopy

	Staging laparoscopy (n = 98)
Median age (yr)	64 (range 31-82)
Male/female	52/46
Mean tumor size ± SD	3.2 ± 1.8 cm
Tumor location	
Head/uncinate process	78 (79%)
Body/tail	20 (21%)
CT resectability	
Potentially resectable	45 (46%)
Locally advanced	53 (54%)

SD = standard deviation.

Staging laparoscopy was performed with patients under general anesthesia. Pneumoperitoneum was established using an open technique, followed by introduction of a 30-degree angled laparoscope. Two 5 mm trocars were placed in the right upper quadrant. A systematic, 360-degree inspection of the abdomen was performed, beginning with the liver and including the peritoneum, omentum, and entire bowel mesentery. Biopsies were obtained of any suspicious lesions and sent for pathologic examination. No attempt was made to examine or perform a biopsy of

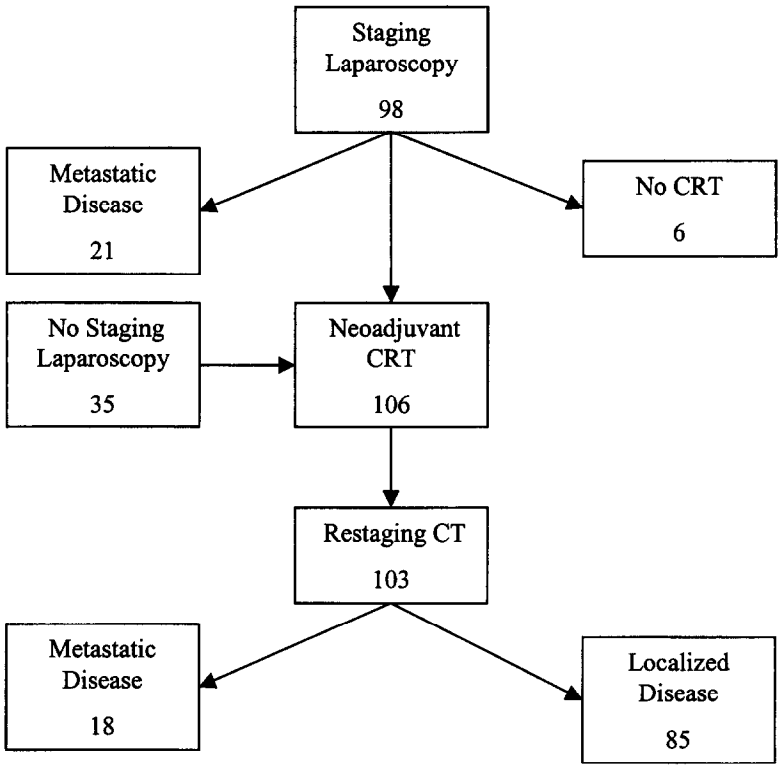

Fig. 1. Staging laparoscopy was performed in 98 patients. A total of 103 patients underwent restaging CT on completion of neoadjuvant chemoradiation therapy. Eighty-five patients with localized disease on restaging CT were considered for surgical exploration.

the primary tumor, and 72 patients underwent placement of a jejunostomy feeding tube. In the 82 patients of one senior author (D.T.), 1 to 2 liters of saline solution was instilled and then collected for peritoneal cytologic examination, if no visible evidence of metastasis was present.

Patients with localized disease were considered candidates for neoadjuvant CRT. Exact regimens varied but consisted of either 5 weeks of daily external beam radiation therapy with concurrent 5-fluorouracil–based chemotherapy (91%) or 3 weeks of twice daily external beam radiation therapy with concurrent gemcitabine chemotherapy (9%). The median total dose of radiation received was 4050 cGy. Following CRT, patients were given a 2- to 3-week break for recovery of blood counts and nutrition, followed by restaging CT. Neoadjuvant CRT and restaging CT were completed in 103 patients: 68 laparoscopically staged patients plus the 35 patients who received neoadjuvant CRT without prior staging laparoscopy (Fig. 1). The decision to perform an exploratory operation following neoadjuvant CRT was made by the attending surgeon and was based on restaging CT information, the patient's fitness for surgery, need for surgical palliation, and—in select cases—endoscopic ultrasound–guided fine-needle aspiration.

Patient records were maintained in a prospective database (Microsoft Access, Microsoft Corp., Redmond, Wash.) and supplemented by information obtained from a retrospective review of hospital and physician records. Data were evaluated for statistical differences by two-tailed Fisher's exact test for comparison of ratios (Statistica for Windows, StatSoft, Inc., Tulsa, Okla.).

RESULTS

Staging Laparoscopy

Of 98 patients with localized disease by CT, 18 (18%) had visible metastatic disease to the liver (12%) or peritoneum (6%) on laparoscopic staging. An additional three patients without visible metastatic disease had positive findings on peritoneal cytologic examination. The yield of staging laparoscopy for metastatic disease was directly related to the size of the primary tumor on initial staging CT (Table II). Eight (18%) of 45 patients with potentially resectable disease manifested metastatic disease as compared to 13 (24%) of 55 patients with locally advanced disease (P = nonsignificant). Metastatic disease was discovered in 7 (35%) of 20 patients with body/tail lesions vs. 14 (18%) of 78 patients with head/uncinate

Table II. Yield of staging laparoscopy

	Metastatic disease on staging laparoscopy
Tumor size	
<1 cm (n = 9)	1 (11%)
≥1 to 2 cm (n = 12)	1 (8%)
≥2 to 3 cm (n = 26)	4 (15%)
≥3 to 4 cm (n = 27)	5 (19%)
≥4 cm (n = 24)	10 (42%)
Tumor location	
Head/uncinate process (n = 78)	14 (18%)
Body/tail (n = 20)	7 (35%)
CT resectability	
Potentially resectable (n = 45)	8 (18%)
Locally advanced (n = 55)	13 (24%)
Indeterminate liver lesions (n = 28)	7 (25%)

process lesions ($P = 0.12$). Indeterminate or "too small to characterize" liver lesions were present on CT in 28 patients (29%), but metastatic disease was subsequently demonstrated in only seven of these patients (25%). For the low-risk subgroup of patients with potentially resectable tumors located in the head/uncinate process without indeterminate liver lesions (n = 31), the yield of laparoscopy for distant metastatic disease was 10%.

Three patients with unsuspected metastatic disease underwent laparoscopic palliative procedures at the time of staging laparoscopy, and only one patient required subsequent laparotomy for palliation. Two thirds of patients in whom metastatic disease was identified received palliative chemotherapy, and 19% did not receive chemotherapy. Three patients (14%) were subsequently treated with palliative CRT for pain or obstruction.

The only complication of staging laparoscopy occurred in one patient with visible metastatic disease who developed supraventricular tachycardia due to hypercarbia; the arrhythmia resolved with release of the pneumoperitoneum. One patient required conversion to laparotomy for placement of a jejunostomy feeding tube.

Restaging CT Following Neoadjuvant Chemoradiation Therapy

Restaging CT scans were performed in 103 patients following completion of neoadjuvant CRT (see Fig. 1). Pre- and post-CRT CT scans were available for a retrospective comparison of primary tumor responses in 84 patients. Significant primary tumor responses were defined as an increase or decrease greater than or equal to 1 cm in at least one tumor dimension. A decrease in primary tumor size was seen in 27% of patients; an increase was seen in 17% of patients (Table III). The majority (56%) demonstrated no significant change, including nine patients without a definite mass on pre- or post-CRT CT scans, and many patients demonstrated distant disease progression on restaging CT despite a favorable primary tumor response (see Table III). The likelihood of a significant decrease in size in response to CRT was related to the initial size of the primary tumor and ranged from 10% in patients with tumors less than 2 cm to 48% in patients with tumors greater than or equal to 4 cm. However, only 6% of patients with locally advanced tumors were downstaged to radiographic resectability on restaging CT (not shown). Furthermore, although a decrease in primary size was not highly predictive of subsequent resectability, no patients with a significant increase in tumor size subsequently underwent resection.

Table III. Radiographic response to chemoradiation therapy

Primary tumor response	Distant metastasis	Subsequent resectability
Decrease (n = 23)	4 (17%)	8 (42%)
No change (n = 47)	7 (9%)	15 (38%)
Increase (n = 14)	4 (29%)	0

Of the total 103 patients who underwent restaging CT, 18 (17%) demonstrated evidence of distant metastasis (see Fig. 1). The 85 patients with localized disease on restaging CT were reclassified as having radiographically potentially resectable (n = 36) or locally advanced (n = 49) disease. Overall 68 (80%) of 85 patients without evidence of distant progression underwent surgical exploration. Three (8%) of 36 patients with potentially resectable tumors on restaging CT were considered to have prohibitive medical conditions. Fourteen (29%) of 49 patients with locally advanced tumors did not undergo surgical exploration because of an unfavorable primary tumor response on CT (e.g., venous occlusion), the patient's medical condition, or additional information obtained from endoscopic ultrasound.

Thirty-three patients with partially resectable tumors on restaging CT underwent surgical exploration; six (18%) were found to be unresectable at surgery, because of local/regional invasion in four patients and distant metastasis in two patients (Table IV). The remaining 27 patients with potentially resectable tumors (82%) underwent resection with negative surgical margins in 20 patients (74%). Since the true resectability of three patients is unknown, the predictive value of CT resectability following CRT is between 75% and 83%.

Eleven of 49 locally advanced tumors on restaging CT (22%) were technically resectable at surgery (Table IV). Whipple pancreaticoduodenectomy without vascular resection was performed in 10 patients, and distal pancreatectomy was performed in one patient. Surgical margins were negative in six patients (55%), a not significantly lower rate than that for radiographically potentially resectable tumors (P = 0.24). All patients had been considered to have locally advanced tumors as shown by restaging CT on the basis of soft tissue abutment or encasement (circumferential involvement) of the SMA or celiac axis. An example of a post-CRT restaging CT demonstrating apparent involvement of the SMA is shown in Fig. 2. Biopsy of this tissue at surgical exploration revealed only fibrotic tissue, and the tumor was resected with negative surgical margins. Only one technically resectable patient (with positive surgical margins) demonstrated vascular narrowing on three-dimensional CT angiography, and none of these patients had CT evidence of SMV or portal vein occlusion. Twenty-four patients with locally advanced tumors on restaging CT underwent surgical exploration and were indeed found to be unresectable because of unsuspected distant disease in 10 patients and local/regional disease in 14. Because an additional 14 patients did not undergo surgical exploration, the exact predictive value of CT unresectability following CRT is unknown but does not exceed 78%.

Table IV. Performance of restaging CT

	CT resectability	
Surgical resectability	**PR (n = 36)**	**LA (n = 49)**
Resectable	27	11
Not explored	3	14
Unresectable	6	24
Distant disease	2	10
Local/regional disease	4	14

LA = locally advanced; PR = potentially resectable.

DISCUSSION

Staging Laparoscopy

Several series have addressed the value of laparoscopic staging in patients with radiographically localized pancreatic cancer prior to laparotomy for attempted resection (Table V). In two of the largest and most recent series from Memorial Sloan-Kettering Cancer Center[4] and Massachusetts General Hospital,[15] visible distant metastases were evident in 26% and 24% of patients by laparoscopy, respectively. Subsequent resectability rates for patients with no evidence of unresectability by laparoscopy range from

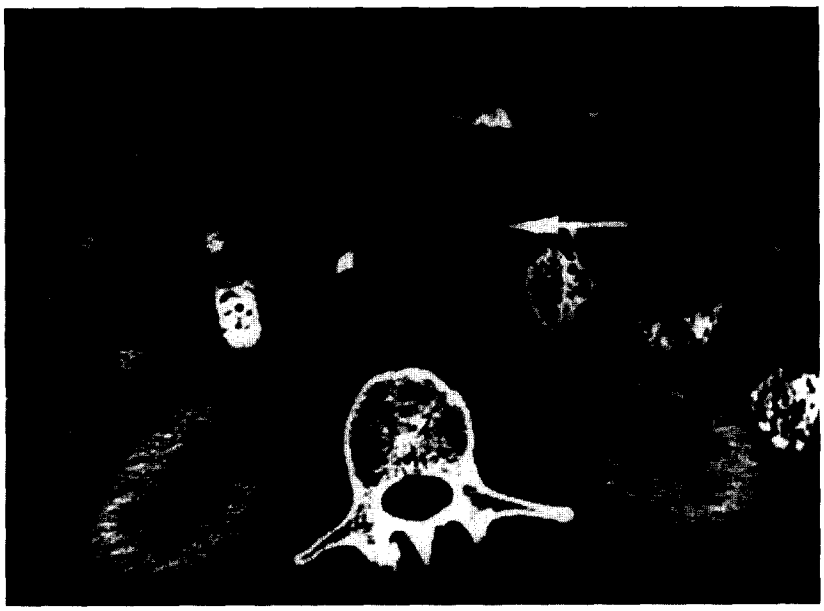

Fig. 2. Post–chemoradiation therapy contrast-enhanced spiral CT scan. The mass in the pancreatic head demonstrated no significant change in size from the pre–chemoradiation therapy study. The mass abuts the medial aspect of the superior mesenteric artery *(arrow)* over approximately 180 degrees. The lateral aspect of the superior mesenteric artery is surrounded by fatty tissue and is spared from tumor. The tumor was subsequently resected with negative surgical margins.

37% to 91%; however, in some of these series, negative standard laparoscopy was followed by laparoscopic ultrasonography,[2] angiography,[3] or examination of the lesser sac[4] to evaluate patients for evidence of local tumor invasion. The earlier series from Duke University Medical Center[5] was dominated by patients with locally advanced tumors on preoperative imaging studies, with expectedly higher rates of unresectability both by laparoscopy and on subsequent laparotomy.

In the Massachusetts General Hospital experience, peritoneal lavage for cytologic testing at the time of staging laparoscopy yielded an additional 5% to 10% of patients with only microscopic metastatic disease.[3,15] Peritoneal cytology is a simple, inexpensive study that is capable of identifying a subgroup of patients whose survival is not significantly different from the survival of patients with visible metastatic disease.[16] Since cytologic study requires more than a day for specimen preparation and pathologic review, it is more useful as an adjunct to laparoscopic staging of patients prior to neoadjuvant therapy than prior to planned immediate laparotomy.

The yield of staging laparoscopy for metastatic disease in our study (21%) is slightly lower than that in previous studies, perhaps because we included more patients with radiographically resectable disease. The more recent Massachusetts General Hospital series[15] excluded tumors smaller than 2 cm because of the low incidence of metastasis for this subgroup in their previous series.[3] In contrast, the series reported by Conlon et al.[4] included only patients with "potentially resectable" tumors. The majority of these studies have included a greater proportion of tumors located in the body or tail of the pancreas than did our study. The yield of laparoscopy in our series was lower for patients with potentially resectable tumors than for patients with locally advanced tumors (18% vs. 24%) and lower for patients with head/uncinate process tumors than for patients with body/tail tumors (18% vs. 35%). Subgroups of patients were identified in whom the yield was as low as 10% (potentially resectable tumors located in the head or uncinate process without indeterminate liver lesions). Although the benefit of staging laparoscopy clearly outweighs its cost in patients with high-risk tumors, its value in patients with relatively low-risk tumors remains an unsettled question, which pits cost-effectiveness against quality-of-life issues.

Table V. Yield of staging laparoscopy for distant metastasis

Reference	No. of patients	No. with visible distant metastasis
John et al.[2] (1995)	40	14 (35%)
Fernandez-del Castillo et al.[3] (1995)	114	27 (24%)
Conlon et al.[4] (1996)	108	28 (26%)
Holzman et al.[5] (1997)	28	14 (50%)
Jiminez et al.[15] (2000)	125	30 (24%)

Restaging CT

The challenges of accurately determining local resectability by CT scans are more pronounced following CRT, and only limited information is available from previous studies of neoadjuvant therapy. The general experience is that radiographic responses to CRT are modest and do not correlate well with pathologic responses.[11-14] The replacement of tumor with fibrosis, which is typically observed on histologic examination of resected surgical specimens, may result in little or no change in radiographic appearance. Indeed, a potential early response of the tumor to CRT may be to increase in size as a result of radiation-induced edema or fibrosis. CT is generally considered to be more than 90% reliable for predicting unresectability; since vascular involvement by tumor was not confirmed pathologically prior to CRT, initial staging CT likely overestimated unresectability in a small number of tumors classified as locally advanced. However, CT was less accurate than expected following CRT, as 22% of tumors considered locally advanced by restaging CT were resected, with negative margins in more than half.

Furthermore, distant disease progression appears unrelated to primary tumor response to CRT. In this series, restaging CT detected distant metastases in 17% of patients, the majority of whom required no additional surgery. However, an additional 18% of patients undergoing exploration were found to have unsuspected distant metastases, two thirds to the liver and one third to the peritoneum. Therefore, although it is not our routine practice, restaging laparoscopy might be considered prior to exploratory laparotomy, unless an indication for a palliative surgical procedure is also present.

Proposed Algorithm

Our protocol for patients with radiographically localized pancreatic adenocarcinoma includes staging laparoscopy to exclude small-volume metastatic disease. Although we encourage all patients on formal protocols to undergo staging laparoscopy, nonprotocol patients with relatively low-risk tumors may be given the option of proceeding directly to neoadju-

vant CRT. Patients with visible metastatic disease on staging laparoscopy may undergo concurrent laparoscopic palliative procedures, if indicated for symptoms. For patients with localized disease, the routine placement of enteral feeding access has not significantly influenced the subsequent morbidity or mortality of resection in our experience.[17] However, selective placement in nutritionally challenged patients may improve tolerance to neoadjuvant CRT.

Following CRT, restaging CT guides the selection of patients for surgical exploration but may need to be interpreted differently in the postradiation setting (Fig. 3). Patients with potentially resectable tumors by restaging CRT undergo surgical exploration unless medical comorbidities prohibit this. Patients with metastatic disease are offered surgical palliation only as needed to alleviate symptoms. Patients with locally advanced tumors on restaging CT represent the largest and most heterogeneous group, as this includes a wide spectrum of disease. CT evidence of SMV or portal vein occlusion generally precludes curative resection, and these patients do not routinely undergo surgical exploration with curative intent. In contrast, the appearance of arterial involvement on CT—typically considered indicative of unresectability—may represent sterile fibrosis. Endoscopic ultrasonography with fine-needle aspiration has been employed in these patients to obtain cytopathologic evidence that viable tumor cells are present. In the absence of clear involvement by tumor—as manifested by results of fine-needle aspiration—patients with arterial abutment and even encasement could be offered surgical exploration, although most of these patients remain unresectable at exploration.

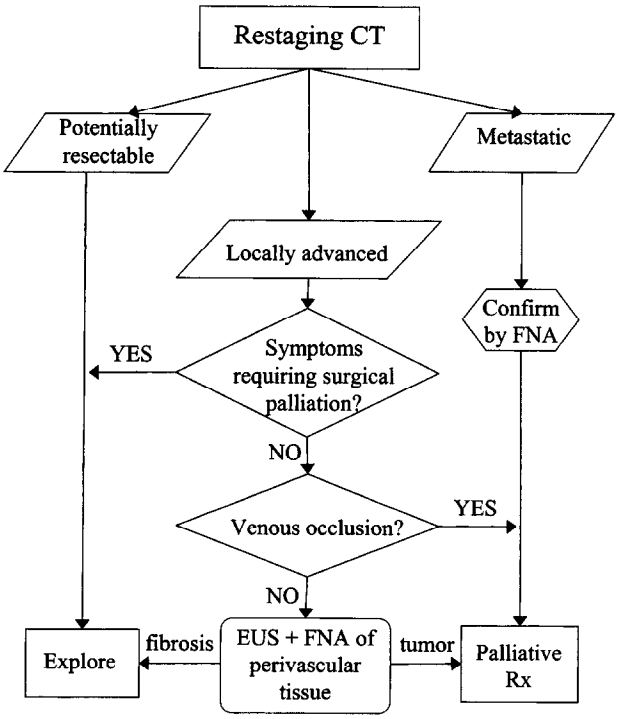

Fig. 3. Algorithm for interpretation of restaging CT after neoadjuvant chemoradiation therapy *(CRT)*. EUS = electronic ultrasound; FNA = fine-needle aspiration.

CONCLUSION

Particularly for the development and assessment of neoadjuvant regimens, staging laparoscopy plays an important role in the selection of appropriate patients for treatment. Use of laparoscopy allows for more accurate characterization of patients entering protocols by eliminating patients with metastatic disease. The exclusion—to the extent possible—of patients with distant metastasis is necessary to fairly compare neoadjuvant approaches to each other and to the standard approach of primary resection followed by adjuvant therapy. Laparoscopy also provides an opportunity to optimize symptomatic patients for neoadjuvant therapy through either laparoscopic palliation or placement of a feeding tube.

High-quality CT is also an important tool for both the staging and restaging of patients undergoing neoadjuvant therapy. Although the radiographic response to therapy tends to underestimate the pathologic response, restaging CT is useful to identify patients with distant metastatic or clearly unresectable local/regional disease who may avoid laparotomy. For the remaining patients, reliance on the standard CT criteria for unresectability will deprive approximately 20% of the opportunity for curative resection. To optimize the radiographic selection of patients for attempted resection, better correlation of radiographic appearance with the pathologic response to therapy is necessary to refine the criteria for unresectability in these patients.

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Pancreatic Epithelial Cyst in an Adult Treated by Central Pancreatectomy

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The infrequent occurrence of benign epithelial cysts of the pancreas is the reason why little is known regarding their clinical relevance and surgical management. We report the case of a 38-year-old woman with a benign epithelial cyst that was resected by the rarely performed central pancreatectomy. The presentation, evaluation, and differences between this and other cystic lesions of the pancreas are discussed. The benefits of central pancreatectomy for this benign lesion are reviewed. (J GASTROINTEST SURG 2001;5:634-637.)

KEY WORDS: Pancreas, epithelial cyst, central pancreatectomy

Benign cystic lesions of the pancreas are rare entities. These non-neoplastic benign pancreatic lesions can be further subclassified under the subheading of simple or solitary true cysts. Tanno et al.[1] recently found only eight reported adult patients with this type of lesion in the English literature and added two patients of their own. Little is known regarding its clinical or pathologic relevance.[1-4] The rarity of this lesion makes it difficult to determine its optimal treatment. In fact, it appears from the literature that operative management of this lesion should be determined on an individual basis. We report the case of a 38-year-old woman with a benign epithelial cyst of the pancreas that was treated by central pancreatectomy.

CASE REPORT

A 38-year-old woman sustained a gunshot wound midway between the xiphoid process and umbilicus in 1994 for which she underwent an exploratory laparotomy. Injuries to the left lobe of the liver, stomach, and transverse colon were repaired. The pancreas apparently was not injured. As part of an infertility workup in 1994, a CT scan was obtained that demonstrated a 2 cm cystic lesion of the pancreas at the junction of the neck and the body. In 1997 a follow-up CT scan demonstrated that the cystic lesion remained approximately 2 cm. The patient began experiencing back pain for which she took nonsteroidal anti-inflammatory agents with moderate success. In June 2000 a third CT scan of her abdomen demonstrated that the cystic lesion had increased in size to approximately 3 cm. Magnetic resonance cholangiopancreatography (MRCP) showed a slightly lobulated, nonseptated 2.8 × 3.0 × 2.3 cm exophytic mass arising from the superior aspect of the pancreatic body and neck with a thin postenhancement rim. It had a high and a moderate-to-high signal intensity on T_2- and T_1-weighted images, respectively. The cystic mass was superior to the main pancreatic duct and was separate from it. Septations or filling defects were not identified by MRCP. The CA19-9 level was normal. The patient denied any history of diabetes or pancreatitis. Her medications included omeprazole, clinidium, chlordiazepoxide, zolpidem, and L-thyroxine. She drank only one to two glasses of wine with her meals. Physical examination findings were unremarkable except for a well-healed midline laparotomy scar. Because the size of the cystic lesion had increased and she had developed symptoms of back pain, a pancreatic resection was recommended to eliminate the possibility of a neoplasm. At operation a 3 cm cystic lesion was identified at the neck of the pancreas (Fig. 1). The benign nature and location of the lesion permitted the removal of this lesion by means of a central pancreatectomy. The proximal pancreas was closed with an intestinal stapler, and an end-to-side pancreaticojejunostomy was performed to the tail. Intraoperatively the cystic structure was noted to be unilocular and filled with fluid. Microscopic examination revealed a benign fibrous epithelial cyst that was smoothly lined with flattened cells

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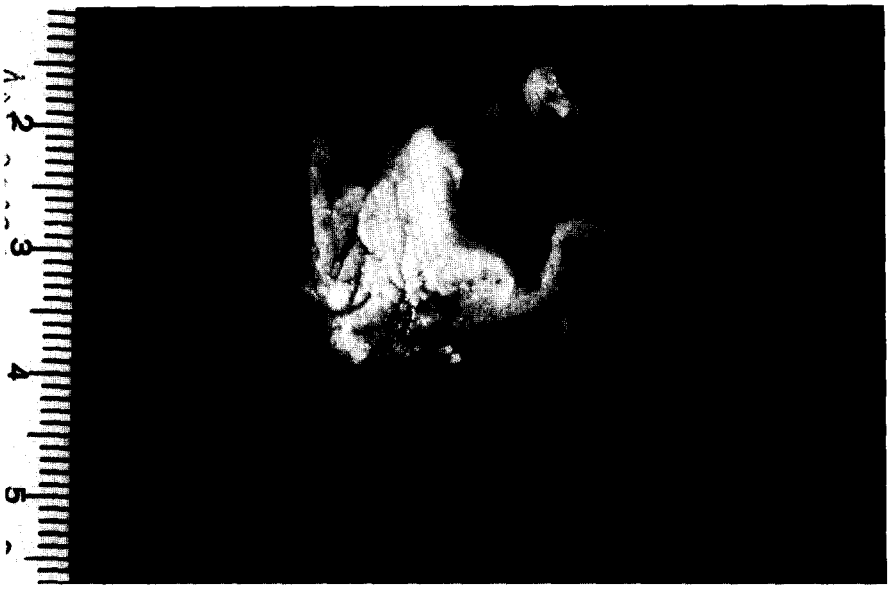

Fig. 1. Gross appearance of the cyst as seen through cross section of the pancreatic specimen.

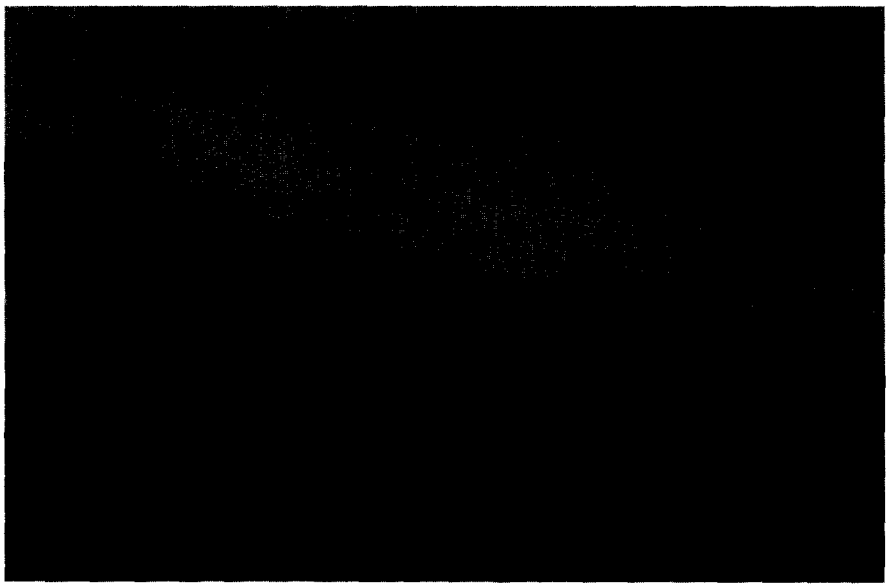

Fig. 2. High-power view (×40) of the cavity demonstrates a single layer of epithelial cells lining the cyst.

that stained well with keratin (Fig. 2). Gross analysis of the cyst fluid did not reveal the presence of mucin. The hospital course was unremarkable and the patient was discharged home 11 days after the operation.

DISCUSSION

The etiology of these rare benign epithelial cysts of the pancreas is unknown. Their discovery in a greater proportion of infants and children than adults suggests that they may be congenital. Absence of a known cause, their distinction from symptomatic acquired cystic disease, and their very rare occurrence in the adult population all lend support to a congenital origin.[1,2,4] Although these lesions are nonneoplastic, their clinical and radiographic presentation raises concern about the presence of a possible malignancy. Therefore surgical intervention for this lesion is warranted. Resection by pancreatectomy, when it can be safely performed, is advocated partic-

ularly because of the uncertainty of whether a malignancy is present.[2]

These lesions may or may not be symptomatic. Review of the literature on solitary true cysts of the pancreas in adults demonstrates that the symptoms which patients have exhibited include a palpable mass, abdominal pain, weight loss, nausea, vomiting, hematemesis, gastric outlet obstruction, biliary stenosis and obstruction, and pancreatitis. Several of these lesions were discovered incidentally by imaging studies or operative procedures for unrelated disease processes.[1,2,4] A recent review of this topic revealed that 3 of the 10 documented cases of this cystic lesion in adults were incidental findings.[1]

Ultrasonography, CT scanning, and magnetic resonance imaging are effective noninvasive tools for identifying these cysts. However, as with virtually all cystic lesions of the pancreas, further differentiation and exclusion of malignancy may be difficult with the use of these modalities alone.[1,4] Although no imaging modality can rule out malignancy with complete certainty, magnetic resonance imaging may be particularly useful in at least differentiating this lesion from other pancreatic cysts.

The fluid in benign epithelial cysts does not contain mucin as is found in the more common mucinous cystic tumors or intraductal papillary mucinous tumors. Some investigators have advocated analysis of cyst fluid by aspiration of the lesion as part of its evaluation. In theory, fluid analysis from a true epithelial cyst should reveal amylase, lipase, carcinoembryonic antigen, and CA19-9 levels that approach or are much lower than serum values. There have been several reports where fluid analysis revealed an elevated amylase, lipase, carcinoembryonic antigen, or CA19-9 level but surgical evaluation found a benign epithelial cyst.[1,4] It is our belief that cyst fluid aspiration has a limited role in evaluating this lesion. Its main use should be when it is desirable to avoid operation in the high-risk patient.[4]

A variety of cysts may be found in the pancreas including pseudocysts, retention cysts, gut duplication cysts, hereditary cysts, and lymphoepithelial and endometriotic cysts. Cystadenomas when serous or mucinous are usually easily distinguished. Pseudocysts are usually without a lining and show fibrosis in the adjacent pancreas. Retention cysts develop behind areas of fibrosis. Gut duplication cysts have gastrointestinal epithelium, typically small intestinal epithelium. Hereditary cysts may form in families with renal cystic disease or in von Hippel–Lindau disease and are essentially multiple. Lymphoepithelial cysts show a mature squamous lining and surrounding lymphocytosis. Endometriotic cysts show endometrial stroma in the wall. Our patient showed none of these features and had a flattened epithelial (keratin AE 1/3 and keratin 8/18 positive) lining with a fine squamous layer with normal adjacent pancreas.

Surgical intervention has been advocated for this cystic, non-neoplastic lesion and is based on its rarity and the preoperative concerns of a possible cystic malignancy. No uniform procedure has been described; enucleation, distal pancreatectomy, and pancreaticoduodenectomy have all been used as surgical treatments.[1,4] Resection rather than enucleation offers the best opportunity to obtain adequate margins in the event of possible neoplastic disease. Resection is associated with a lower incidence of pancreatic leak or fistula compared with enucleation.[2] Central pancreatectomy using a Roux-en-Y limb of jejunum for anastomosis to the distal pancreas and closure of the proximal pancreas has been advocated for benign or low-malignant-potential lesions of the pancreas, some of which have included this type of cystic lesion. This approach preserves maximal pancreatic parenchyma and the continuity of the upper gastrointestinal tract.[5]

Another approach is duodenum-preserving pancreatic head resection, which was originally described by Beger and associates and later used by Proposito et al.[6] in a patient with a cystadenoma of the pancreas. This procedure has yielded good results in terms of preserving exocrine and endocrine function for other pancreatic problems and should be applicable to benign epithelial cysts. This operation involves two side-to-side pancreaticojejunal anastomoses, and is more technically challenging than the single anastomosis used with central pancreatectomy. Rotman et al.[3] advocated the use of a central pancreatectomy for benign tumors of the pancreas from their experience with 14 patients. They attributed their favorable outcomes in endocrine and exocrine function to the limited resection of the pancreas with preservation of the stomach and duodenum. The description by Warshaw et al.[5] of their middle-segment pancreatectomy also yielded no evidence of diabetes or exocrine insufficiency among the 12 patients who had undergone this procedure. Thus several series have shown that lesions of the neck and proximal body of the pancreas can be safely managed by central pancreatectomy.

Our patient had prior abdominal trauma that may have predisposed her to develop a pseudocyst. However, she denied any history of pancreatitis and had no pancreatic complications after her initial laparotomy. The lesion caused relatively few symptoms. More than likely it was congenital and increased slowly over time. Other than identifying a cystic lesion and excluding a pseudocyst, the imaging studies employed did not add much to her management. The final histopathologic

findings and the uneventful postoperative course justify the use of the central pancreatectomy.

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Multimodality Treatment for Patients With Hepatocellular Carcinoma: Analysis of Prognostic Factors in a Single Western Institution Series

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There are few Western studies evaluating prognostic factors for survival in patients with hepatocellular carcinoma (HCC) and the influence on survival of various therapeutic options including orthotopic liver transplantation (OLT). A retrospective analysis was performed of 122 patients with HCC treated at the University of Alabama at Birmingham from January 1990 through December 1999. Clinicopathologic and treatment factors were analyzed with overall survival as the main outcome variable. Median age was 62 years. Most patients were male (74%) and white (79%). Eighty patients (66%) had associated cirrhosis. Sixty-three percent of patients presented with American Joint Committee on Cancer (AJCC) stage III or IV tumors. The median follow-up for survivors was 22 months. The 1-, 3-, and 5-year actuarial survival rates for the entire cohort were 46%, 24%, and 17%, respectively. On multivariate analysis, ablative surgery ($P = 0.003$), AJCC stages I and II ($P = 0.0012$), and absence of vascular invasion ($P = 0.0001$) were found to be independent favorable characteristics. Forty-four patients underwent surgical resection (including OLT, n = 20) or a surgical ablative procedure. All but two nonsurgical patients died of disease. The actuarial 1-, 3-, and 5-year survival rates for this group were 80%, 71%, and 61%, respectively. On multivariate analysis of the surgical group, only vascular invasion was associated with poor prognosis ($P = 0.001$). OLT was associated with a favorable prognosis on univariate analysis ($P = 0.02$). Forty percent of patients who received transplants underwent local/regional treatment before transplantation and the outcome in these patients was no different from that in other transplant patients. Surgical treatment is the only potential curative option for HCC, and qualifying for liver transplantation may be a favorable prognostic factor in surgical patients. Local/regional therapy prior to transplantation may provide a bridge to OLT without an increase in tumor-related mortality. (J GASTROINTEST SURG 2001;5:638-645.)

KEY WORDS: Hepatocellular carcinoma, liver resection, liver transplant, prognosis

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies throughout the world, causing more than one million deaths every year.[1] The worldwide geographic distribution of HCC varies greatly, likely because of differences in the major etiologic factors. Whereas the incidence of HCC is low among white populations in northern Europe, the United States, and Australia, it remains high among black populations in Mozambique and sub-Saharan Africa, and Asians in the Far East.[2] In the United States the American Cancer Society estimated 15,300 new cases of primary liver cancer for the year 2000, representing the 3% of estimated cancer deaths in men.[3] In Asia, where the disease has a much higher prevalence, the epidemiology of HCC is distinct. Patients are younger and chronic hepatitis is the most common risk factor, as opposed to alcoholism in the United States.[4] The current treatment options and their success rates vary significantly.

Most reported series studying prognostic indicators and therapeutic options for HCC have been performed in Asian countries with few reports from

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Western medical centers.[4-8] Most studies have focused on surgically treated patients, but there is little in the recent literature describing outcome related to the baseline patient characteristics among all patients with HCC seen at a single Western institution.

In the present study we examined the clinicopathologic characteristics of patients presenting with HCC to the University of Alabama at Birmingham, a tertiary referral center, during the past 10 years. We examined the prognostic factors associated with overall survival and the results of diverse nonsurgical and surgical treatment approaches including orthotopic liver transplantation (OLT).

MATERIAL AND METHODS

All patients with a histologic diagnosis of HCC recorded by the Tumor Registry at the University of Alabama at Birmingham in the 10-year period between January 1990 and December 1999 were included in the present analysis. We reviewed their medical records and examined demographics (i.e., age, sex, and race), clinical or pathologic stage, the presence of cirrhosis, hepatitis serology, serum biochemical values (albumin, bilirubin, lactate dehydrogenase, alkaline phosphatase, and alpha-fetoprotein), type of treatment, and outcome. The date of last follow-up was January 31, 2000.

Ablative surgery was defined as resection, including OLT, as well as cryotherapy and radiofrequency ablation (RFA) of liver lesions (surgical group). Nomenclature for the extent of resection is that defined by Goldsmith and Woodburne.[9] All resections less than a right or left lobectomy were referred to as a wedge resection. Nonsurgical therapies included systemic chemotherapy, intra-arterial chemotherapy, chemoembolization, ethanol injection, palliative radiation therapy, and supportive care (nonsurgical group). Chemotherapy was considered an adjuvant treatment when it was administered after complete surgical ablation of liver lesions in the absence of macroscopic residual disease.

Liver function was evaluated according to Child's classification, and clinical staging of disease was performed using American Joint Committee on Cancer (AJCC) staging criteria.[10] In cirrhotic patients, the presence of hepatitis C antibody and hepatitis B surface antigen was considered diagnostic for the corresponding virus as the etiologic factor of cirrhosis.

Overall survival was the end point of the study and was determined from the time of diagnosis. Perioperative mortality was defined as death within 30 days of operation. The chi-square test or Fisher's exact test, where appropriate, was used for comparisons. Actuarial survival was calculated using the methods of Kaplan and Meier,[11] and the curves were compared using the Cox-Mantel log-rank test. Multiple regression analysis was performed on all variables found significant by univariate analysis with the aid of an SPSS statistical package (SPSS Inc., Chicago, Ill.). Differences were considered significant at $P < 0.05$.

RESULTS

All Patients

During the 10-year study period, 122 patients with HCC were identified. The median age was 62 years (range 8 to 81 years). Patient characteristics are listed in Table I. There were 90 men (74%) and 32 women. Most patients were white (79%) and less than 2% were of Asian heritage. Eighty patients (66%) had associated cirrhosis. In contrast to other Western series, hepatitis C infection was the most common cause of underlying liver disease; 56% of cirrhotic patients were positive for hepatitis C virus (HCV) antibody. Sixty-three percent of patients had AJCC stage III or IV disease at presentation. Mean tumor size was 6.1 cm (range 0.7 to 20 cm). Only 10% of patients had tumors smaller than 2 cm. Seventy-one patients (58.2%) had tumors larger than 5 cm.

Fifty-seven patients (47%) underwent surgical exploration; of these, 44 (77%) underwent some type of ablative procedure and constitute the surgical group. Eleven patients underwent celiotomy and biopsy, one patient had a cholecystectomy, and one patient had a peritoneovenous shunt placed for palliation of ascites. For survival analysis, these patients were considered together with the nonsurgical group.

Median follow-up time for survivors was 22 months (range 1 to 71 months). At the time of last follow-up, 84 patients (69%) had died as a result of HCC, 27 patients (22%) were alive with no evidence of disease, six patients (5%) were alive with recurrent or persistent disease, and five patients (4%) had died of non–tumor-related causes. The median survival for all patients was 10 months (95% confidence interval [CI] = 7 to 13). The 1-, 3-, and 5-year actuarial survival rates were 46%, 24%, and 17%, respectively.

By univariate analysis, the factors associated with long-term outcome are depicted in Table II. Factors not influencing outcome were sex, ethnicity, age, cirrhosis, and Child's status. Multivariate analysis was performed using those variables found significant on univariate analysis. Ablative surgery ($P = 0.003$), AJCC stages I and II ($P = 0.0012$), and the absence of vascular invasion ($P = 0.0001$), were found to be independent favorable characteristics. Survival as related to therapy and AJCC stage are depicted in Figs. 1 and 2, respectively.

Table I. Characteristics of patients with hepatocellular carcinoma

Characteristics	All patients (n = 122)	Surgical group (n = 44)	Nonsurgical group (n = 78)	*P* value
Age (mean ± SD)	58.1 ± 15.0	55.9 ± 14.7	59.1 = 15.1	NS
Median age (range)	62 (8-81 yr)	59 (11-75 yr)	63 (8-81 yr)	NS
Sex (M/F)	90/32	31/13	59/19	NS
Race				NS
White	96 (78.7%)	33 (75%)	63 (80.8%)	
Black	21 (17.2%)	9 (20.5%)	12 (15.4%)	
Asian	2 (1.6%)	0	2 (2.5%)	
Other	3 (2.4%)	2 (4.5%)	1 (1.3%)	
Cirrhosis	80 (65.6%)	33 (75%)	47 (60.3%)	
Child's status				NS
A	18	9	9	
B	25	12	13	
C	37	12	25	
Cause of cirrhosis				NS
Hepatitis B	11	2	9	
Hepatitis C	32	14	18	
Both	1	0	1	
ETOH	10	2	8	
ETOH + HCV	12	5	7	
Other	14	10	4	
AJCC stage				0.0001
I	7	7	0	
II	38	24	14	
IIIA	11	4	7	
IIIB	18	4	14	
IVA	39	5	34	
IVB	9	0	9	
No. of lesions				0.0001
1	71	37	34	
>1	51	7	44	

ETOH = ethanol; HCV = hepatitis C virus; NS = nonsignificant; SD = standard deviation.

Table II. Factors affecting prognosis of hepatocellular carcinoma (all patients: N = 122)

Factor		Median survival (mo)	95% CI	*P* value
Ablative surgery	Yes	84		0.00001
	No	7	5-9	
AJCC stage	I-II	37	19-55	0.00001
	III	9	7-11	
	IV	4	1-7	
Lobe	Unilobar	13	9-17	0.003
	Bilobar	3	0-12	
No. of lesions	1	15	8-22	0.00001
	>1	7	3-11	
Size of tumor	<5.0 cm	14	11-17	0.004
	>5.0 cm	8	6-10	
Vascular invasion	No	84		0.00001
	Yes	7	5-9	
Satellite lesions	No	84		0.00001
	Yes	7	4-10	
Alkaline phosphatase	<150 IU/ml	14	8-20	0.0007
	>150 IU/ml	7	5-9	
LDH	<200 IU/ml	14	6-22	0.01
	>200 IU/ml	8	5-11	

CI = confidence interval; LDH = lactate dehydrogenase.

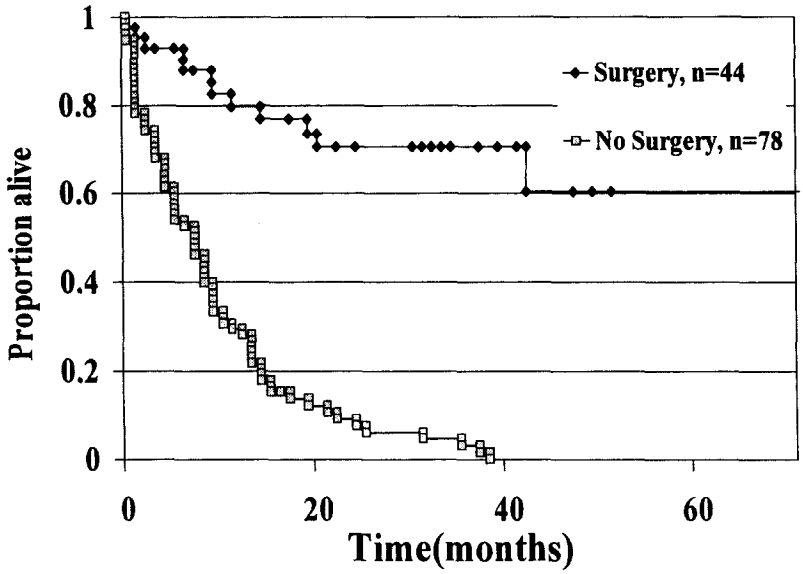

Fig. 1. Survival of patients according to therapy (N = 122) (P = 0.00001).

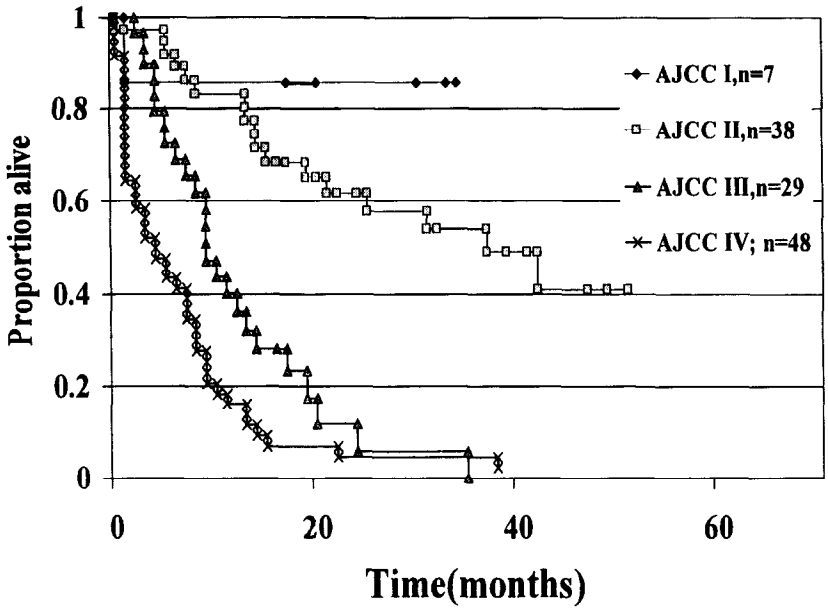

Fig. 2. Effect of stage of disease on survival for all patients (N = 122) (P = 0.00001).

Table III. Type of surgical procedure for treatment of hepatocellular carcinoma (n = 44)

Procedure	No. of patients	%
Resection		
Trisegmentectomy	3	6.8
Lobectomy	10	22.7
Wedge	4	9.1
Orthotopic liver transplantation	20	45.5
Radiofrequency ablation	5	11.4
Cryotherapy	2	4.5

Table IV. Favorable prognostic factors in surgical patients (n = 44)

Factor	*P* value
Univariate analysis	
No vascular invasion	0.0001
Tumor size <5 cm	0.0007
AJCC stage I or II	0.002
Negative margin	0.008
Orthotopic liver transplantation	0.02
Multivariate analysis	
No vascular invasion	0.002
Tumor size <5 cm	0.06*

*Marginally significant.

Surgical Group

Forty-three patients underwent surgical ablative procedures. Clinicopathologic characteristics of these patients are depicted in Table I. Similar to the entire population, most patients were male (70%) and white (75%). The proportion of patients with cirrhosis was higher in the surgical (75%) than in the nonsurgical group (60%), but the difference was not statistically significant ($P = 0.11$). The Child's status of cirrhotic patients did not differ between surgical and nonsurgical groups ($P = 0.18$). The proportion of patients with AJCC stage IV at presentation was significantly lower than in the nonsurgical group (11% vs. 55%; $P = 0.0001$), as was the proportion of patients with more than one liver lesion (16% vs. 56%; $P = 0.0001$).

The surgical procedures performed are listed in Table III. Twelve patients (27%) had some type of surgical complication, including two patients who required repeat transplantation because of hepatic artery thrombosis. At the time of last follow-up, both patients were alive with no evidence of disease 34 and 37 months after diagnosis. The median hospital stay was 8 days (range 1 to 48 days). Three patients died in the postoperative period for a surgical mortality rate of 6.8%.

Twenty patients underwent OLT. In seven there was an incidentally discovered HCC in the explanted liver. Eight transplant patients (40%) received some type of treatment before liver transplantation: four were treated with chemoembolization, two with ethanol injection, one with intra-arterial chemotherapy, and one with cryotherapy. Four patients (9.1% of the surgical group) received adjuvant chemotherapy.

The actuarial 1-, 3-, and 5-year survival rates for the surgical group were 80%, 71%, and 61%, respectively. Fifteen patients (34.1%) had a recurrence. Eleven patients had recurrences in the liver only, one had a synchronous liver and lung recurrence and the three remaining sites of recurrence were bone, lung, and central nervous system. Two of the transplanted patients presented with recurrences in the liver and died of disease 9 and 33 months after transplantation. The median time to recurrence for the entire surgical group was 9 months (range 3 to 37 months).

Factors significantly associated with outcome in surgical patients are listed in Table IV. The single most important factor associated with adverse outcome was the presence of vascular invasion. Alpha-fetoprotein values, per se, were not associated with overall survival; however, they were significantly correlated with vascular invasion ($P = 0.01$, chi-square test). OLT was significantly associated with a favorable prognosis on univariate ($P = 0.015$) but not on multivariate ($P = 0.08$) analysis (Fig. 3). Clinical and pathologic characteristics of transplant patients were similar to those of the other surgical patients, except for the higher rate of cirrhosis and smaller tumors (95% vs. 58% and 4.0 cm vs. 6.4 cm, respectively; $P < 0.03$) in transplant patients. The actuarial 3-year survival for transplant patients was 85%, despite 25% having tumors larger than 5 cm. Survival rates did not differ between transplant patients with known HCC before transplantation (mean 40 months; 95% CI 30 to 51) and those with HCC discovered incidentally in the explanted liver (mean 64 months, 95% confidence interval 51 to 77; log-rank test $P = 0.52$).

On univariate analysis, positive histopathologic margins were significantly associated with adverse outcome ($P = 0.01$). No demographic characteristics were associated with poor prognosis. Adjuvant therapy was associated with decreased survival but it was not statistically significant ($P = 0.2$). Neither the presence of cirrhosis nor Child's status was found to be a significant predictor of outcome in the surgical group.

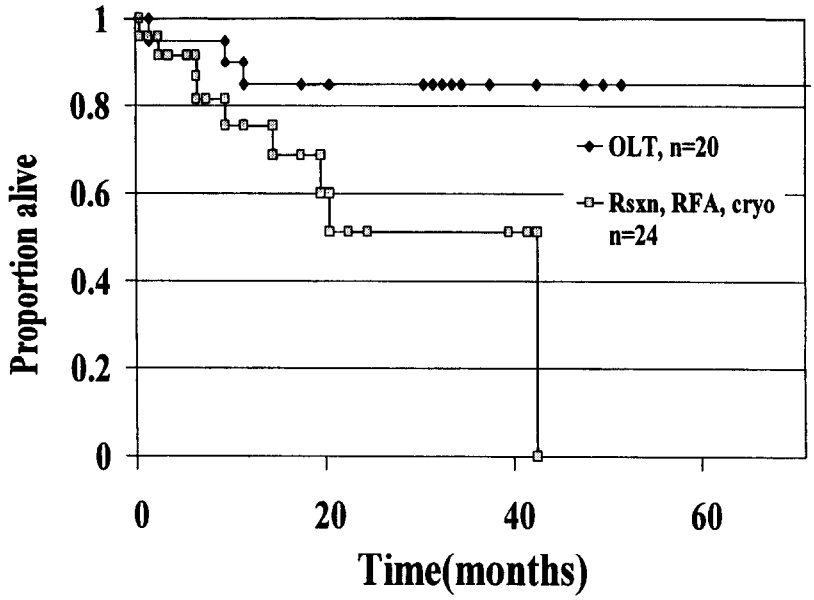

Fig. 3. Survival of transplant and nontransplant patients in the surgical group (n = 44) ($P = 0.02$). OLT = orthotopic liver transplantation; RFA = radiofrequency ablation; Rsxn = resection; cryo = cryoablation.

Table V. Favorable prognostic factors in nonsurgical patients (n = 78)

Factor	*P* value
Univariate analysis	
Chemoembolization/ OH injection	0.0004
AJCC stage I or II	0.001
Child's status A or B	0.02
Multivariate analysis	
AJCC stage I or II	0.02
Chemoembolization/ OH injection	0.02

Nonsurgical Group

Seventy-eight patients underwent nonsurgical therapy or supportive care. Demographic and histopathologic characteristics of these patients are listed in Table I.

Thirty-three patients (42%) received some type of treatment. The most common treatment was chemoembolization (14 patients), followed by systemic chemotherapy (9 patients) and intra-arterial chemotherapy (6 patients). Three patients were treated by ethanol injection and one patient received palliative radiation therapy.

The median survival in this group of patients was 7 months (95% CI 5 to 9). The actuarial 1-year survival was 29% and only one patient survived longer than 3 years. Factors significantly associated with outcome in the nonsurgical group are depicted in Table V. Tumor size was not a significant predictor of outcome, whereas poor Child's status was significant, which is different from the surgical group. Treatment with chemoembolization, chemotherapy, or ethanol injection was significantly better than supportive care (median survival of 12 months vs. 3 months; $P = 0.0004$).

DISCUSSION

Risk factors contributing to the development of HCC have been well described. Worldwide, the most common of these are chronic infections with hepatitis B (HBV) and C viruses (HCV), aflatoxin exposure, and hemochromatosis. In the United States, alcoholic liver damage has been described as the most likely etiology. Stuart et al.[4] reported a history of alcohol abuse in 43% of their patients. In this series, 66% of patients presented with cirrhosis and the most common cause was hepatitis C infection (56% of cirrhotic patients). Alcohol abuse was reported by only 18% of this group, but more than half of these patients had evidence of concomitant HCV infection. Viral infection has been described as the most common cause of cirrhosis in patients with HCC in other Western series. In the series of Fong et al.,[5] 67% of cirrhotic patients had evidence of HCV or HBV infection, but the latter was much more frequent

(53%). These epidemiologic differences are likely due to the fact that the present series encompasses more recent years, where HCV infection has become more easily determined.[12] In fact, serologic testing for hepatitis C has been widely available only since 1991.[13] Our lower incidence of HBV infection compared with other Western series is likely due to the low number of Asian patients in the present series (1.6%).

Few Western studies have examined the clinical prognostic factors of HCC in all patients regardless of treatment.[4,5,14] We recognize that there is a clear selection bias in favor of surgical patients, but we do not believe that a prospective randomized trial that included a control arm would be feasible or ethical. We found that prognostic factors affecting survival are different between surgical and nonsurgical patients. Although the stage of disease was different in the two populations, other demographic and clinical characteristics were similar.

Ablative surgery (resection including OLT, RFA, or cryotherapy), stage of disease, and vascular invasion were found to be independent predictors of outcome in the entire population. The survival of patients who underwent ablative surgery (5-year survival = 61%) compares favorably with that in other Western[4,5,14] and Eastern[15,16] series. We were not able to demonstrate a significant difference between resection and other ablative techniques, but the number of patients who underwent RFA or cryotherapy was too small to analyze separately.

The AJCC staging system has not been universally adopted for staging of HCC, probably because it does not incorporate liver function as a staging criterion.[17] However, in the present study we found it to be a significant predictor of outcome in the entire study population and in nonsurgical patients. Alternatively, Child's status as a measure of liver function was found to be a predictor of survival only in the nonsurgical group.

HCC has a great tendency for intravascular extension.[18] This pathologic characteristic has been found to be an independent predictor of adverse outcome in the present series and others.[4,5,15] Similar to Fong et al.,[5] we found a significant correlation between alpha-fetoprotein level and vascular invasion. Alpha-fetoprotein level has been reported to be significantly associated with outcome in other series.[4,5,15] In the present series it was not significantly associated with overall survival. It may be that alpha-fetoprotein level is a surrogate marker of vascular invasion and not an independent prognostic factor. However, it remains very useful because it can be measured preoperatively. Because of its prognostic significance for a poor outcome, patients with vascular invasion should be considered for adjuvant therapy trials.

OLT is a potentially curative option in patients with cirrhosis and HCC.[6-8] The observed 3-year survival of 85% in the present analysis compares favorably with other recent reports of OLT in patients with HCC.[7,19,20] In fact, OLT was found to be a favorable prognostic factor for survival in surgical patients on univariate analysis. In the present series, as in others, the median size of the tumor was significantly smaller in transplanted than in resected patients. However, other clinicopathologic characteristics were similar between these groups. Alternatively, more than 40% of transplant patients underwent treatment before transplantation (chemoembolization, cryotherapy, or intraarterial chemotherapy) and the outcome in these patients was no different from that in other transplant patients. Based on these observations, the size of the tumor, per se, may not represent an absolute contraindication for liver transplantation, and the use of other therapeutic options in a neoadjuvant setting is a feasible option for bridging cirrhotic patients with HCC to potential transplantation.

The recurrence rate of 34% in the surgical group is consistent with that in other series.[4,5,14-16] Most recurrences are in the liver only, according to the model of multifocal carcinogenesis in the liver, and liver transplantation eliminates this "field cancerization" effect. Effective adjuvant therapies are sorely needed. In the present series, adjuvant chemotherapy was associated with a trend for decreased survival, but it was because patients who received adjuvant treatment had more advanced disease.

The outcome of patients who were not candidates for surgical treatment was dismal. The median survival in this group of patients was only 7 months. In this group, different from surgical patients, factors associated with outcome were more related to the functional status of the liver. Patients who were candidates for nonsurgical therapy appeared to have a significantly better outcome, but this was likely due to better performance status and ability to undergo any therapy. The small number of patients who received each therapeutic modality did not allow us to reach any definite conclusions regarding their efficacy.

CONCLUSION

These data showed a changing epidemiology regarding the cause of cirrhosis at this Western medical center. Factors associated with outcome are different between surgical and nonsurgical candidates, and this should be taken into account when stratifying patients who enter studies evaluating the effectiveness of therapeutic procedures or adjuvant therapies. Surgical treatment is the only potential curative option for HCC, and selective liver transplantation seems to be

an effective alternative for those with underlying cirrhosis. Effective adjuvant treatment is needed because of the continued high rate of recurrence.

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Effect of Interstitial Laser Hyperthermia in a Murine Model of Colorectal Liver Metastases

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Interstitial laser hyperthermia (ILH) is an in situ ablative technique used for the treatment of colorectal liver metastases. At present, few data exist concerning the optimum power settings required to maximize tissue necrosis.The aim of this study was to establish the dose-response relationship between the laser power setting and the extent of tissue necrosis produced in liver and tumor tissue, as well as the pattern of necrosis in a murine model of liver metastases. An intrasplenic induction model of liver metastases in 4- to 6-week-old male inbred CBA mice was used. Laser hyperthermia was applied to liver and tumor tissue using a bare optical quartz fiber from a Laserex SLY500 Nd:YAG surgical laser generator. Two-watt and 5-watt power settings were used at specific time intervals. The livers were then excised, fixed in formalin, and the extent and degree of necrosis were measured. Results were expressed as mean ± standard deviation and were normally distributed. Analysis of variance was performed, and the least significant difference was used for post hoc tests. A P value of less than 0.05 was considered significant. Interstitial laser hyperthermia at 5 watts of power produced larger diameters of necrosis than did 2 watts for specific exposure times in normal liver tissue. However, when the total energy applied was compared, there was no significant difference in the diameters of tissue necrosis produced by the two power settings. The diameter of tissue necrosis in the normal liver increased from 2 mm at 10 joules to 8 mm at 600 joules of energy. Within tumor tissue, ILH at 2 and 5 watts produced similar diameters of necrosis for specific exposure times. When amounts of total energy applied were compared, ILH at the lower power setting (2 watts) produced a significantly larger diameter of necrosis than the higher power setting (5 watts). The diameter of necrosis achieved in tumor tissue was significantly larger than that in normal liver tissue at both power settings, for an equivalent amount of applied energy. The difference was more pronounced when ILH was performed at the lower power setting. The maximum diameter of necrosis achieved was 6.8 ± 0.7 mm in normal liver tissue and 7.7 ± 0.8 mm in tumor tissue. Charring of the fiber tip was delayed when the lower power setting was used, occurring after 20 seconds of exposure, compared to 5 seconds at the higher power setting. Similarly, cavitation occurred initially at 50 seconds at 5 watts of power and was delayed until 90 seconds of exposure at 2 watts of power. Histopathologic findings revealed an elliptical area of homogeneous necrosis, with a central acellular coagulum surrounded by intact but nonviable tissue. ILH is capable of producing highly reproducible, uniform, and complete tissue necrosis. The diameter of necrosis is related to the total energy applied. At low-power settings at any given amount of applied energy, a significantly larger diameter of tissue necrosis was achieved in tumor tissue compared to normal liver tissue. (J GASTROINTEST SURG 2001;5:646-657.)

KEY WORDS: Colorectal carcinoma, liver metastases, interstitial, laser, hyperthermia

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There is an increasing trend toward the use of in situ ablative techniques such as interstitial laser hyperthermia (ILH) in the treatment of focal liver metastases.[1,2] Although ILH for focal colorectal liver metastases has been in clinical use for more than a decade, there is a lack of information on long-term survival. In addition, information on the characteristics and pattern of tumor destruction, the differential response of normal liver and tumor tissue to ILH, and the most efficient mode of energy application is limited. This study characterizes the different responses of tumor and liver tissue to ILH in a murine model of colorectal liver metastases.

MATERIAL AND METHODS

Liver Metastases Model

A murine model of colorectal liver metastases created by intrasplenic injection of dimethylhydrazine-induced colorectal cancer cells was used in this study. This model has been previously fully characterized in our laboratory and utilizes 4- to 6-week-old inbred male CBA mice.[3,4] Optimum tumors greater than 10 mm in diameter were chosen for this experiment. Experiments were carried out with approval from the Monash University Animal Experimentation and Ethics Committee. Mice were maintained on irradiated standard mouse feed and water ad libidum.

Preparation of Cell Suspension

Mice with flank tumors were killed by anesthetic overdose, after which the tumors were dissected out, minced, and dissociated by incubation with 0.1% trypsin in 0.1% glucose for 10 minutes. Trypsin was inactivated by an equal volume of Dulbecco modified eagle medium and centrifuged for 5 minutes at 1000 rpm (model TJ-6 centrifuge, Beckman Coulter, Inc., Palo Alto, Calif.). The pellet was resuspended in 5 ml 0.02% EDTA in phosphate-buffered saline and centrifuged again in a similar manner. The second pellet was resuspended in Ringer's solution with 0.1% glucose, a viable cell count was performed by trypan blue exclusion (0.05% trypan blue in distilled water), and the cell concentration was adjusted to 1×10^6 cells/ml.

Induction of Metastases

Mice were anesthetized with a mixture consisting of 2% ketamine and 0.1% xylazine in 0.9% saline solution at a dosage of 0.05 to 0.1 ml/10 gm body weight administered intraperitoneally. The spleen was exteriorized through a left subcostal incision, and 0.1 ml of tumor cell suspension (100,000 cells) was slowly injected into it using a 30G needle. The spleen was compressed for 2 minutes to prevent spillage and allow the tumor cells to enter the portal circulation. Splenectomy was then performed with cautery, hemostasis was achieved, and the laparotomy was closed in layers. Animals were observed until they recovered and were then monitored daily for 21 days.

Interstitial Laser Hyperthermia

Animals with established liver metastases on day 21 following induction of tumors and a control group of normal animals had anesthesia induced by intraperitoneal injection of 2% ketamine and 0.1% xylazine. Suitable tumors (>10 mm) were chosen and a 600 μm diameter bare optical quartz fiber was inserted into the center of the tumor for application of laser hyperthermia. In the control mice, the fiber was inserted into normal liver tissue. The optical fibers were connected to an SLY5100 Nd:YAG laser source (Laserex International, Adelaide, Australia), which produced laser with a wavelength of 1064 nm. Three separate study groups were used in this study.

Group A. Animals with liver metastases used in this group were killed immediately after laser hyperthermia. Following cessation of treatment, the livers were removed for quantitative and histologic evaluation. In this group two power settings—2 watts and 5 watts—were used. At each power setting the laser was activated for specific time exposures of 5, 10, 15, 20, 50, 70, 90, and 120 seconds. A total of 10 experimental results were obtained for each specific setting.

Group B. In this group the effect of ILH on normal liver tissue was assessed by means of power and exposure settings equivalent to those used in group A.

Group C. In this group the delayed tissue effects of ILH were assessed. Animals with normal livers were subjected to laser hyperthermia at a power setting of 5 watts for 15 seconds.

Following cessation of therapy, the animals were allowed to recover; they were subsequently killed after 7 or 21 days and assessed in a similar manner to those in group A. Several measurements of the diameter of each thermal injury were taken and the mean was calculated. The relationship between the total energy applied and the extent of tissue necrosis in normal liver and tumor tissue was determined.

Laser Doppler Flometry

Following anesthesia and laparotomy, blood flow in the liver and tumor was measured using a Laserflo blood perfusion monitor (model 403A, TSI, Inc., St.

Paul, Minn.). After initial stabilization for 10 minutes, blood flow measurements were obtained from normal liver and tumor tissue at 1-minute intervals. In tumor tissue, readings were taken from the center and periphery. Eight readings were taken from each point and the mean was calculated. The relative blood flow of the tumor was expressed as a percentage of normal liver blood flow in each animal.

Statistical Analysis

Results were expressed as mean ± standard deviation and were normally distributed. They were compared using a multiple analysis of variance, and a post hoc comparison was carried out using a least significant difference test (Statsplus, StatSoft, Inc., Tulsa, Okla.). Probability values less than 0.05 were considered significant.

RESULTS

Relationship of Power, Exposure Time, and Size of Necrosis

In *normal liver tissue* there was an initial rapid increase in the size of the area of necrosis, which tapered off after 20 seconds of exposure. A power setting of 5 watts produced a significantly larger diameter of necrosis than 2 watts for equivalent exposure times (Fig. 1, *A*). Maximum diameters of necrosis achieved after 120 seconds of exposure at 2 watts and 5 watts power were 6.8 ± 0.7 mm and 7.8 ± 1.3 mm, respectively ($P < 0.05$). However, when the energy applied was calculated (power × time) and its relationship to the size of tissue necrosis compared, no significant difference was noted between the 2-watt and 5-watt power settings (Fig. 1, *B*).

In *tumor tissue* a similar rapid increase followed by a more gradual one was noted. That is, the maximum diameter of necrosis at 120 seconds was 7.7 ± 0.8 mm and 8.5 ± 0.6 mm at 2 watts and 5 watts of power, respectively ($P < 0.05$) (Fig. 2, *A*). In contrast to normal liver tissue, when the relationship between applied energy and size of tissue necrosis was correlated, ILH at 2 watts of power produced a larger diameter of necrosis than ILH at 5 watts (Fig. 2, *B*).

When the size of tissue necrosis in normal liver and tumor was compared, necrosis in tumor tissue was significantly larger than that in normal liver tissue for equivalent power and energy settings ($P < 0.05$) (Fig. 3, *A* and *B*).

Laser Doppler Flowmetry

Blood flow in tumor tissue was lower than that in normal liver tissue, and decreased with increasing tumor size. In tumors less than 5 mm in diameter, tumor blood flow was 52.63% ± 18.46% of normal liver blood flow. This decreased to 29.83% ± 17.10% of normal liver blood flow in tumors 6 to 10 mm in diameter ($P = 0.0003$).

Macroscopic Characteristics of the Effect of Interstitial Laser Hyperthermia

The characteristic macroscopic changes in normal liver tissue during ILH are shown in Fig. 4. Initial coagulative changes were seen as a concentric white region, which increased progressively in size. This was delineated by an outer dark ring of hyperemic tissue, which separated normal-looking liver tissue from the white coagulum (Fig. 5, *A*). Cavitation was first noted to occur after ILH treatment at 5 watts of power for 20 seconds of exposure and at 2 watts of power for 70 seconds. After 120 seconds of exposure, the resultant progressive cavitation rendered further ILH treatment ineffectual because of the loss of contact between the fiber and the tissue. Charring was noted at both power settings and at all exposure times, resulting in the formation of a rim of charred tissue in contact with the optical fiber. Sections performed along the axis of the position of the quartz fibers revealed similar zones, but in an elliptical distribution pattern. When examined for late changes after ILH, at 7 and 21 days, the peripheral ring of hyperemia was absent. The central white coagulated region remained unchanged. The central cavity was partially filled with tissue. No charred tissue was visible when examined at this time.

In tumor tissue a similar sequence of events was seen during ILH. The main exception was the absence of the hyperemic zone between the coagulated and viable tumor tissue. The transition from coagulated tumor to normal tumor was therefore more gradual and not well defined. Charring was noted at all power settings and exposure times. Cavitation occurred after exposure for 10 seconds at 5 watts and 50 seconds at 2 watts (Fig. 5, *B*).

Histopathologic Characteristics of the Effect of Interstitial Laser Hyperthermia

On hematoxylin and eosin staining, normal liver architecture in the mouse closely resembles that of the human liver. Metastatic tumor was seen as discrete nodules of poorly differentiated cells. Characteristically, large areas of dilated vascular lakes were found within the center of the tumors.

Following ILH, normal liver tissue displayed a number of discreet zones, centered on the site of fiber insertion, which correlated with the macroscopic

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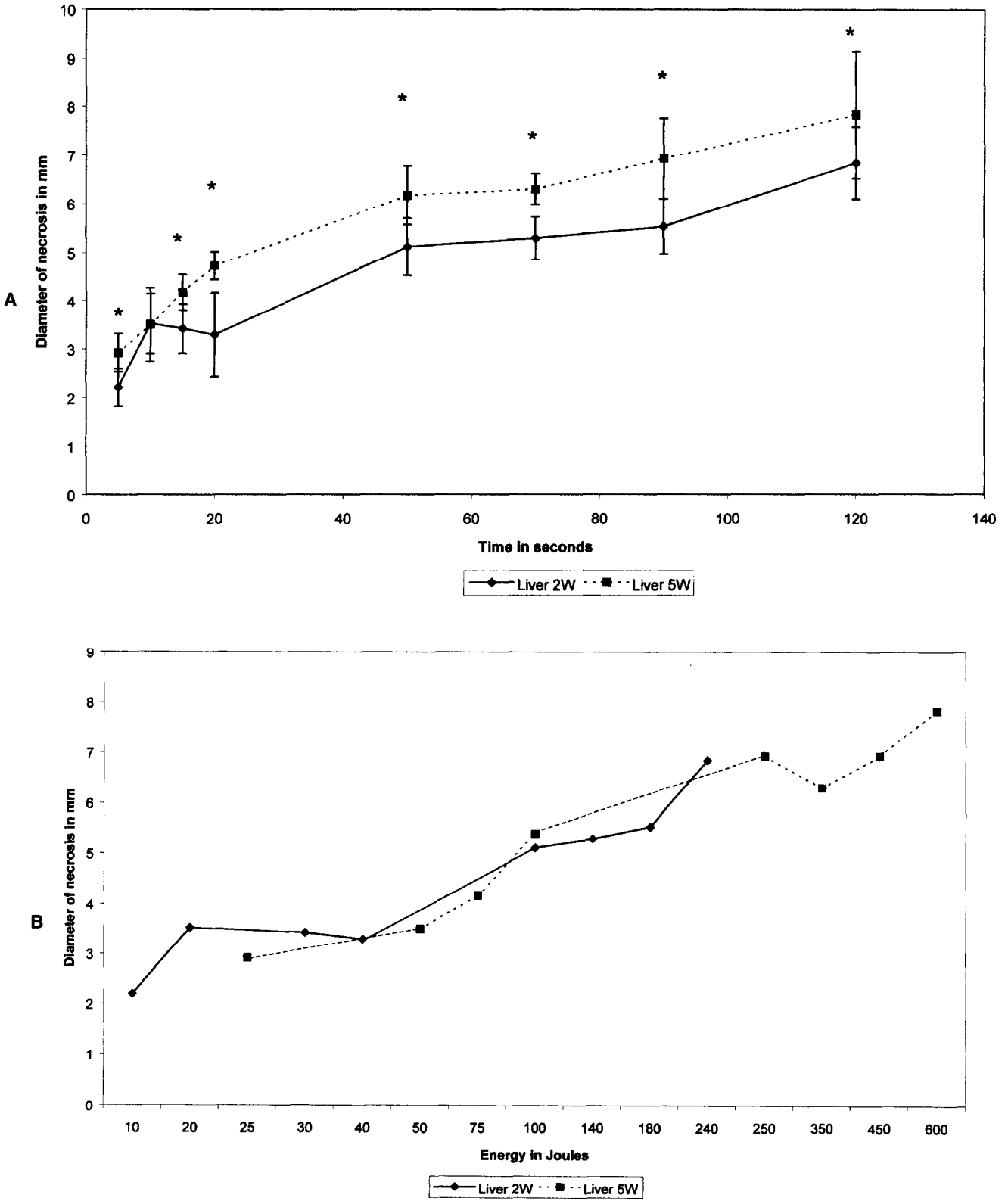
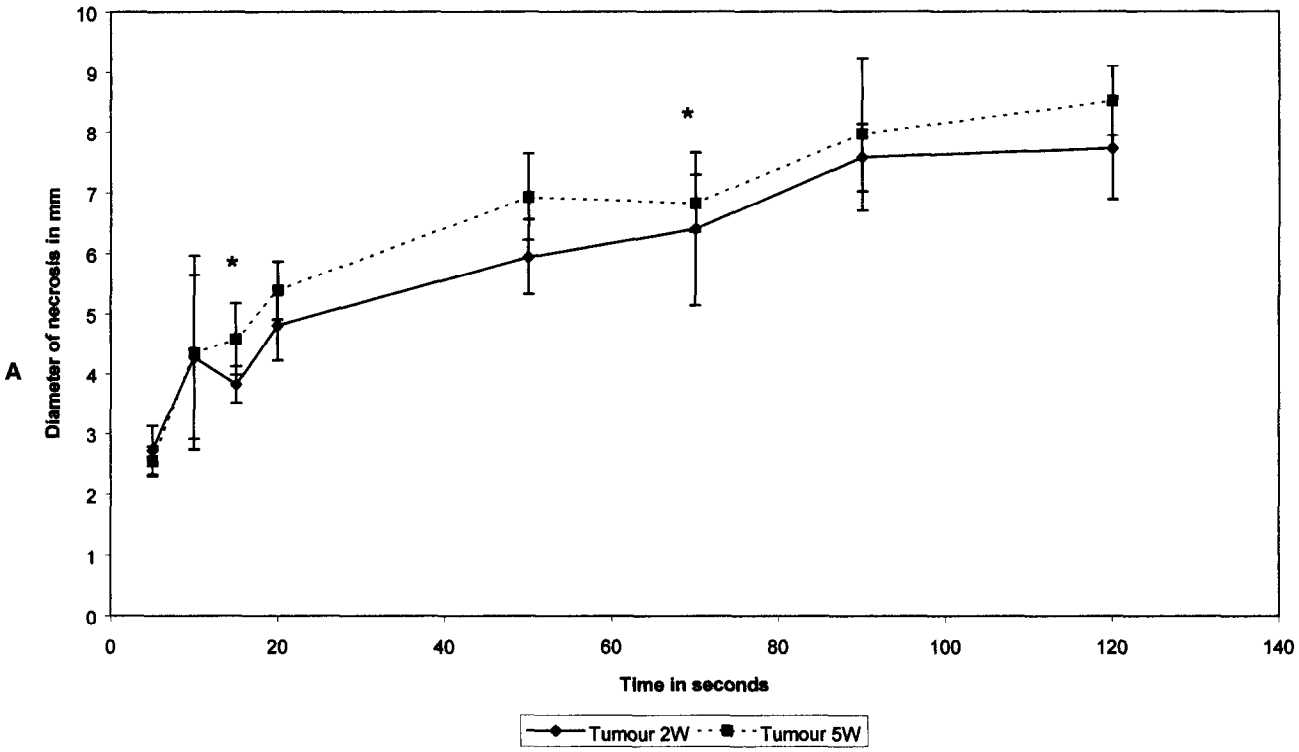


Fig. 1. Relationship of the duration of exposure to the size of necrosis at both 2- and 5-watt power settings in liver tissue. Although 5 watts of power produced significantly larger lesions **(A)**, when total energy applied was considered, the power of applied ILH was not found to be significant **(B)** (*$P < 0.05$).



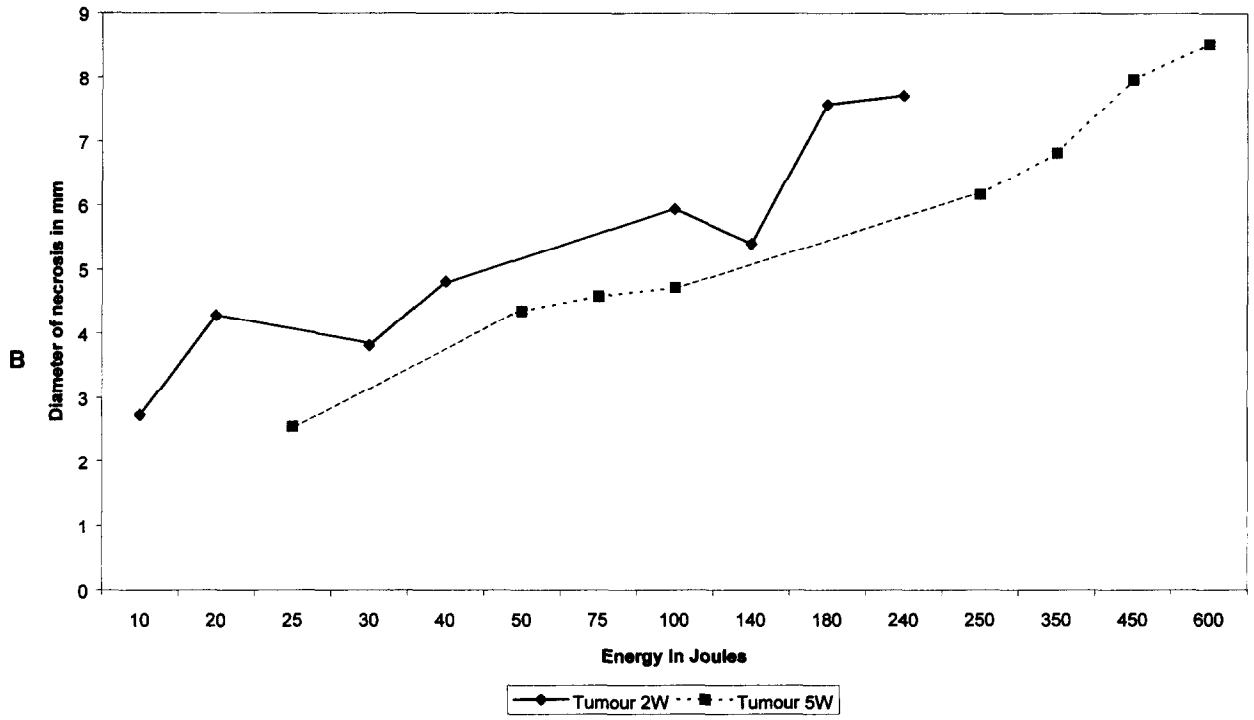


Fig. 2. Relationship of the duration of exposure to the size of necrosis at both 2- and 5-watt power settings in tumor tissue. Although 5 watts of power produced significantly larger lesions **(A)**, when total energy applied was considered, this trend was reversed, with low-power ILH producing larger lesions **(B)** (*$P < 0.05$).

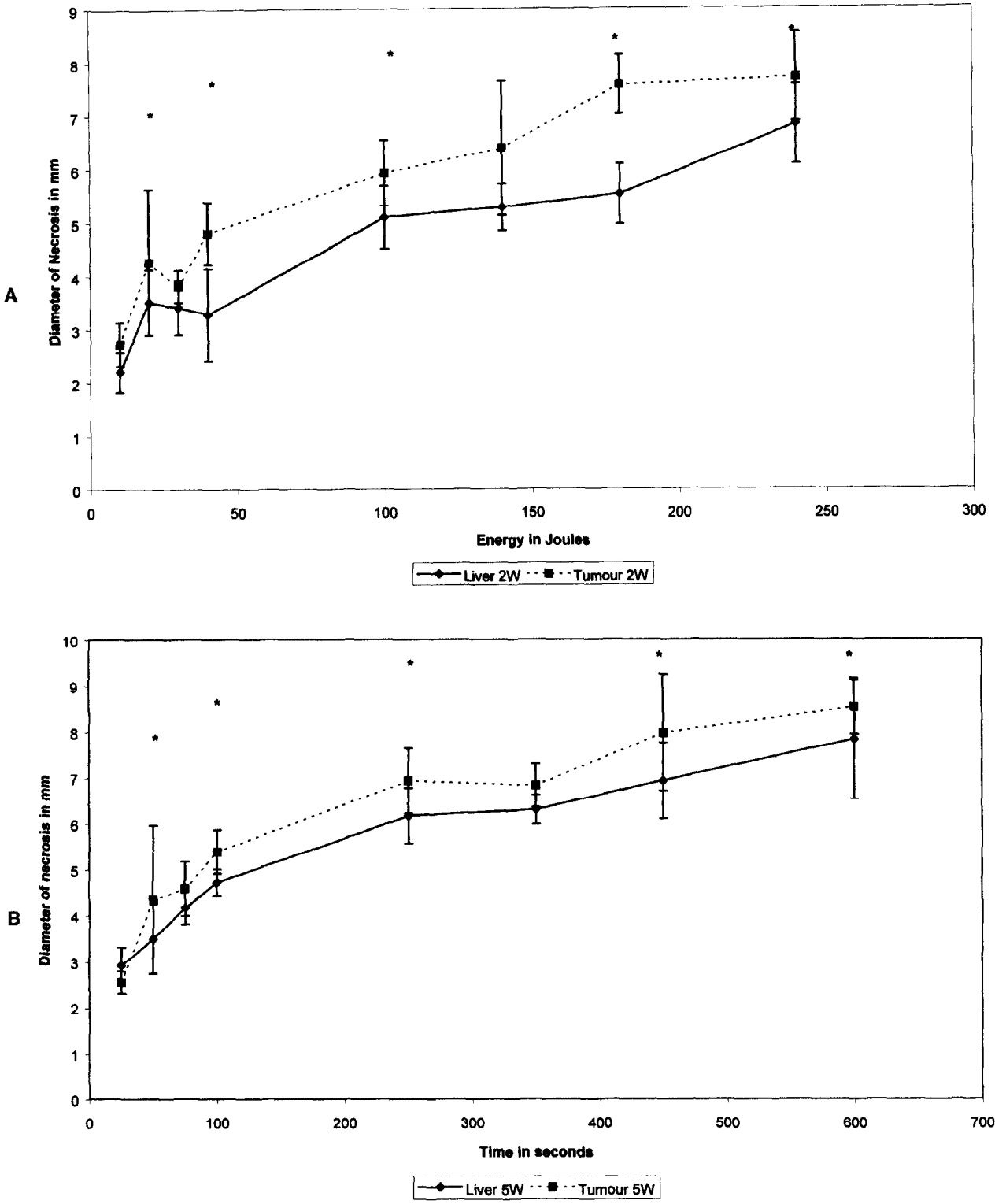


Fig. 3. Comparison of the effects of ILH on normal liver and tumor tissue. ILH produced larger lesions in tumor tissue at both 2-watt **(A)** and 5-watt **(B)** power settings (*$P < 0.05$).

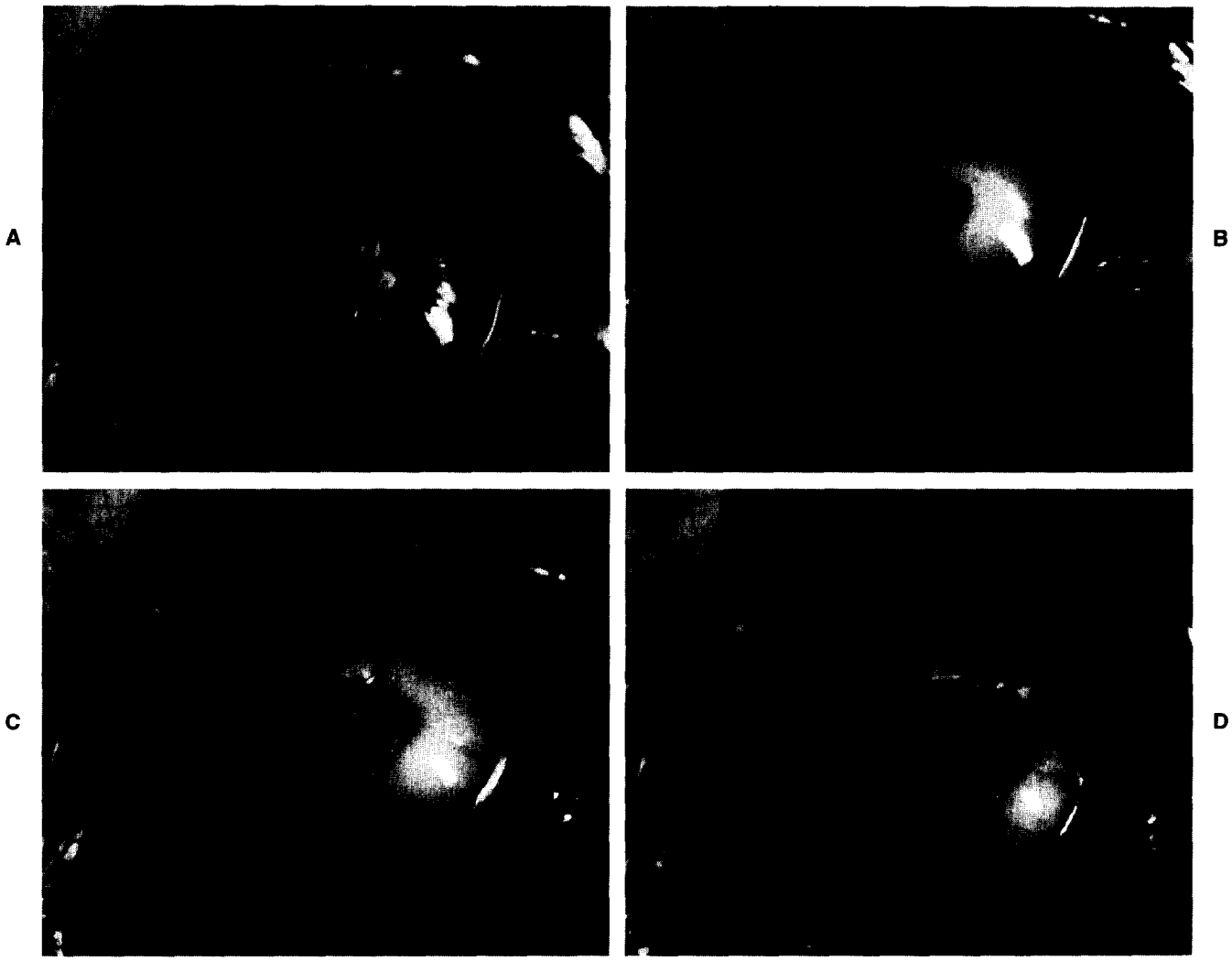


Fig. 4. Macroscopic changes seen during application of interstitial laser thermotherapy to a liver metastasis are seen in this series of photographs. **A,** Initial thermal necrosis is seen as an increasing area of white coagulum. **B,** Occurrence of carbonization can be seen. **C,** Cavitation has begun. **D,** A large cavity, resulting from tissue ablation, covered by carbonized tissue is clearly seen.

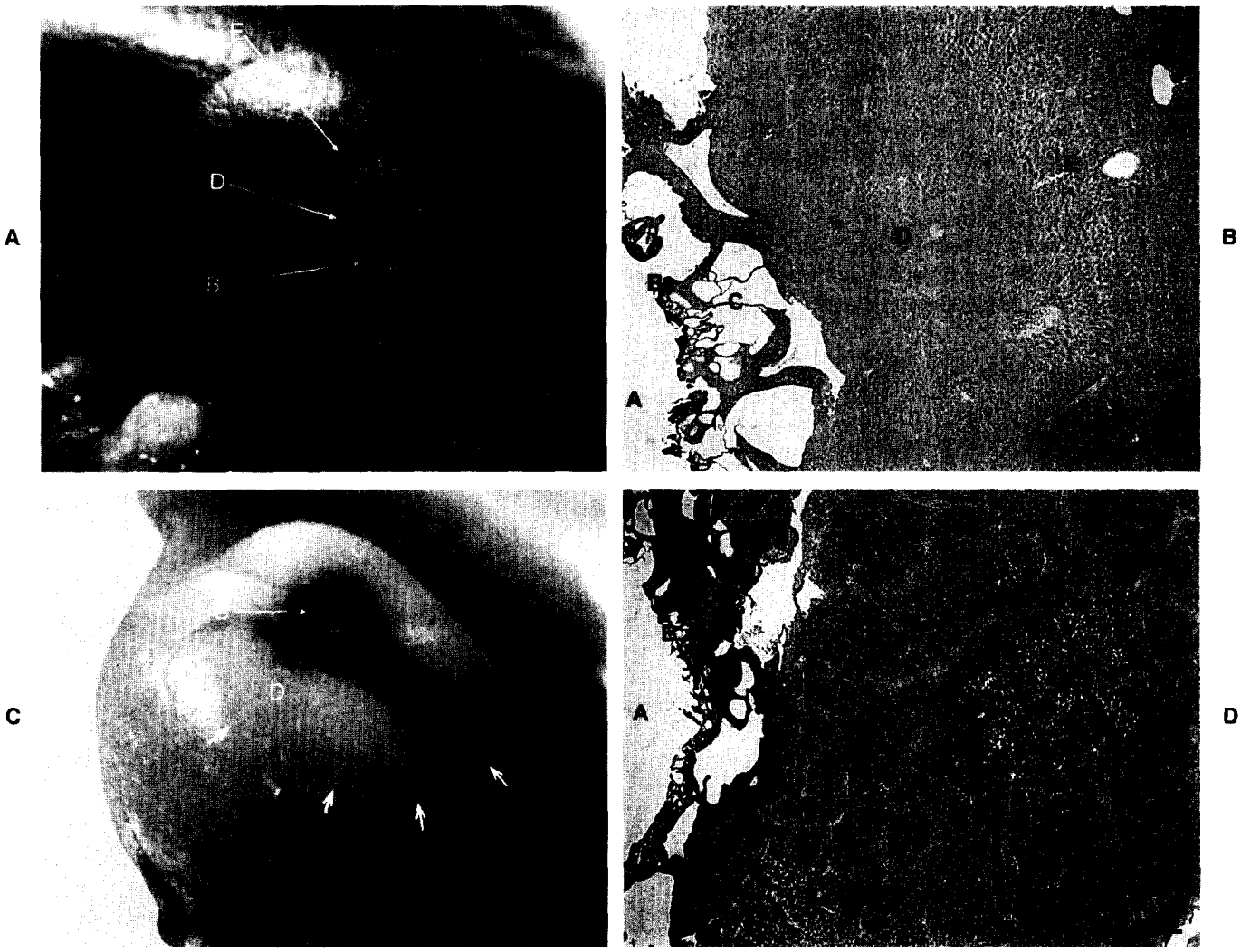


Fig. 5. ILH of normal liver tissue results in concentric areas of change. **A,** Zone I = central cavitation *(A);* zone II = layer of carbonized tissue *(B);* zone IV = coagulative necrosis *(D);* zone V = ring of hyperemia *(E).* These are confirmed under microscopy **(B),** where a further zone III, consisting of acellular ablated tissue can be identified *(C).* Similar zones and changes are seen in tumor tissue after ILH (**C** and **D**) with the notable absence of zone V. The change from coagulated tumor tissue *(D)* to viable tumor *(T)* is more gradual *(arrows).*

findings (see Fig. 5, *A* and *B*). Zone I constituted a cavity. Zone II was a narrow area of carbonization adjacent to the cavitation. Zone III was a thin rim consisting of a poorly staining, acellular coagulum. Moderate-sized vacuoles were evident. Zone IV was greatest in depth and consisted of intact cells with disordered cytoplasmic architecture. Cell borders were not visible and cytoplasmic staining was poor. This correlated with the white coagulated zone seen macroscopically (see Fig. 5, *A*). Zone V formed a thin band separating zone IV from normal liver tissue. It was seen as a pale halo zone at low magnification (see Fig. 5, *B*). This consisted of congested and dilated liver sinusoids, which correlated with the zone of hyperemia seen macroscopically.

Seven days after thermal injury, the hyperemic zone V was absent in normal liver tissue and the central cavity was filled with acellular material. Zone III (acellular coagulum) had completely replaced zone IV. fibroblast migration was noted at the surface as well as the interface with viable liver tissue. Zone V, the ring of hyperemia, was absent. These changes persisted after 21 days, with increasing fibroblast migration. No cellular structures were seen in the area of thermal injury, except for fibroblasts at the periphery (see Fig. 3, *C* and *D*).

Similar zones of varying degrees of injury were also seen in tumor tissue with two exceptions. Zone IV was consistently larger in tumors and the hyperemic zone V was absent. In contrast to normal liver tissue, there appeared to be a more gradual transition between necrotic tissue and viable tumor (see Fig. 5, *C* and *D*). A photomicrograph of a section where laser injury has been produced at the tumor-liver interface highlights this difference clearly (Fig. 6, *A* and *B*). The halo zone is seen to extend on both sides of the tumor, with its absence and the relatively larger zone IV in the tumor being clearly shown. Vacuoles were

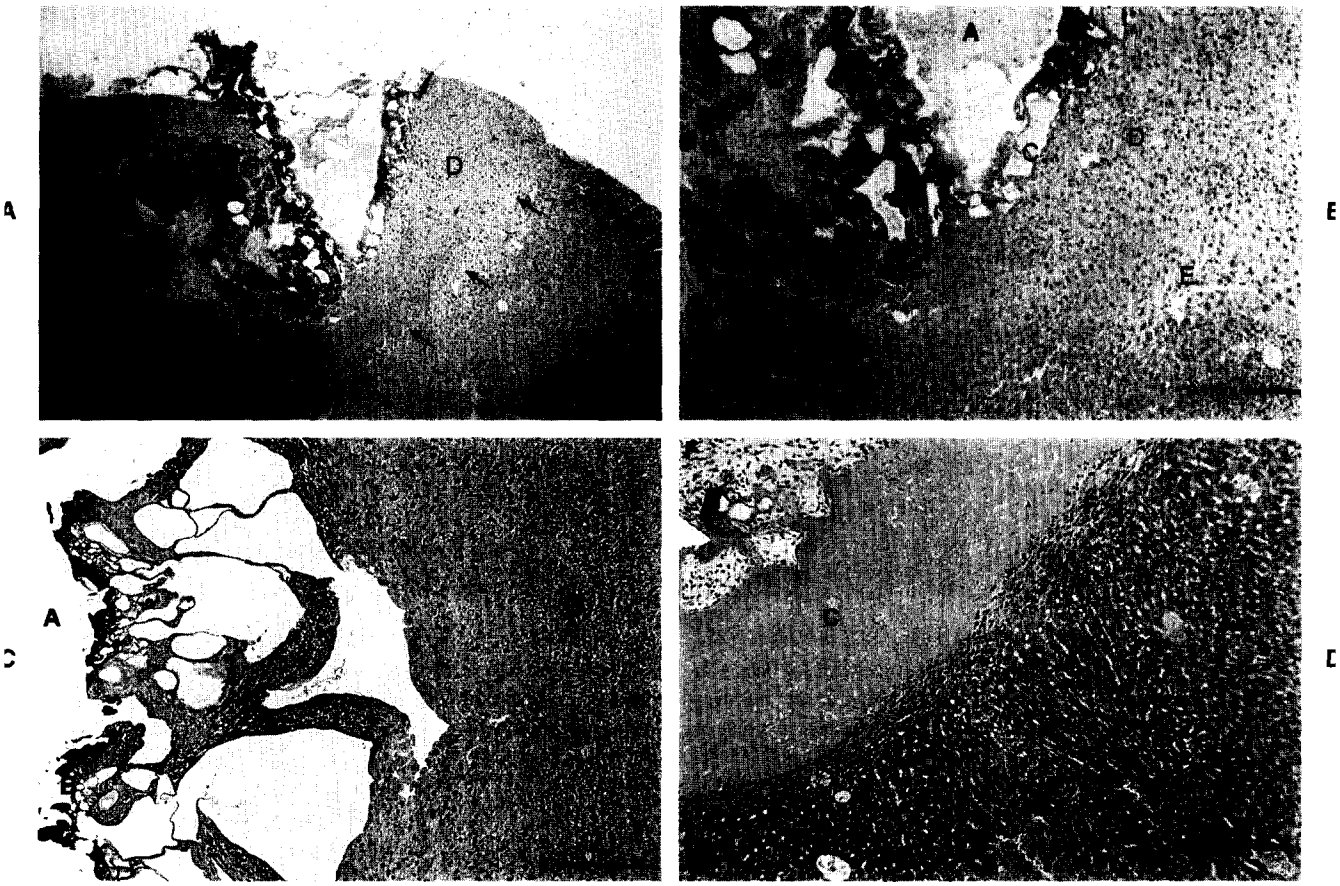


Fig. 6. Longitudinal section showing the effect of ILH at the tumor-liver interface allowing direct comparison. **A** and **B,** Note the presence of zone V in normal liver tissue and its absence in tumor tissue *(arrows)*, as well as the occurrence of vacuoles to a greater depth within tumor tissue. Comparison of sections obtained immediately after ILH **(C)** and 21 days later **(D)** shows conversion of zone IV *(D)* to acellular coagulum *(C)* with fibroblast proliferation *(F)* at the interface and surface.

also noted to form to a deeper level in tumor tissue than in normal liver tissue.

DISCUSSION

Colorectal cancer is the third most common cause of cancer deaths in Western society. The major determinant of mortality is the development of liver metastases.[5-12] Surgical resection for a suitable group of patients (10%) provides the only potential for cure at present,[13] with 5-year survival for this group approaching 40% to 50%.[14] There is an additional group of patients (10% to 15%) in whom focal disease confined to the liver is present, but resection is not possible for a number of reasons. In this group, minimally invasive technology may be applicable. ILH is one such technique. One of the major limitations of these techniques, including ILH, is the size of the tissue necrosis that can be achieved with a single optical fiber. Despite its use in the clinical setting, experimental data on the power settings used to achieve optimum tumor necrosis and the macroscopic and histologic changes achieved by ILH are limited.

The earliest studies aimed at examining the relationship between applied energy and size of tissue necrosis achieved were based on in vitro experiments conducted on a variety of animal tissues. These experiments produced tissue necrosis with diameters as large as 44 mm using low-power settings (4 to 7 watts) and long exposure times (9 to 30 minutes).[15-17] Although these studies established the capacity of ILH to produce defined areas of tissue necrosis, they lacked the physiologic responses that are present in living tissue. The earliest in vivo study conducted in normal liver tissue by Matthewson et al.[18] defined an initial exponential increase in the diameter of tissue necrosis up to 400 joules of applied energy and the subsequent plateau effect using low-power ILH (0.5 to 2.0 watts). Tissue necrosis was assessed 3 to 4 days after ILH, with a maximum diameter of 16 mm being achieved. Subsequent studies produced similar results in normal liver tissue.[19] The limited number of studies to date on the effect of ILH on tumor tissue have been conducted on in vivo models of tumor implanted into liver. These have demonstrated the ability of ILH to achieve complete tumor necrosis and a maximum diameter of necrosis of 11 mm.[20,21]

The liver metastases model used in our study was an in vivo model created by intrasplenic injection of a suspension of tumor cells. Previous characterization of this model has shown that it closely resembles the pathophysiology of the development and growth of liver metastases as well as the structure and vascularity in humans.[3,4] Tumor vasculature has been shown to consist of large disordered lakes that acquire inflow by means of multiple channels directly communicating with liver sinusoids.[4] Blood flow within tumor tissue was found to be significantly less than that in normal liver tissue. Relative blood flow at the center of the tumor decreased as the size of the tumor increased. In tumors 10 mm or more in diameter, central tumor blood flow was 29.8% ± 16.9% of normal liver blood flow ($P = 0.05$).

In the clinical situation, treatment involves ablation of larger tumors than those used in this study. The tissue that is to undergo ablation falls into three distinct categories: (1) solid tumor tissue, (2) the surrounding normal tissue, and (3) the less distinct and much smaller area in between—that is, the tumor-host interface. The aim of clinical treatment is to achieve ablation of the tumor along with a 1.0 cm margin of surrounding normal tissue. There are significant differences in the histology, growth pattern, and pathophysiology of the tumor and that of the surrounding normal liver tissue. Therefore the essential aim of this study is to establish the differences between normal liver tissue and tumor tissue in their thermal response and sensitivity to ILH.

In this study we investigated the relationship between the power setting used in ILH, the length of exposure, and the total energy applied against the size of the resultant tissue necrosis achieved in both normal liver and tumor tissue. When the size of necrosis in response to increasing exposure times was compared, for any given exposure times, 5 watts of power produced significantly larger areas of necrosis than 2 watts in normal liver tissue. However, when the size of necrosis was compared to the total energy applied, the power of the ILH was seen not to make a significant difference. The size of tissue necrosis in normal liver was therefore seen to depend on the total energy applied to the tissue regardless of the power of the ILH.

In tumor tissue a comparison of the effect of exposure time on tissue necrosis revealed that unlike in normal liver tissue, there was no significant difference. Conversely, when the total energy applied was compared to the size of necrosis, low-power ILH (2 watts) was found to produce larger areas of necrosis than high-power ILH (5 watts). Charring and cavitation were noted to occur earlier in tumor tissue when compared to normal liver tissue for equivalent energy levels. It was also noted to occur earlier with higher power ILH. When the effects of ILH on normal liver and tumor tissue were compared, at both the 2-watt and 5-watt power settings, we found that ILH was more effective in tumor tissue at any given amount of applied energy. The difference was statistically significant ($P < 0.05$) but the difference was more pronounced at the lower power setting of 2 watts.

This high rate of blood flow in normal liver tissue contributes to the rapid dissipation of heat and thereby limits the size of tissue necrosis when compared to tumor tissue. The use of higher power settings results in the application of energy at a faster rate for any given time exposure, thereby reducing the effect of heat dissipation, as seen in our experiments. Within tumor tissue, blood flow is sluggish and therefore heat dissipation is minimal. In our study we noted that carbonization and cavitation occurred earlier when ILH was applied at the higher power setting. In the absence of a "heat sink," rapid buildup of thermal energy results in a rapid increase in tissue temperature, first causing coagulation, then dessication and carbonization. The occurrence of carbonization reduces light penetration and thereby impedes heat transfer, whereas cavitation reduces interstitial contact of the probes with similar results. These effects were delayed by applying ILH at lower power settings. This results in the greater efficiency of ILH at lower power settings in tumor tissue. The absence of blood flow would account for the much larger diameters of necrosis achieved in in vitro studies.[16,17]

The most striking feature in the histopathologic study was the uniformity of tissue necrosis achieved with ILH. No viable liver or tumor cells were found in the coagulated (zone IV) area when examined immediately after ILH, 7 days later, and 21 days later. Significant differences between tumor tissue and liver tissue were noted. The central cavitation area (zone I) and the surrounding thin region of acellular coagulum (zone III) were present in both types of tissue. Carbonization was also noted in both tissue types. This was in keeping with findings in previous studies.[18,21] Zone III was deeper and vacuoles occurred deeper in tumor tissue than in normal liver tissue. This was consistent with the absent heat sump effect and resultant increase in temperature within tumor tissue. The most characteristic difference was the presence of the hyperemic zone V in normal liver tissue, separating coagulated tissue from viable liver tissue. The dilatation of these sinusoids with increased blood flow at the periphery of the treated area may play an additional part in the heat sump effect. This zone was absent in tumor tissue, signifying the loss of normal physiologic responses in tumor vasculature and contributing to the efficiency of ILH. Results of histologic examination at 7 and 21 days after ILH of both tumor and liver tissue showed fibroblast migration and healing by secondary intention. Zone IV, which consisted of poorly staining cells with intact cell outlines, was replaced by an acellular coagulum on examination at 7 and 21 days. No viable cells were found within the coagulated region.

In conclusion, interstitial laser thermotherapy is capable of achieving highly reproducible, uniform, and complete tissue necrosis. The size of the tissue necrosis is closely related to the total amount of energy applied. Laser used at low-power settings is more efficient at producing larger areas of destruction in tumor tissue. A potential problem is the heat sink effect at the tumor-host interface. Blood flow occlusion may be potentially beneficial in achieving complete tumor destruction.

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Concomitant Sclerosing Mesenteritis and Bile Duct Fibrosis Simulating Klatskin's Tumor

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Sclerosing mesenteritis is an uncommon benign condition that should be included in the differential diagnosis of abdominal masses. We present the first reported case of this condition in association with idiopathic bile duct fibrosis simulating Klatskin's tumor. A review of the literature regarding both clinical entities is presented. (J GASTROINTEST SURG 2001;5:658-660.)

KEY WORDS: Sclerosing mesenteritis, bile duct fibrosis, cholangiocarcinoma, Klatskin's tumor

Sclerosing mesenteritis is an uncommon, idiopathic, benign condition that has been reported in both adult and pediatric populations.[1,2] It causes thickening and shortening of the mesentery, often presenting as a palpable abdominal mass. Occasionally, patients with this condition may present with bowel obstruction and, rarely, bowel ischemia. Systemic symptoms are uncommon. Microscopically, sclerosing fibrosis, fat necrosis, chronic inflammation, and lipid-laden histiocytes are found.[1] We report the first case of a patient presenting with jaundice caused by fibrosis of the bile duct in association with an abdominal mass histologically consistent with sclerosing mesenteritis.

CASE REPORT

A 64-year-old white man had a 1-month history of pruritus and jaundice, which were followed by abdominal pain as well as blood per rectum. The patient had a history of hypertension and benign prostatic hypertrophy, as well as an episode of gastrointestinal bleeding due to ischemic colitis 2 years previously. Surgical history included appendectomy, tonsillectomy, and excision of bilateral submandibular glands for sialadenitis. At physical examination he was found to be well developed, with jaundice and evidence of scratching; no palpable masses were felt in the abdomen. Laboratory workup revealed the following: a normal white blood cell count, hematocrit, 37; total bilirubin, 12.8 mg/dl; direct bilirubin, 7.3 mg/dl; alkaline phosphatase, 358 U/dl; gamma-glutamyl transpeptidase, 102 U/dl; alanine aminotransferase, 54 U/dl; and aspartate aminotransferase, 55 U/dl. Serum electrolytes and blood chemical values were normal. Abdominal ultrasound showed intrahepatic biliary dilatation without extrahepatic dilatation, a collapsed gallbladder, heterogeneity of the liver, and splenomegaly. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated a hiliar stricture compatible with cholangiocarcinoma (Klatskin's tumor) rather than hiliar metastases and pancreas divisum (Fig. 1). Brushings from the bile duct were obtained and were negative for malignancy. A biliary stent was placed in the right ductal system. The patient continued to have severe pruritus, and a repeat ERCP was performed with stents placed in both the right and left ductal system. Over the next 7 to 10 days, the jaundice showed marked improvement but the itching continued. Dynamic contrast-enhanced helical CT scans revealed focal thickening of extrahepatic biliary ducts around the biliary stent as well as an infiltrative mass measuring 8.4 × 4.6 cm in the root of the mesentery (Fig. 2). Insignificant abdominal lymphadenopathy was noted, but the pancreas, spleen, kidneys, and small and large bowel were unremarkable. Diagnostic considerations included lymphoma as the most likely diagnosis, but metastatic cholangiocarcinoma was considered in the differential diagnosis. A colonoscopy was performed and the findings were normal.

The patient underwent a right upper quadrant laparotomy. An exploratory abdominal operation revealed a dense infiltrate into the mesentery that was biopsied in four separate places, and significant material was sent for both frozen section analysis and flow cytometry. No evidence of lymphoma or malignancy was found. This appeared to be

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Fig. 1. ERCP showing a hiliar stricture compatible with Klatskin's tumor.

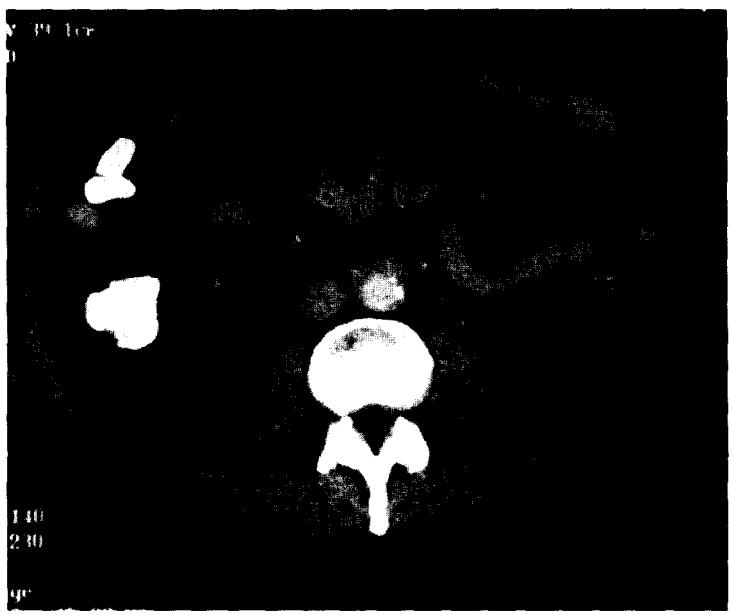

Fig. 2. CT scan demonstrating an infiltrative mass in the root of the mesentery.

an inflammatory process. The rest of the abdomen did not show any evidence of malignancy. The gallbladder was taken down out of the liver bed and the dissection was continued along the bile duct, which was found to be very thick and more prominent in the area between the cystic duct and the hilium. Because of the concern about malignancy, the bile duct was excised from the head of the pancreas up to above the level of the bifurcation. Frozen section analysis of the superior margin showed no evidence of malignancy. A Roux limb was created and a hepaticojejunostomy was performed over 6.6 Fr Silastic stents. The patient recovery was uneventful. One week after the operation, the patient was asymptomatic and laboratory evaluation revealed the following: total bilirubin, 6.2 mg/dl; direct bilirubin, 3.5 mg/dl; alkaline phosphatase, 153 U/dl; and gamma-glutamyl transpeptidase, 44 U/dl.

On microscopic examination, the section from the mesenteric mass showed densely sclerotic tissue with chronic inflammation encasing few residual adipocytes, which is consistent with a diagnosis of sclerosing mesenteritis. The bile duct was extensively encased by sclerotic thickening and inflammation similar to that seen in the mesenteric specimen. The gallbladder showed chronic cholecystitis and cholesterolosis.

DISCUSSION

Sclerosing mesenteritis produces tumor-like masses of the mesentery composed of varying degrees of fibrosis, chronic inflammation, and fat necrosis. In related conditions such as mesenteric panniculitus, there is a predominance of chronic inflammation and in mesenteric lipodystrophy there is a predominance of fat necrosis. However, sclerosing mesenteritis such as that found in this patient is characterized by a predominance of fibrosis.[3] The etiology of this process is unknown, and most cases have been reported in white men in their seventies.[3] The clinical differential diagnosis included carcinoma, lymphoma, and fat necrosis. In the series from the Armed Forced Institute of Pathology, no case was diagnosed correctly based on gross appearance.[3]

In most cases reported in the literature, patients presented with an abdominal mass that was either palpable or incidentally discovered during celiotomy for unrelated causes.[1,3] To our knowledge, ours is the first reported case of sclerosing mesenteritis in which a patient presented with jaundice due to fibrosis of the bile duct simulating a cholangiocarcinoma. This presentation is more commonly seen in patients with primary sclerosing cholangitis, but our patient had no other radiologic or clinical characteristics of this disease. In our patient, the ERCP findings were more consistent with cholangiocarcinoma at the confluence of the hepatic ducts. The differential diagnosis of Klatskin's tumor should include idiopathic benign focal stenosis. This clinical entity has been previously described[4] and closely resembles the pathologic findings of the bile duct in the present case. In addition, sclerosing mesenteritis may be histologically indistinguishable from retroperitoneal fibrosis, but the location, clinical presentation, and surgical appearance usually separate these two clinical entities.[3] In our patient the clinical characteristics were more consistent with sclerosing mesenteritis than retroperitoneal fibrosis. Some investigators, however, have proposed that these two processes are part of a spectrum that includes other clinical entities characterized by inflammation and fibrosis in different organs.[5] We can hypothesize that our patient had an idiopathic benign focal stenosis of the confluence of the bile ducts as one manifestation of a more systemic process characterized by inflammation and fibrosis.

Although it appears to be a benign process on microscopic examination, progressive fibrosis of the intrahepatic bile ducts may lead to future jaundice. To this end, intrahepatic placement of Silastic stents (pediatric Broviac catheters) brought through the Roux limb allows for serial examination and dilatation or definitive stenting if necessary.

In summary, we present the first known case of concomitant idiopathic benign focal stenosis of the confluence of the bile ducts simulating Klatskin's tumor in a patient with sclerosing mesenteritis.

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Cholinergic Stimulation of Rat Acinar Cells Increases *c-fos* and *c-jun* Expression via a Mitogen-Activated Protein Kinase-Dependent Pathway

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Acetylcholine release from cholinergic neurons regulates pancreatic exocrine function through pathways that are still under investigation. Pancreatic AR42J acinar cells were studied to determine intracellular calcium ($[Ca^{2+}]_i$) release, enzyme activation, and gene expression in response to the acetylcholine analog carbachol (CCh). CCh stimulated dose-dependent increases in $[Ca^{2+}]_i$ that were inhibited by atropine and by specific inhibitors to the muscarinic receptor subtypes m1 and m3. Polymerase chain reaction analysis was performed, which sequenced products corresponding to the m1 and m3 receptor subtypes but not the m2 subtype. CCh also stimulated mitogen-activated protein kinase activity. CCh induced time- and dose-dependent increases in the *c-fos* and *c-jun* early-response genes, which were blocked by m1 and m3 inhibition but not by m2 inhibition. (J GASTROINTEST SURG 2001;5:661-672.)

KEY WORDS: Pancreas, acinar cells, AR42J, acetylcholine, *c-fos*

The exocrine pancreas, the major site of production of digestive enzymes, is under both hormonal and neural control. The role of cholinergic pathways in short-term regulation of pancreatic exocrine function has been extensively studied.[1] Acetylcholine release from cholinergic neurons is stimulated by ingestion of nutrients. The neurotransmitter binds to muscarinic receptors on acinar cells to stimulate enzyme secretion. Acetylcholine is well known to increase intracellular Ca^{2+} and diacylglycerol through activation of phospholipase C. Changes in intracellular second messengers activate a series of cascade pathways that cause enzyme secretion by altering patterns of cellular protein phosphorylation.

Recent studies suggest that the pathways involved in acute regulation of pancreatic exocrine secretion may also exert longer term functional control. A number of hormones and neurotransmitters that stimulate enzyme secretion have also been shown to regulate pancreatic growth and development.[2] The secretagogue cholecystokinin (CCK) stimulates pancreatic growth in vivo and acinar cell proliferation in vitro.[3-5] Exposure of acinar cells to CCK stimulates enzyme gene expression, and Lu and Logsdon[6] have demonstrated that the hormones CCK and bombesin, as well as the cholinergic agonist carbachol (CCh), increase nuclear oncogene expression in rat pancreatic acini. Although these observations suggest that long-term cellular responses may be induced by exposure to enzyme secretagogues, the cellular mechanisms involved remain incompletely defined.

Muscarinic acetylcholine receptors are members of a family of G protein–linked seven transmembrane cell surface receptors.[7] Five subtypes (m1 to m5) have been identified to date.[8] Muscarinic receptor subtypes may be categorized according to their signaling mechanisms. Receptor subtypes m1, m3, and m5 couple preferentially to pertussis toxin–insensitive Gq/G11 proteins and stimulate phosphoinositide hydrolysis and Ca^{2+} mobilization.[8] The m2 and m4 receptor subtypes couple to Gi/Go proteins that inhibit adenylate cyclase.[8] Most studies have designated the acinar cholinergic receptor as an m3 subtype, although a recent report by Schmid et al.[9] indicated the presence of both m1 and m3 receptor subtypes by polymerase chain reaction (PCR) analysis. In other cell types, muscarinic acetylcholine receptors can activate transcription of immediate-early genes such as *c-fos* and

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c-jun.[10] In nonpancreatic cells, mitogen-activated protein (MAP) kinases may act as a convergence pathway for cell surface signals that regulate cellular growth and differentiation.[11] In nonpancreatic tissue, muscarinic stimuli have been reported to regulate expression of nuclear oncogenes via MAP kinase–dependent pathways.[12]

The current study was designed to investigate the pathways linking muscarinic receptor activation and *c-fos* and *c-jun* expression in rat pancreatic AR42J cells. This cell line is an acinar cell line that has regulated exocrine enzyme secretion and has been routinely used to examine mechanisms of pancreatic signaling.[13,14] We show here that AR42J cells express both m1 and m3 receptors. Receptor occupancy activates pathways that release inositol trisphosphate–sensitive calcium stores via a mechanism that is pertussis toxin insensitive and linked to phospholipase C. Cholinergic receptor occupancy also stimulates increased expression of *c-fos* and *c-jun* messenger RNA through mechanisms that involve calmodulin-dependent kinase II and protein kinase C. The signaling pathway involves MAP kinase as a downstream mediator.

MATERIAL AND METHODS

AR42J cells and plasmids containing probes for *c-fos*, *c-jun*, and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from the American Type Culture Collection (Manassas, Va.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, Hanks balanced salt solution, Trizol, DNAse I (amplification grade), buffer-saturated phenol, and phenol:chloroform: isoamyl alcohol (25:24:1) were purchased from Life Technologies, Inc. (Gaithersburg, Md.). Penicillin/streptomycin, atropine sulfate, and sonicated salmon sperm DNA came from Sigma (St. Louis, Mo.). CCh, U73122, U73343, GF109203X, chelerythrine chloride, PD 98059, KN 93, tyrphostin A23, and herbimycin A were purchased from Calbiochem (San Diego, Calif.). Pertussis toxin, 4-DAMP, methoctramine-4-HCl, and pirenzepine-2-HCl were obtained from RBI (Natick, Mass.). α-^{32}Pd-CTP (3000 Ci/mmole) was purchased from NEN Research Products (Boston, Mass.). Midi select G-50 Sephadex spin columns came from 5 Prime→3 Prime (Boulder, Colo.). Expresshyb and rat brain total RNA were purchased from Clontech (Palo Alto, Calif.). Qiagen Maxi-Prep isolation kits and Qiaex II gel isolation kits were purchased from Qiagen (Santa Clara, Calif.). Phospho-$p^{44/42}$ MAP kinase antibody and $p^{44/42}$ control proteins came from New England Biolabs (Beverly, Mass.). Antirabbit immunoglobulin G horseradish peroxidase was obtained from Promega Corp. (Madison, Wis.). Nitrocellulose membranes and Ready Gels were purchased from Bio-Rad Laboratories, Inc. (Hercules, Calif.). The Redi-Prime labeling kit, the enhanced chemiluminescence Western blot reagents, and Hyperfilm H were purchased from Amersham Pharmacia, Inc. (Piscataway, N.J.). BCA protein assay reagents came from Pierce (Rockford, Ill.). Nytran membrane was purchased from Shleicher & Schuell (Keene, N.H.). The ProStarUltra HF RT-PCR kit came from Stratagene (La Jolla, Calif.). Fura 2-AM was purchased from Molecular Probes (Eugene, Ore.).

Cell Culture

AR42J cells were seeded at a density of 4 million cells/100 mm dish and maintained at 37° C under 10% CO_2/90% air for 48 hours as a subconfluent monolayer in media consisting of DMEM supplemented with 10% FBS, 2 μmol/L L-glutamine, and 100 U penicillin/100 μg streptomycin. Cells were grown in DMEM/0.1% FBS for 24 hours before treatment. Cells from passage 20 to 30 were used experimentally.

Intracellular Calcium Concentration Measurement

AR42J cells were plated on collagen-coated coverslips and cultured for 48 hours as described earlier. Cells were incubated at 37° C for 45 minutes in DMEM containing 2 to 3 μmol/L fura-2 AM. The coverslips were washed, resuspended in control buffer (118 mmol/L NaCl/4.7 mmol/L $CaCl_2$/10 mmol/L HEPES/1.5 mmol/L $NaHCO_3$/11 mmol/L glucose/0.9 mmol/L NaH_2PO_4/0.8 mmol/L $MgSO_4$ at pH 7.4), and placed in a lucite superfusion chamber. The superfusion rate of control and experimental solutions was 1 ml/min at 37° C.

A Zeiss Axiovert inverted microscope and an Attofluor digital imaging system (Rockville, Md.) were used for single-cell $[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ was calculated from the ratios of the fluorescence intensities of fura-2 at 334 and 380 nm wavelengths with an emission wavelength of 500 nm. Calibration of the system was performed with the following two-point standardization equation using fura-2 free acid: $[Ca^{2+}]_i = K_d[(R-R_{low})/(R_{high}-R)]\beta$, where K_d = the dissociation constant of the Ca^{2+}–fura-2 complex (225 nm), $R = F_{334/380}$, that is, the fluorescence at 334 nm excitation, R_{low} = the ratio at zero Ca^{2+} (the buffer contains 1 mmol/L EGTA), R_{high} = the ratio at high Ca^{2+} (the buffer contains 1 mmol/L $CaCl_2$), and $\beta = F_{380}$ (zero Ca^{2+})/F_{380} (saturating Ca^{2+}).

Frames were not averaged. A ratio pair was taken every second.

Results were calculated only for cells having basal $[Ca^{2+}]_i$ between 50 and 100 nmol/L. One microscopic field was examined per coverslip. Each experimental condition was assayed on three different coverslips. Peak intracellular $[Ca^{2+}]_i$ was measured at the highest $[Ca^{2+}]$ achieved during agonist exposure. In inhibitory studies, the percentage of inhibition was calculated using the equation 100-100X, where X = the $\Delta[Ca^{2+}]_i$ of cells exposed to antagonist /$\Delta[Ca^{2+}]_i$ level of control.

Polymerase Chain Reaction and Sequencing

Total RNA was isolated from AR42J cells with Trizol reagent according to manufacturer's directions. The reverse transcriptase reaction was carried out at 42° C for 50 minutes followed by heat inactivation at 70° C for 15 minutes. The mixture was treated with DNAse I, then reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) provided in the ProStar Ultra HF RT-PCR system kit (Stratagene). Total rat brain RNA (Clontech) was treated in the same manner.

Fifty microliters of PCR mixture contained 1 μl of reverse transcriptase products, 1× cDNA PCR reaction buffer, 400 nmol/L of each primer, 200 μmol/L of dNTP mix, and 1× Advantage cDNA polymerase mix. The PCR was carried out using a Perkin-Elmer Thermal Cycler (Norwalk, Conn.). Samples were denatured initially at 94° C for 1 minute and the PCR was performed as follows: 25 cycles of 35 seconds at 94° C, 1 minute at 62° C, and 1 minute 40 seconds at 72° C, followed by the final extension at 72° C for 7 minutes.

Portions of the sequences for the rat muscarinic type 1 and type 3 receptors, designed to include significant amounts of the sequence for the third intracellular loop, were amplified by PCR from the cDNAs derived from the total RNAs from rat brain and AR42J cells according to manufacturer's directions. The primers used were:

m1 receptor
Sense: 5'CCTGTGACCTCTGGCTGGCCCTG-GACTAT 3'
Antisense: 5'CCTTTCTTGGTGGGCCTCTT-GACTGTATTTG 3'

m3 receptor
Sense: 5'AACGATGCTGCTGCCTCCCTGGAA-AACTCTGC 3'
Antisense: 5'AGCGTCTGGGCGGCCTTCTTC-TCCTTGATGA 3'

PCR products were electrophoretically analyzed on 1.5% agarose gel containing ethidium bromide. DNA bands were excised from the gel and purified as follows: gel slices were crushed in the tube, and sample DNA was extracted twice with buffered phenol and once with phenol:chloroform:isoamyl (25:24:1) followed by ethanol precipitation with 0.1 volume 3 mol/L sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol and washing with 70% ethanol. The purified DNA fragments were directly sequenced by the DNA sequencing core at the University of Michigan using Applied Biosystems DNA sequencers.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from harvested cells using Trizol following manufacturer's directions. RNA samples (15 to 20 μg) were electrophoresed in 2.2 mol/L formaldehyde/1% agarose gels, transferred to Nytran nylon membrane overnight with 10× SSPE, then UV cross-linked with an ultraviolet Stratalinker 2400 (Stratagene).

Plasmids containing probes were isolated from bacterial cultures with Qiagen Maxi-Prep isolation kits. The plasmids were cut and the fragments containing probes isolated from agarose gels using Qiaex II gel isolation kits. Probes were labeled with ^{32}P-dCTP using the Redi-Prime kit. Unincorporated ^{32}P was removed with a G50 Sephadex spin column.

Labeled probe supplemented with 100 μg salmon sperm DNA was added to the Expresshybe solution after a 30-minute period of prehybridization at 68° C. Hybridization proceeded for 1 hour at 68° C. Membranes were washed twice in 2 × SSPE/0.1% SDS at room temperature followed by a final wash for 30 minutes at 60° C in 0.1 × SSPE/0.1% sodium dodecyl sulfate (SDS). After exposure to Kodak X-AR film with intensifying screens at −70° C, the films were developed and band intensity was quantitated with a Personal Densitometer (Molecular Dynamics). Membranes were stripped by boiling in 0.1 × SSPE/0.1% SDS and reprobed. All signals were normalized to GAPDH. Each experiment was performed at least four times.

Western Blot Analysis

AR42J cells were exposed to carbachol, carbachol plus antagonist, or vehicle for 30 seconds. The cells were immediately placed on ice, washed with ice-cold phosphate-buffered saline containing 1 mmol/L sodium orthovanadate, lysed with 100 μl of SDS sample buffer (62.5 mmol/L Tris, pH 6.8/2% SDS/10% glycerol/50 mmol/L DTT/ 0.1% bromophenol blue/

2 mmol/L sodium orthovanadate), sonicated, boiled for 2 minutes, and centrifuged (refrigerated) for 5 minutes. After saving a portion for the BCA protein assay, samples were transferred to another tube and stored at −70° C until use.

Equivalent amounts of protein (250 μg) were denatured and resolved by 10% SDS/PAGE. The resolved proteins were transferred to nitrocellulose membrane with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Laboratories). The membranes were incubated with a phosphospecific $p^{44/42}$ polyclonal MAP kinase antibody at a 1:1000 dilution and subsequently incubated with horseradish peroxidase–conjugated antirabbit immunoglobulin G at a 1:5000 dilution. Membranes were washed six times between incubations. Enhanced chemiluminescent substrates and autoradiography were used to visualize the reactive bands on the membranes. Band intensity was quantitated with a Personal Densitometer. Each experiment was performed twice.

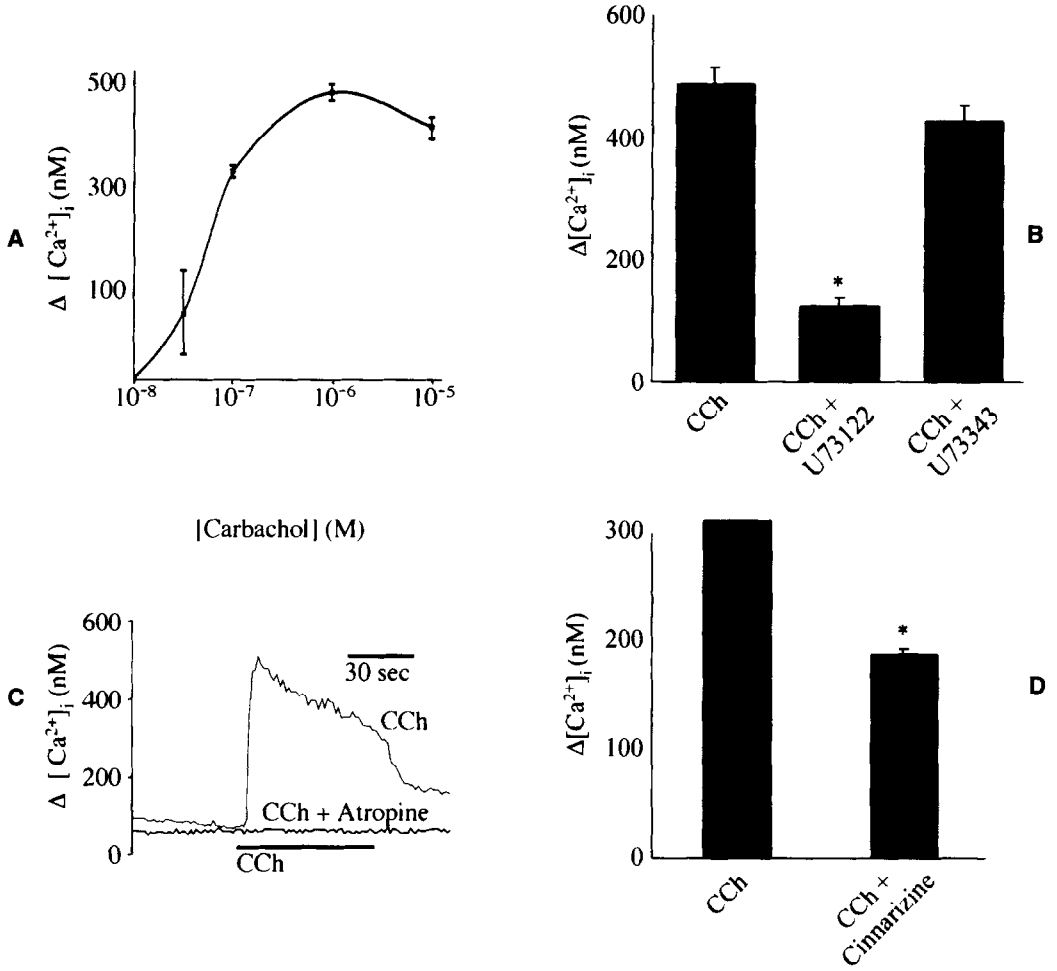

Fig. 1. A, Dose-dependent increments in $[Ca^{2+}]_i$ in AR42J cells exposed to carbachol *(CCh)*. Cells were exposed to only one concentration of CCh. For each condition, at least four separate coverslips were used. More than 50 cells were examined for each concentration. **B,** Typical response for 24 AR42J cells to superfusion of CCh, 10 μmol/L, for 60 seconds. In separate experiments, fura-loaded cells were exposed to CCh in the presence of 10 μmol/L atropine. Calcium concentration was determined in individual cells using the ratio of intensities of fura-2 at 334 nm and 380 nm. **C,** Effects of phospholipase C inhibitor U73122 (10 μmol/L, n = 36) or its inactive analog U73343 (10 μmol/L, n = 35) on CCh-stimulated increases in $[Ca^{2+}]_i$ in AR42J cells. Cells were exposed to U73122 or U73343 for 30 seconds before CCh (10 μmol/L). In parallel experiments, cells were exposed to CCh alone (n = 41). * = $P < 0.05$ vs. CCh. **D,** Fura-loaded cells were exposed to either CCh alone (10 μmol/L) or CCh plus cinnarizine (50 μmol/L, n = 29).

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Data were analyzed using analysis of variance, with Fisher's post hoc test. Significance was accepted as $P < 0.05$ (95% confidence level). For calcium studies, n equals the number of cells examined. At least three coverslips were used for each experimental condition. Results have been calculated only for those cells having basal $[Ca^{2+}]_i$ levels below 100 nmol/L. Cells were considered to be responsive if peak $[Ca^{2+}]_i$ was at least 50 nmol/L higher than the baseline value. Cells with a high $[Ca^{2+}]_i$ before the addition of agonist were considered damaged or leaky and were excluded from the study. $\Delta[Ca^{2+}]_i$ represents the difference between peak and basal $[Ca^{2+}]_i$.

RESULTS

Effect of Carbachol on Ca^{2+} Signaling

Exposure of fura-loaded AR42J cells to carbachol (CCh 10^{-8} to 10^{-5} mol/L) produced dose-dependent increases in $[Ca^{2+}]_i$ (Fig. 1, *A*). In 27 AR42J cells exposed to CCh (10 μmol/L), $[Ca^{2+}]_i$ rose from a basal level of 89 ± 5 nmol/L to a peak of 487 ± 19 nmol/L. The onset of this peak was rapid, occurring within 5 seconds of perfusion by agonist (see Fig. 1, *B*). The $[Ca^{2+}]_i$ responses were maximal at 1 μmol/L. The addition of atropine (10 μmol/L) to the perfusate solution abolished $[Ca^{2+}]_i$ increases (see Fig. 1, *B*). Preincubation with pertussis toxin (100 ng/ml for 18 hours) did not affect CCh-stimulated Ca^{2+} increments. AR42J cells were fura loaded and perfused with the aminosteroid U73122 (10 μmol/L), an inhibitor of phospholipase C. Compared to 71 control cells, U73122 significantly inhibited CCh-stimulated (10 μmol/L) release of Ca^{2+} in 36 test cells (Fig. 1, *C*). U73433 (10 μmol/L), an inactive enantiomer of U73122, did not inhibit Ca^{2+} release. AR42J cells were also perfused with cinnarizine (50 μmol/L), a cell-permeable antagonist of the intracellular inositol trisphosphate receptor. Cinnarizine inhibited the CCh-induced change in $[Ca^{2+}]_i$ by 39% versus control values (Fig. 1, *D*).

Muscarinic Receptor Expression

To assess the participation of muscarinic receptor subtypes in AR42J signaling, RT-PCR was performed using primers generated from known rat coding sequences. AR42J cells expressed mRNA corresponding to the m1 and m3 receptor subtypes (Fig. 2). Sequencing of the m1 and m3 PCR product demonstrated identity to the predicted rat coding sequences.

Antagonism of Ca^{2+} Signaling

Dose-dependent inhibition of CCh-stimulated Ca^{2+} signaling was produced by coperfusion of muscarinic subtype–specific antagonists. Pirenzepine, an

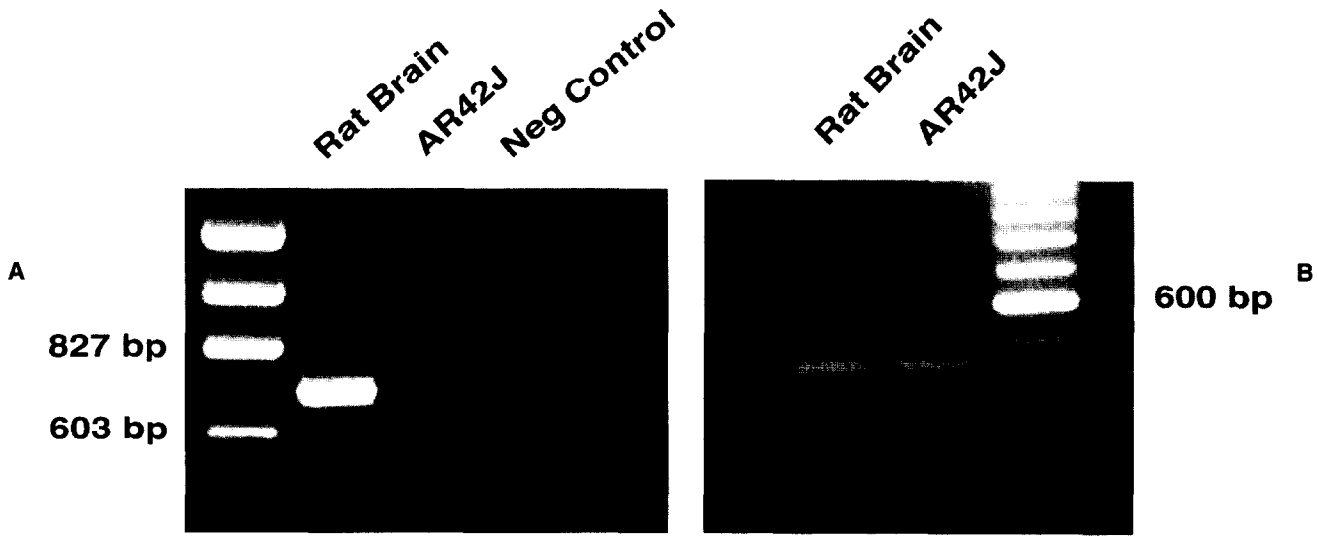

Fig. 2. Expression of rat m1 and m3 mRNAs in AR42J cells. **A,** RT-PCR product from AR42J cell cultures corresponding to m1 coding sequence is shown in lane 3. Lane 1 corresponds to size markers, whereas lane 2 is a positive control from rat brain. Lane 4 represents the negative control (RT-PCR performed without extracted RNA). **B,** RT-PCR product from AR42J cells corresponding to m3 coding sequence.

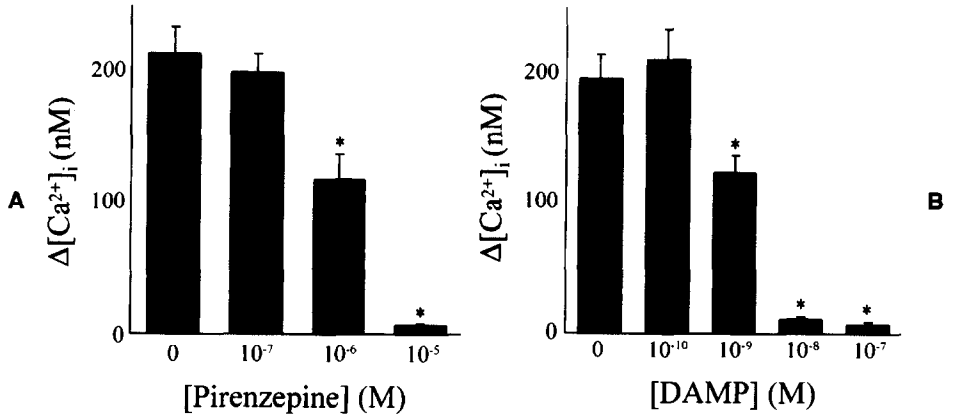

Fig. 3. A, Dose-dependent inhibition of CCh-stimulated $[Ca^{2+}]_i$. Cells were exposed to a single concentration of pirenzepine (10^{-7} to 10^{-5} mol/L) beginning 60 seconds before CCh (10 μmol/L). Each experimental group contained more than 50 cells. * = $P < 0.05$ vs. control. **B,** Dose-dependent inhibition of CCh-stimulated $[Ca^{2+}]_i$ by 4-DAMP (10^{-10} to 10^{-7} mol/L). n = >50 cells for each group. * = $P < 0.05$ vs. control.

m1 antagonist, significantly decreased CCh-induced changes in $[Ca^{2+}]_i$ at 1 μmol/L, and abolished responsiveness at 10 μmol/L (Fig. 3). 4-DAMP, a muscarinic antagonist with m3 selectivity in the micromolar range, abolished CCh-induced increments in $[Ca^{2+}]_i$ at 10 nmol/L.

Effects of Carbachol on *c-fos* and *c-jun* Expression

CCh (10 μmol/L) stimulated the expression of *c-fos* mRNA in a time-dependent manner in pancreatic AR42J cells (Fig. 4). *c-fos* expression was 10-fold greater than control values at 15 minutes, maximal (24-fold higher) at 30 minutes, and returned to baseline by 60 minutes. CCh induced *c-jun* expression with similar time dependence, with maximal induction (7.5-fold) at 30 minutes. CCh-induced *c-fos* and *c-jun* expression were both dose dependent over concentration ranges from 10^{-8} to 10^{-4} mol/L (Fig. 5). Northern blot analysis of AR42J cells exposed to CCh in the presence of atropine (10 μmol/L) demonstrated abolition of incremental *c-fos* and *c-jun* expression (Fig. 6). Preincubation of AR42J cells with pertussis toxin (100 ng/μl) for 18 hours had no effect on CCh-stimulated (10 μmol/L, 30 minutes) *c-fos* or *c-jun* expression. The m1 receptor antagonist pirenzepine and the m3 receptor antagonist 4-DAMP produced dose-dependent inhibition of CCh-stimulated (10 μmol/L, 30 minutes) *c-fos* or *c-jun* expression (Fig. 7). The m2-selective antagonist methoctramine was without an inhibitory effect over a concentration range of 10^{-10} to 10^{-5} mol/L.

Role of Inositol Triphosphate–Sensitive Ca^{2+} Stores

Cholinergic m1 and m3 receptors have been reported to couple to phospholipase C_β, which in turn causes release of Ca^{2+} from inositol triphosphate–sensitive stores in the endoplasmic reticulum. Northern blot analysis demonstrated that *c-fos* expression decreased by 87 ± 1%, relative to control values, when AR42J cells were coincubated with both CCh (10 μmol/L) and cinnarizine (10 μmol/L) (Fig. 8). Calmodulin-dependent protein kinase II has been observed to be activated by increments in intracellular calcium. When the calmodulin-dependent protein kinase II inhibitors KN 62 or KN 93 (10 μmol/L each) were coincubated with CCh, *c-fos* expression was decreased by 92 ± 1% and 88 ± 1%, respectively (see Fig. 8).

Protein Kinase C Effects on *c-fos* Expression

Diacylglycerol, which activates the isoforms of protein kinase C present in AR42J cells, is also produced by the actions of phospholipase C_β. Two inhibitors of protein kinase, GF109203X and chelerythrine, had similar effects on *c-fos* expression in AR42J cells exposed to CCh (Fig. 9). Coincubation of 10 μmol/L CCh with 3.5 μmol/L GF109203X caused *c-fos* ex-

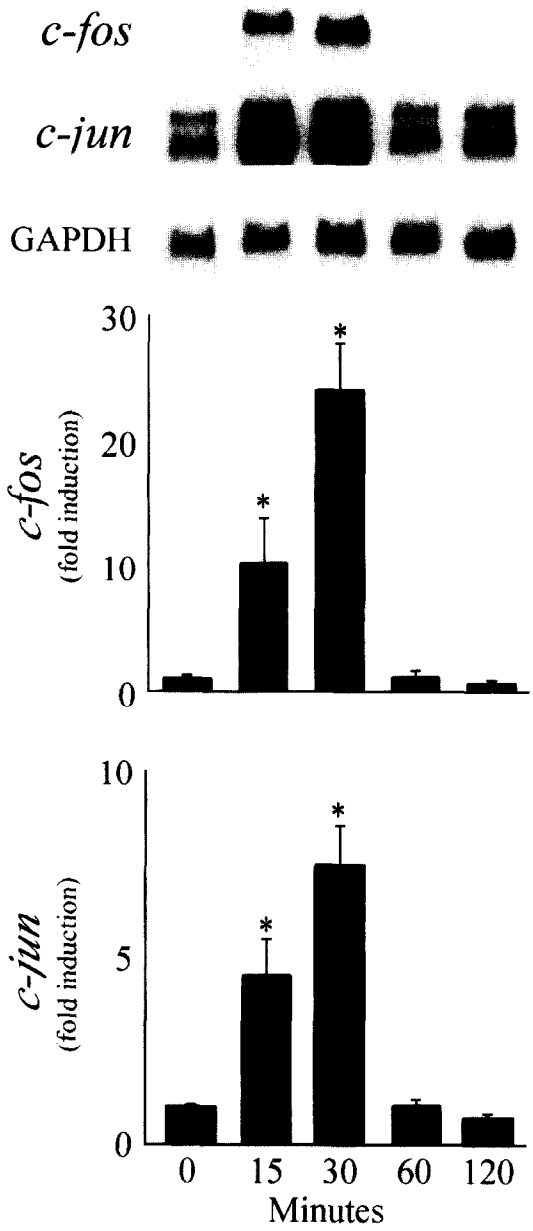

Fig. 4. Northern blot analysis of time-dependent increases in *c-fos* and *c-jun* mRNA expression on exposure to CCh. Serum was replaced with 0.1% bovine serum albumin 24 hours prior to each experiment. AR42J cells were treated with CCh (10 μmol/L) for 15, 30, 60, and 120 minutes. Time intervals of 15 and 30 minutes were significantly different from basal levels. Blot shown is representative of data from four separate experiments. After *c-fos* or *c-jun* hybridization, membranes were rehybridized using a cGAPDH probe to control for variations in gel loading and transfer efficiency.

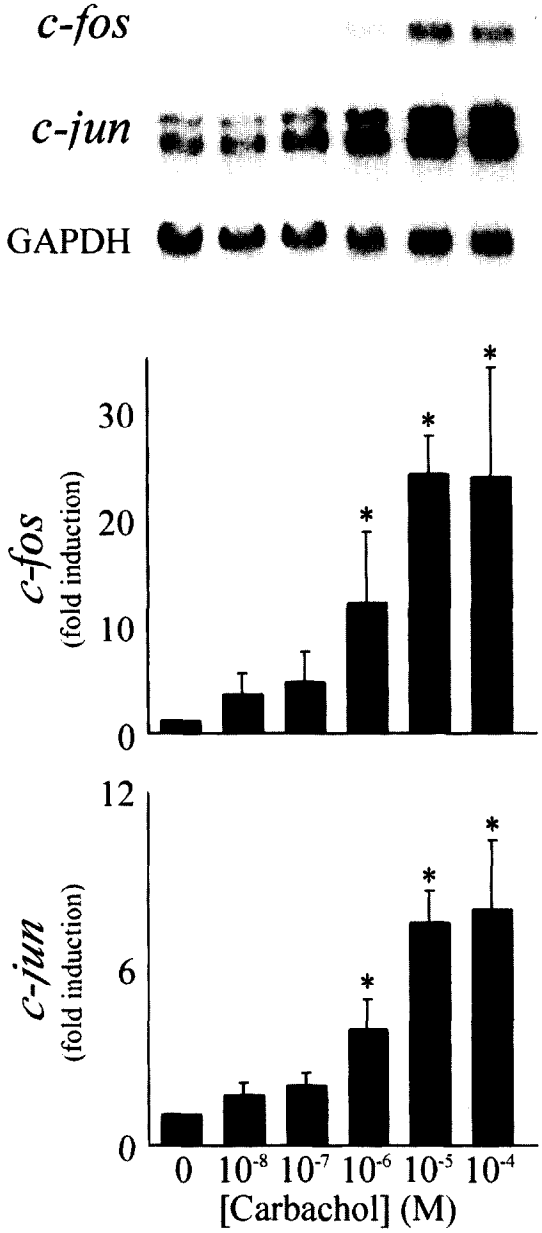

Fig. 5. Dose-dependent expression of *c-fos* and *c-jun* mRNA. AR42J cells were exposed to CCh in a concentration range of 10^{-8} to 10^{-4} mol/L. Data are expressed as an average of four separate experiments. Concentrations of 10^{-6}, 10^{-5}, and 10^{-4} mol/L were significantly different from basal levels.

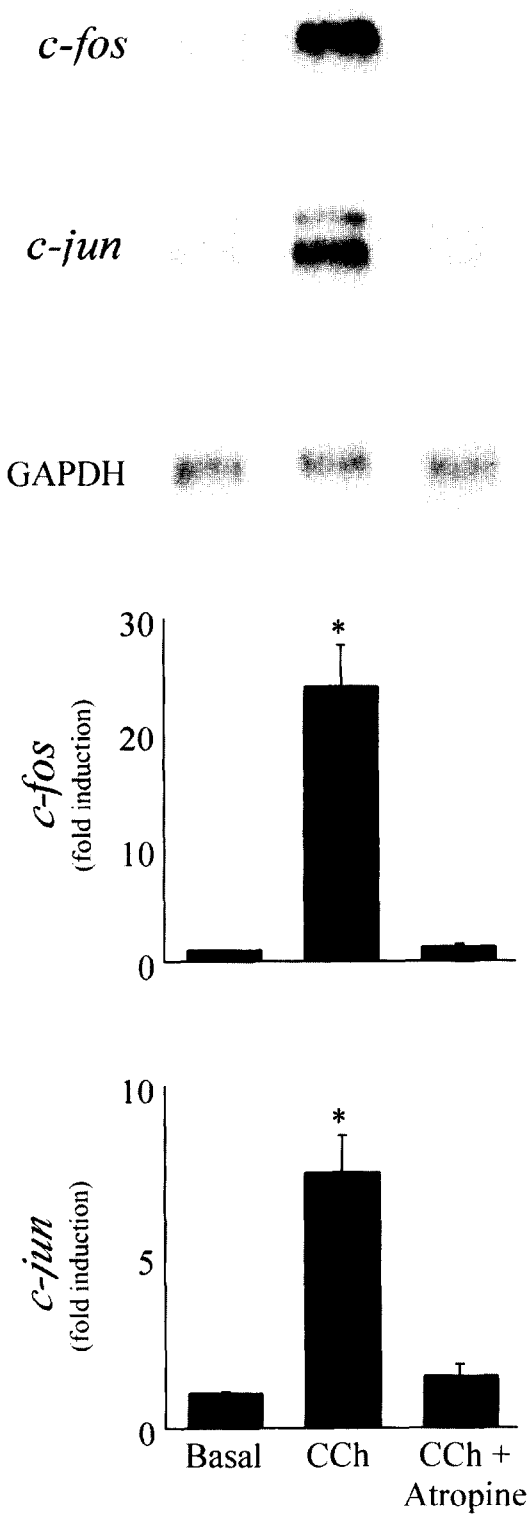

Fig. 6. Effects of atropine (10 μmol/L) on CCh (10 μmol/L)–stimulated *c-fos* and *c-jun* expression. AR42J cells were exposed to atropine 30 minutes before and during a 30-minute incubation with CCh. Data are expressed as an average of four separate experiments. * = $P < 0.05$ vs. basal or CCh plus atropine.

pression to decrease by 63 ± 1%. Coexposure to CCh with 1 μmol/L chelerythrine inhibited *c-fos* expression by 66 ± 1%.

MAP Kinase Pathway in *c-fos* Expression

In separate experiments, AR42J cells were incubated with CCh in the presence or absence of PD 98059, an inhibitor of MAP kinase kinase. PD 98059 (50 μmol/L) caused a 76 ± 1% decrease in *c-fos* expression relative to control cells treated with CCh (10 μmol/L) alone (Fig. 10). Exposure of AR42J cells to CCh (10 μmol/L) for 30 seconds was associated with a significant increase in MAP kinase activation as determined by Western blotting with a phosphospecific antibody (Fig. 11). The experiment was repeated, and the blot was incubated with an antibody that did not differentiate between the phosphorylated and nonphosphorylated forms of the ERK 1 and 2 proteins. The overall level of ERK expression was not changed by CCh (10 mmol/L) treatment for 30 seconds (data not shown). MAP kinase activity was decreased by more than 80% by coincubation with atropine (10 μmol/L), GF109203X (3.5 μmol/L), cinnarizine (10 μmol/L), KN 93 (10 μmol/L), or PD 98059 (50 μmol/L) (see Fig. 11).

The following compounds did not affect CCh-stimulated expression of *c-fos* mRNA: tyrosine kinase inhibitors herbimycin A (3 μmolL) or tyrphostin (5 μmol/L), protein kinase A type I inhibitor Rp-8-Cl-cAMPs (100 μmol), or protein kinase G inhibitor Rp-8-Br-cGMPs (100 μmol/L).

DISCUSSION

The current studies demonstrate that CCh stimulates *c-fos* and *c-jun* expression in AR42J cells via a mechanism that involves m1 and m3 receptors acting through the MAP kinase pathway. The signaling pathway includes phospholipase C, protein kinase C, and calmodulin-dependent kinase II. The data that support these conclusions are six fold: (1) CCh stimulates pertussis toxin–insensitive increases in $[Ca^{2+}]_i$ that are inhibited by the m1- and m3-specific inhibitors pirenzepine and 4-DAMP and by inhibitors of the inositol triphosphate receptor and phospholipase C; (2) PCR analysis demonstrated m1 and m3 receptor subtype reaction products identical to predicted rat coding sequences; (3) CCh stimulated time- and dose-dependent increases in *c-fos* and *c-jun* expression; (4) *c-fos* and *c-jun* expression was suppressed by pirenzepine and 4-DAMP but not by methoctramine, an m2-specific inhibitor; (5) *c-fos* mRNA expression was significantly suppres-

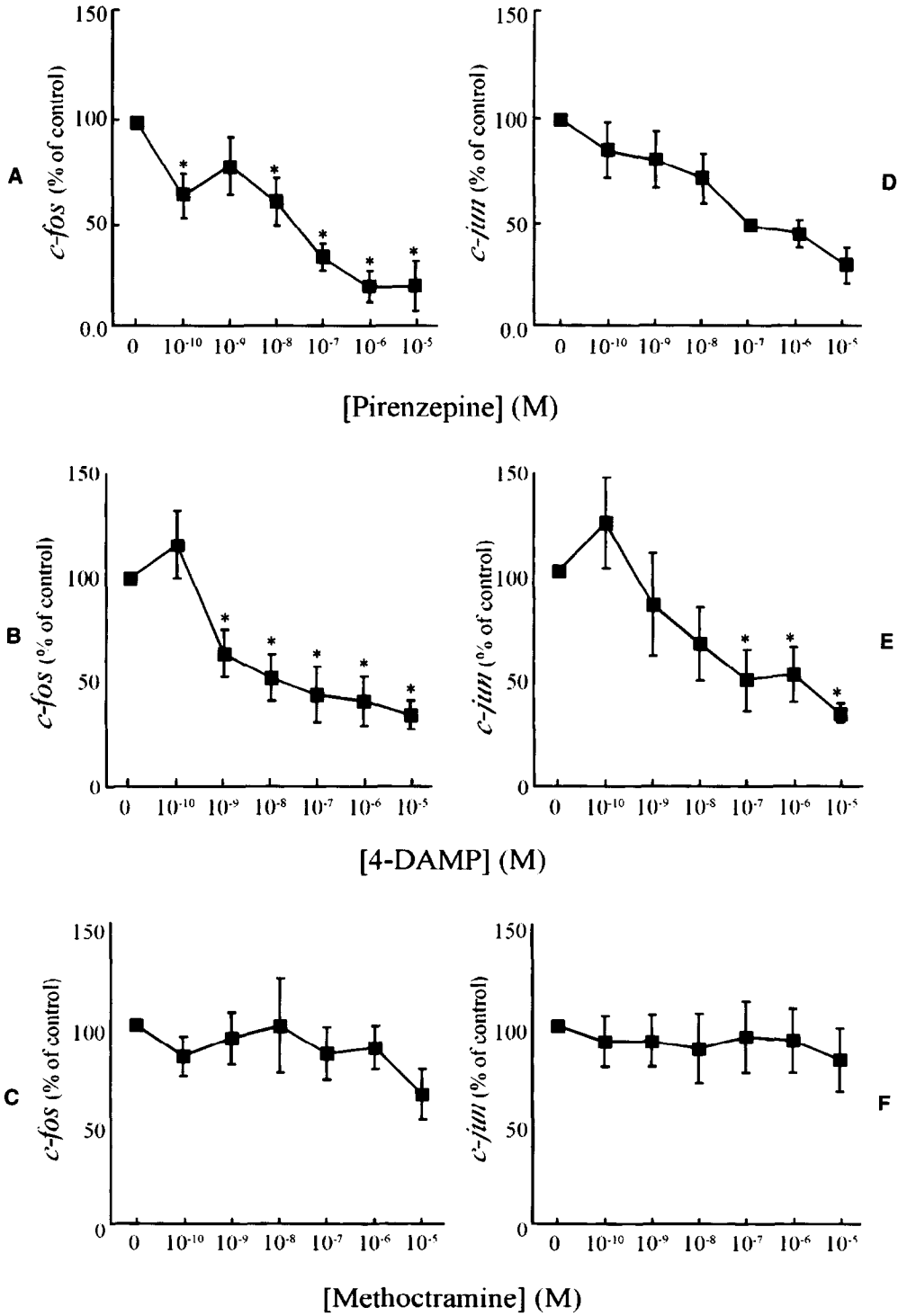

Fig. 7. Effects of muscarinic receptor subtype–specific inhibitors on *c-fos* (**A, B,** and **C**) and *c-jun* (**D, E,** and **F**) expression. AR42J cells were exposed to pirenzepine, 4-DAMP, or methoctramine at concentrations ranging from 10^{-10} to 10^{-5} mol/L for 30 minutes before and during exposure to CCh at 10 μmol/L. Each point reflects the mean of four separate experiments. * = $P < 0.05$ vs. control.

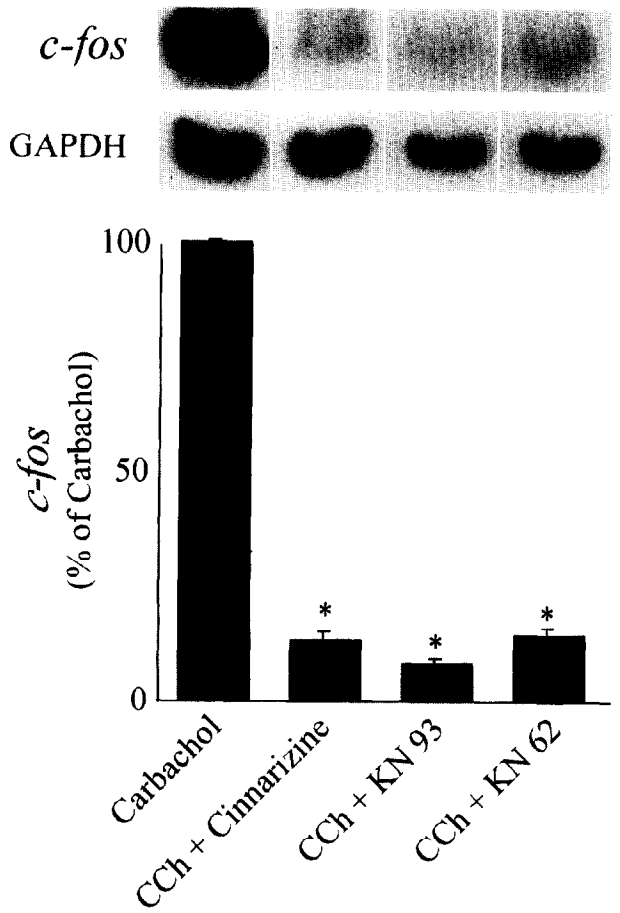

Fig. 8. Inhibition of 10 μmol/L CCh-stimulated *c-fos* expression by cinnarizine (10 μmol/L), KN 93 (10 μmol/L), and KN 62 (10 μmol/L). * = $P < 0.05$ vs. carbachol alone.

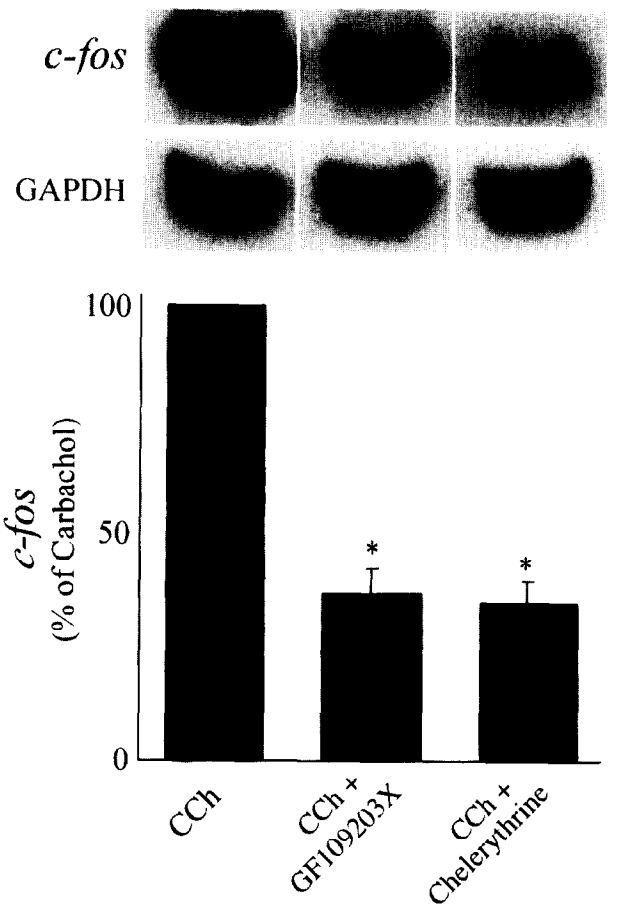

Fig. 9. AR42J cells were incubated with CCh alone (10 μmol/L) or CCh plus GF109203X (3.5 μmol/L) or CCh plus chelerythrine (1 μmol/L). Antagonists were added 30 minutes before and during exposure to CCh. Data are expressed as the mean of four experiments. * = $P < 0.05$ vs. CCh.

sed by inhibitors of protein kinase C, calmodulin-dependent kinase II, and MAP kinase kinase; and (6) MAP kinase activity was increased in cells briefly exposed to CCh.

Neural control of pancreatic exocrine function has long been recognized to be of primary physiologic significance. Cholinergic pathways are activated on ingestion of a meal; the resultant release of acetylcholine at the surface of acinar cells initiates a well-characterized signaling pathway. Binding of acetylcholine to muscarinic receptors on acinar cells causes increases in intracellular Ca^{2+} consequent to production of inositol trisphosphate by phospholipase C. The presence of acetylcholine m1 and m3 receptors in pancreatic acinar cells has been reported based on pharmacologic studies with receptor subtype–specific antagonists.[9] In a study utilizing [N-methyl-^{3}H]-scopolamine, investigators also reported the presence of m2 receptors on rat pancreatic acini.[15] Direct evidence for m1 and m3 receptors in rat pancreatic tissue has come from PCR analysis; the presence of the m2 subtype could not be confirmed in this study.[9] Using PCR analysis, the present study confirmed the presence of m1 and m3 receptor subtypes in rat AR42J cells. CCh-stimulated *c-fos* and *c-jun* expression was pertussis toxin insensitive. Pertussis toxin insensitivity is consistent with mediation of CCh signaling via m1 and m3 receptor subtypes.[7]

The short-term effects of pancreatic secretagogues on enzyme secretion have been well studied. Recent studies also demonstrate that long-term regulation of exocrine pancreatic function is crucial in control of dietary enzyme gene expression, dietary adaptation, and acinar cell growth.[6] A number of gastrointestinal peptide hormones, initially investigated for their effects on enzyme secretion, have been shown to regulate pancreatic growth or differentiation.[4-6] The peptide hormones gastrin, CCK, bombesin, and secretin have each been shown to stimulate pancreatic growth.[4-6] Endogenous CCK release, stimulated by

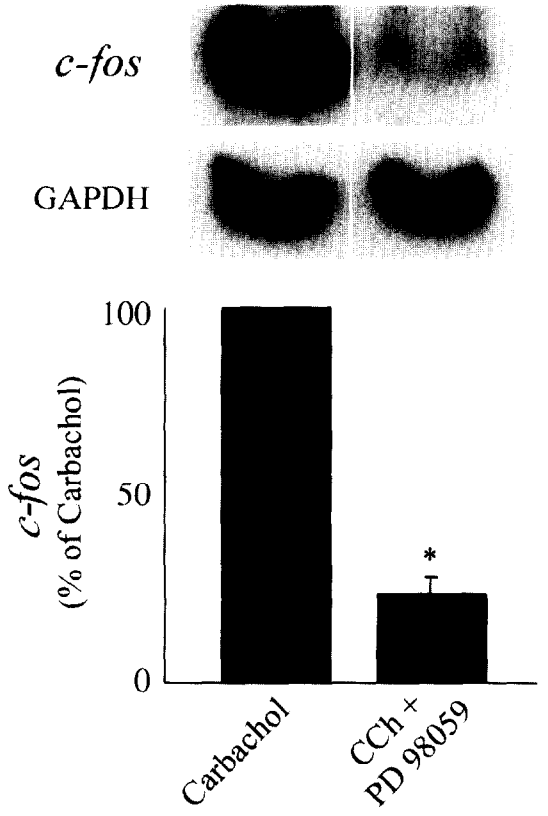

Fig. 10. Inhibition of carbachol-stimulated *c-fos* expression by the MAP kinase kinase inhibitor PD 98059 (50 μmol/L).

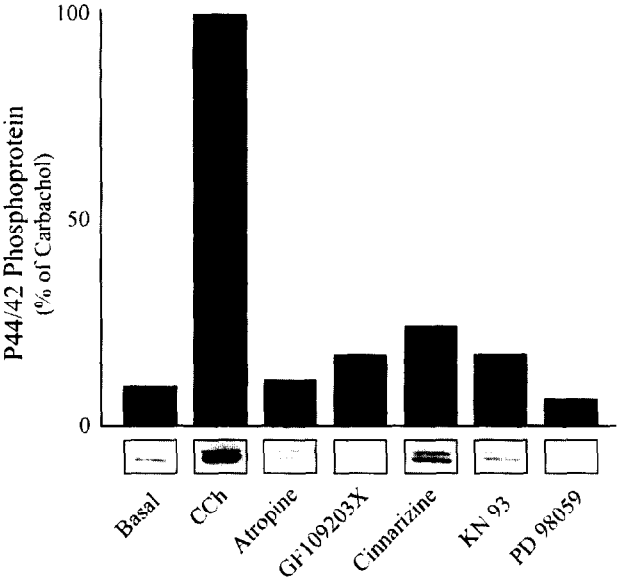

Fig. 11. Western blot of MAP kinase activity in AR42J cells. Data are represented graphically as the mean of two separate experiments. Representative blots are depicted underneath. Cells were exposed to vehicle, CCh alone (10 μmol/L), or CCh plus atropine (10 μmol/L), GF109203 (3.5 μmol/L), cinnarizine (10 μmol/L), KN 93 (10 μmol/L), or PD 98059 (50 μmol/L).

intraduodenal infusion of amino acids, stimulates pancreatic growth, suggesting a physiologic role for this peptide.[16]

In vitro studies of cultured rat pancreatic acini have shown that peptides such as CCK and bombesin and the cholinergic agonist CCh increase levels of the nuclear oncogenes *c-fos*, *c-jun*, and *c-myc*.[6] The present study demonstrates that brief exposure of AR42J cells to CCh produces significant increases in expression of *c-fos* and *c-jun* mRNA. The activation pathway involves MAP kinase mediation, as the actions of CCh were sensitive to PD 98058, a selective inhibitor of the upstream MAP kinase kinase.

Many hormonal and neural signals received at the cell surface initiate the MAP kinase cascade.[12] Receptors that couple to G proteins, including those for bombesin, lysophosphatidic acid, α-thrombin, angiotensin, dopamine, and acetylcholine, have been shown to active MAP kinases.[17,18] The MAP kinase family includes serine/threonine kinases such as ERK1 and ERK2, the Jun N-terminal kinase/stress-activated protein kinase, and p^{38mapk}.[19]

Changes in $[Ca^{2+}]_i$ levels have crucial roles in regulation of cellular processes mediated by m1 and m3 cholinergic receptors. An important mediator of Ca^{2+} signaling is the Ca^{2+}-binding protein, calmodulin. The Ca^{2+}-calmodulin complex interacts with and modulates the activities of multiple signaling enzymes, including calmodulin-dependent protein kinases and calmodulin-dependent protein serine/threonine phosphatases. In a number of cell types MAP kinases are a point of convergence for Ca^{2+}-dependent signals associated with cell growth and division. In rat hippocampal neurons, exposure to the muscarinic agonist CCh caused a prolonged Ca^{2+}-dependent activation of ERK1/2.[20] Calcium-dependent transcription of immediate early genes has been described in neuronal PC12 cells, an effect that is mediated by calmodulin kinase and MAP kinase.[21,22]

The effects of Ca^{2+}–dependent signals on gene expression appear to be cell type specific. In AR42J cells, Stepan et al.[23] reported that gastrin 17 stimulated cell growth through activation of the MAP kinase pathway and *c-fos* gene expression. In contrast, in GH3 cells, gastrin 17 induced cell proliferation, although it failed to activate MAP kinase. In the present study, MAP kinase activation was inhibited by the inositol triphosphate receptor antagonist, cinnarizine, and by KN 93, an antagonist of Ca^{2+}-calmodulin kinase.

CCh-induced *c-fos* expression was also inhibited by these agents and by the MAP kinase kinase antagonist PD 98059.

In summary, AR42J cells express both m1 and m3 cholinergic receptors. Exposure to the cholinergic agonist CCh stimulated the expression of the immediate early genes *c-fos* and *c-jun*. In these cells, cholinergic control of gene activation was Ca^{2+} dependent and mediated via a calmodulin kinase pathway acting through MAP kinase.

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Expression of 17-1A Antigen and Complement Resistance Factors CD55 and CD59 on Liver Metastasis in Colorectal Cancer

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Despite radical surgery, the prognosis for colorectal cancer patients with liver metastases has not changed markedly. Furthermore, no standard adjuvant therapeutic regimen has been developed. Adjuvant therapy with monoclonal antibodies (e.g., against 17-1A), which has been shown to be effective in preventing metastatic relapse in patients with Dukes' C colorectal cancer, might be a promising approach for these patients. However, the cytotoxic effects of monoclonal antibodies can be blocked by coexpression of complement resistance factors that inhibit antibody-dependent complement-mediated cytotoxicity. We therefore analyzed immunohistochemically the expression of 17-1A and the membrane-bound complement resistance factors CD55 and CD59 on metastatic tumor cells in the livers of 71 patients with colorectal carcinoma who had undergone resection of their metastases with curative intent. In 67 (94%) of 71 patients, liver metastases with homogeneous expression of 17-1A was seen. Heterogeneous expression of 17-1A was seen in four patients (6%). Heterogeneous expression of CD55 or CD59 was observed in 8 (11%) of 71 patients and 4 (6%) of 71 patients, respectively. None of the patients showed homogeneous expression of either CD55 or CD59. All patients with CD55 or CD59 expression showed homogeneous 17-1A expression, whereas none of the four patients with heterogeneous 17-1A expression was positive for CD55 or CD59. Our data indicate that 17-1A is widely expressed on liver metastases of patients with colorectal carcinoma. Therefore patients with completely resected liver metastases might be suitable candidates for adjuvant therapy with anti–17-1A antibody since only a few of these lesions showed coexpression of complement resistance factors. (J GASTROINTEST SURG 2001;5:673-679.)

KEY WORDS: 17-1A, complement resistance factors, colorectal cancer, liver metastases

Colorectal carcinoma is still one of the leading causes of cancer deaths in the Western world. Despite advances in screening, early diagnosis, and radical tumor removal, the prognosis of colorectal cancer has not changed markedly in recent decades. In this tumor entity, spread of tumor cells to the liver is a remarkably common event and represents the major determinant of outcome following apparently successful curative resection of the primary tumor. More than 50% of all patients with colorectal cancer develop liver metastases after initial colorectal resection.[1-5] This indicates that disseminated tumor cells, which are undetectable by current staging procedures, may already have been present at the time of the primary surgery. In the past few decades, immunocytochemical and nucleic acid–based assays have been developed that are able to detect individual disseminated tumor cells in lymph nodes, bone marrow, or blood.[6-13] In colorectal cancer, detection of single tumor cells in pericolic lymph nodes classified as tumor free on routine histopathologic[6,7] or bone marrow[10] examination correlates with a significantly worse prognosis. Most of these minimal residual tumor cells seem be dormant or noncycling and may therefore be resistant to antiproliferative drugs such as 5-fluorouracil, which is the standard adjuvant regimen in colorectal cancer

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patients with International Union Against Cancer (UICC) stage III disease.[14,15] Therefore adjuvant therapeutic modalities that target and destroy postoperatively remaining tumor cells independently from their cell cycle are of special interest. One promising approach in adjuvant cancer treatment seems to be the application of monoclonal antibodies directed against tumor-associated antigens resulting in subsequent tumor cell lysis. An essential event in the humoral immune response, which is therefore partly responsible for antitumoral cytotoxicity in patients undergoing antibody therapy, seems to be activation of complement. Induction of the complement cascade results in the formation of the factor C5b-C9 membrane attack complex. In addition, the anaphylatoxins C3a, C4a, and C5a support the cellular immune response by chemotactic attraction of phagocytes, an increase in capillary permeability, and tumor vascularization.[16] Furthermore, the C3 cleavage product C3b (opsonin) plays an important role in phagocytosis of tumor cells via target opsonization.

CD55 and CD59 are important membrane-bound inhibitors of the complement cascade. CD55 (decay-accelerating factor) is a widely expressed phosphoinositol-linked glycoprotein that dissociates the C3/C5 convertase independently from other proteins, thereby blocking the complement cascade at an early stage including prevention of anaphylatoxin release and cytolysis by the membrane attack complex.[17,18] CD59, an 18 to 25 kDa widely expressed phosphoinositol-linked glycoprotein, prevents cytolysis in the last step of the complement cascade via binding to C8, thereby blocking the C9-polymerization process as the most important step for the function of the membrane attack complex.[19]

We therefore analyzed the expression of 17-1A, CD55, and CD59 on 71 liver metastases and nine autologous primary tumors in patients with colorectal carcinoma who had undergone resection of their metastases and primary tumors with curative intent.

MATERIAL AND METHODS

Patients and Study Design

The study was approved by the ethics committee of the University of Hamburg. Informed consent was obtained from all patients. Tumor samples were collected from 71 colorectal cancer patients with resectable liver metastases, who had undergone radical resection of their disease between March 1992 and February 1998. Patients were reexamined every 3 months after surgery for 2 years and at 6-month intervals thereafter. The evaluation included physical examination, chest x-ray examination, endoscopy, abdominal CT scans, abdominal ultrasound, and analysis of tumor markers (CEA and CA 19-9).

Immunohistochemical Analysis

Representative tumor samples were snap frozen in liquid nitrogen immediately after resection and stored at −80° C. From each tissue sample, 5 to 7 μm cryostat sections were obtained, transferred onto glass slides prepared with 3-triethoxysilyl-propylamin (Merck, Darmstadt, Germany), and stored at −80° C until they were used. Cryostat sections were air dried, fixed in 96% ethanol, rehydrated with tris-phosphatase buffered saline (pH 7.4), and stained using the alkaline phosphatase–antialkaline phosphatase (APAAP) technique as previously described.[9,12] Blocking of nonspecific binding was performed with normal rabbit serum (Dako, Hamburg, Germany) diluted 1:10 in TBS, and applied for 30 minutes. As primary antibodies we used monoclonal antibody BRIC 110 (IgG1; Serotec, Oxford, U.K.) directed against CD55, diluted 1:50 (20 μg/ml) in TBS, monoclonal antibody MEM-48 (IgG2a; Serotec) directed against CD59, diluted 1:500 (2 μg/ml) in TBS, and monoclonal antibody 3B10-C9 (IgG2a; kindly provided by K. Pantel, Department of Gynecology, Hamburg, Germany) directed against 17-1A, diluted 1:100 (10 μg/ml) in TBS. The primary antibodies were applied for 45 minutes. This was followed by incubation with a linking antibody (Z0259, Dako, Hamburg, Germany) for 30 minutes and subsequent application of the APAAP complex for another 30 minutes. After each incubation, washing in TBS was done. Antibody-linked alkaline phosphatase activity was visualized with Fast Red TT (Sigma, Deisenhofen, Germany). Endogenous alkaline phosphatase activity was blocked by levamisole (L9756, Sigma). Sections of normal colonic mucosa served as positive staining controls for 17-1A; cytocentrifuged PHA-stimulated peripheral blood lymphocytes were used as positive staining controls for CD55 and CD59. The sections were counterstained with Gill's hematoxylin. To exclude nonspecific antibody binding, negative control testing was performed with irrelevant mouse myeloma proteins of identical isotypes (MOPC-21 for IgG1 and UPC10 for IgG2a, Sigma). All incubations were performed at room temperature in a humidified chamber.

The slides were evaluated in a double-blind manner by two observers using light microscopy. Tumors were divided into the following four groups according to the percentage of tumor cells expressing 17-1A, CD55, or CD59: negative (0 positive cells), weakly positive (1% to 35% positive cells), moderately positive (36% to 75% positive cells), and homogeneously positive (>75% positive cells) expression. For statistical analysis, patients with weak or moderate expression were combined into one group of heterogeneously positive tumors (i.e., 1% to 75% positive cells).

In 90% of the cases, both observers obtained the same results; the remaining slides were reevaluated and a consensus was reached.

Statistical Analysis

Associations between categorical variables were assessed by Fisher's exact and chi-square tests. Differences between groups were considered statistically significant if the *P* values were less than 0.05 in a two-tailed test.

RESULTS

Tumor samples were collected from 71 patients with resectable liver metastases. The median age was 61 years (range 31 to 80 years). There were 44 (62%) male and 27 (38%) female patients. Twenty (28%) patients had synchronous and 51 (72%) patients had metachronous hepatic metastases. A majority of the 65 (92%) metastatic lesions showed moderate differentiation (G2), two (3%) metastases were well differentiated (G1), and four (6%) metastases were poorly differentiated (G3). Forty (56%) patients had solitary liver metastases, 13 (18%) had two metastases, and 18 (25%) had three or more isolated metastases within the liver. In 66 (93%) patients hepatic resection with tumor-free resection margins (R0) was performed. In five cases histopathologic examination revealed incomplete resection (R1).

Immunohistochemical analysis yielded a distinct membrane-bound and cytoplasmic staining pattern for 17-1A and a predominant membrane-bound staining for both CD55 and CD59. All patients showed a homogeneous (67 of 71) or heterogeneous (4 of 71) expression of 17-1A on their metastases (Fig. l). All tumors with heterogeneous 17-1A expression displayed immunostaining on more than two thirds of their cells. Heterogeneous expression (1% to 75% positive cells) of CD55 was seen on 8 (11%) of 71 tumors (Fig. 2, *A*). Three of these CD55-expressing tumors showed weak expression (1% to 35% positive tumor cells) and five showed moderate expression (36% to 75% positive tumor cells). Regarding CD59, 4 (6%) of 71 metastases showed weak expression (Fig. 2, *B*). Taken together, both CD55 and/or CD59 expression in 12 (17%) of 71 patients displayed at least one of these complement resistance factors on their cell surfaces. None of the patients displayed CD55 or CD59 homogeneously (>75% positive cells) on their metastases (Table I).

All patients with CD55- and/or CD59-expressing metastases showed homogeneous 17-1A expression, whereas none of the four patients with heterogeneous 17-1A expression showed CD55 and/or CD59 expression (Table II).

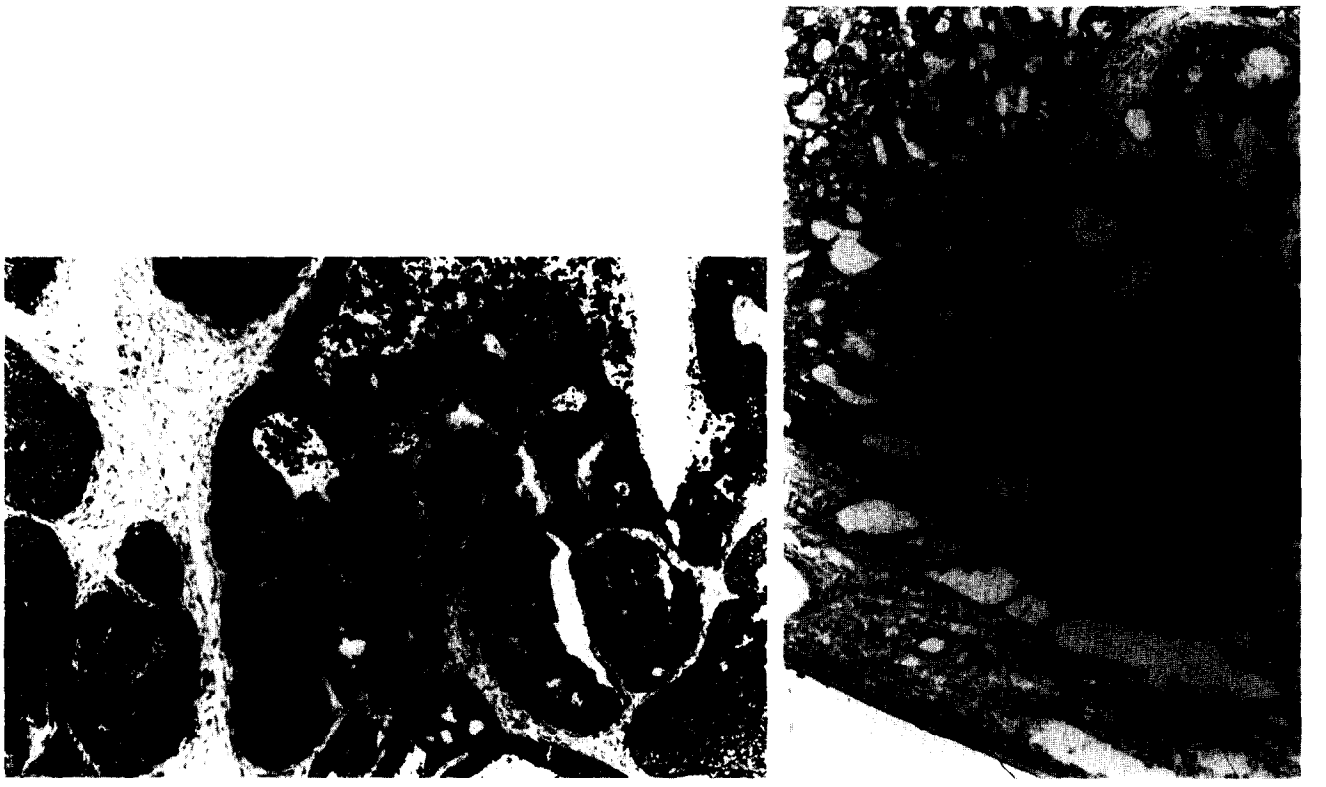

Fig. 1. Homogeneous **(A)** and heterogeneous **(B)** expression of 17-1A antigen on colorectal liver metastases.

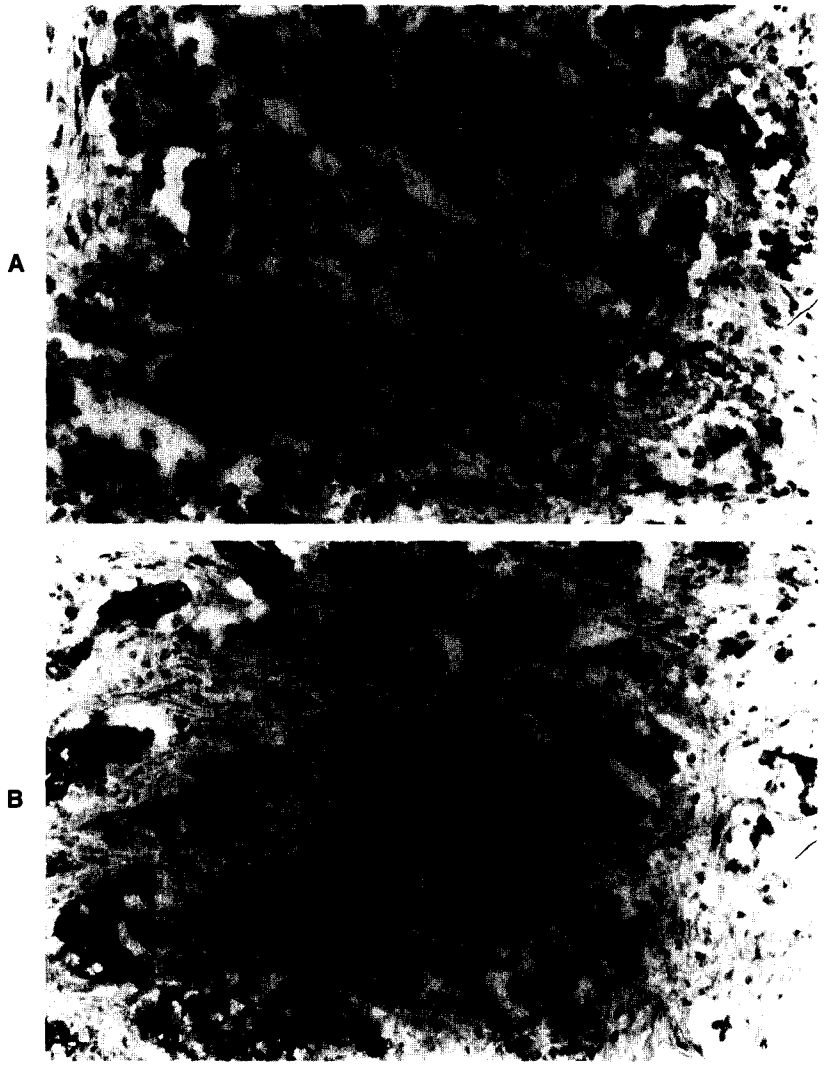

Fig. 2. Heterogeneous expression of CD55 **(A)** and CD59 **(B)** on colorectal liver metastases.

Table I. Expression of 17-1A, CD55, and/or CD59 on colorectal liver metastases

	17-1A	CD55	CD59	CD55/CD59
Negative	0/71	63/71 (89%)	67/71 (94%)	59/71 (83%)
Heterogeneous	4/71 (6%)	8/71 (11%)	4/71 (6%)	12/71 (17%)
Homogeneous	67/71 (94%)	0/71	0/71	0/71

Negative = 0 positive cells; heterogeneous = 1% to 75% positive cells; homogeneous >75% positive cells.

Table II. Correlation of 17-1A with CD55 and CD59 expression*

17-1A	CD55 Positive	CD55 Negative	CD59 Positive	CD59 Negative
Homogeneous (n = 67)	8/8 (100%)	59/63 (94%)	4/4 (100%)	64/67 (96%)
Heterogeneous (n = 4)	0/8	4/63 (6%)	0/4	3/67 (4%)
TOTAL (n = 71)	8	63	4	67

Negative = 0 positive cells; heterogeneous = 1% to 75% positive cells; homogeneous >75% positive cells.
*Differences were not significant.

In univariate analysis, no significant correlations between tumor grading, synchronous/metachronous occurrence, or number of metastases and expression of 17-1A, CD55, and/or CD59 were found (data not shown).

Expression analysis of nine consecutive primary colorectal tumors of patients with synchronous liver metastases revealed homogeneous 17-1A expression on eight tumors (89%) and heterogeneous 17-1A expression on one tumor. All tumors were negative for CD55, whereas heterogeneous CD59 expression was seen in one tumor. When expression patterns of liver metastases and autologous primary tumors were compared, similar results for 17-1A and CD55 were observed in all cases, whereas CD59 expression differed in three cases (data not shown).

DISCUSSION

In this study we examined the distribution of 17-1A, which is the target of the therapeutic antibody 17-1A, and the membrane-bound complement resistance factors CD55 and CD59 on liver metastases in 71 patients with colorectal cancer who had undergone resection of their liver metastases with curative intent, with tumor-free resection margins in 66 cases (93%). In all cases hepatic metastases showed 17-1A expression on more than two thirds of the cells. Only a few of these patients displayed expression of complement resistance factors CD55 and CD59.

Metastasis to the liver in resectable colorectal cancer is a remarkably common event and a major determinant of longtime survival in these patients. More than 50% of all patients with completely resected colorectal carcinoma develop liver metastases in the course of their disease. In addition, synchronous liver metastases are present in 10% to 25% of patients at the time of initial colorectal resection. Resection of solitary or localized liver metastases with curative intent can achieve 5-year survival rates of up to 30%.[1,2,20] On the other hand, approximately 75% of these patients develop recurrences within the liver and/or lung. This observation indicates that most colorectal cancer patients with apparently localized and resectable primary or metastatic lesions may already have occult tumor cell dissemination, which is undetectable by current staging methods. Therefore more sensitive immunocytochemical or nucleic acid–based assays[6-10,21] have been established that are able to identify disseminated single tumor cells in lymph nodes or bone marrow. The extended latency period between detection of disseminated single tumor cells at the time of primary surgery and the occurrence of overt metastatic relapse suggests that these cells remain in a state of dormancy and therefore initially may not have the potential to form clinically detectable metastases. This dormant state of micrometastatic cells is supported by the observation that only a small amount of these cells express proliferation-associated proteins such as Ki-67 and p120.[22] Significantly higher rates of tumor relapse after radical and complete tumor resection (R0) in cancer patients with micrometastasis to lymph nodes or bone marrow[6-13] indicate that these cells may revert to a proliferative state under certain environmental conditions and are therefore potentially precursors of subsequent macrometastases. Because of their dormant state, micrometastatic tumor cells may not be effectively treated with antiproliferative drugs such as 5-fluorouracil and levamisole. In this context the introduction of monoclonal antibodies raises hope for new cancer treatment possibilities. In the past decade many studies have been conducted to evaluate the treatment of cancer with monoclonal antibodies directed against tumor-associated antigens; these usually involve tagging these monoclonal antibodies with radioisotopes, toxins, or drugs. Alternatively, clinical trials using native unconjugated monoclonal antibodies have been conducted. In vitro and in vivo experiments with these native monoclonal antibodies were able to achieve tumor cell destruction via complement-mediated lysis, antibody-dependent cellular cytotoxicity, and/or opsonization of cells with subsequent phagocytosis.[23,24] However, most clinical trials using native monoclonal antibodies in cancer therapy, that is, in pancreatic cancer[25-27] and other gastrointestinal tumors,[28,29] revealed no significant benefit in terms of patient outcome, although the antitumoral effectiveness of monoclonal antibodies in model systems has been demonstrated.[24,30,31] However, the majority of these trials have usually recruited patients with unresectable advanced metastatic disease,[25] where physiologic barriers with subsequent poor or incomplete monoclonal antibody delivery and a large antigenic load might limit the antitumoral effects of therapeutic monoclonal antibodies. Another reason for the generally poor results achieved with antibody therapy seems to be the expression of complement resistance factors on tumor cells, such as CD55 and CD59, which are able to inhibit the ability of monoclonal antibodies to activate complement.[17,19,32]

Despite these generally poor results, one clinical trial applying anti–17-1A monoclonal antibody 17-1A in an adjuvant therapeutic setting in colorectal cancer patients with UICC stage III disease has been shown to be effective in preventing distant metastases in a certain subset of patients.[33] Thus one may speculate that the efficacy of therapeutic monoclonal antibodies depends on complete tumor resection with a subsequent low residual tumor load.[23,24] In this situation low numbers of potentially remaining single cancer cells or small tumor cell clusters are located in an un-

protected area in mesenchymal or reticuloendothelial tissue and might therefore be easily reached by therapeutic monoclonal antibodies via diffusion.

So far it is not known whether patients with colorectal liver metastases may also be suitable candidates for adjuvant antibody treatment after complete resection of their metastases. Reported 5-year survival rates of up to 30% after hepatic resection of colorectal metastases,[1,2,20] as well as high local recurrence rates for liver metastases within the liver, suggest that dissemination of relevant tumor cells is restricted to the liver in certain patients. Generalized dissemination in this tumor entity occurs with less frequency compared to other gastrointestinal carcinomas. Moreover, the liver seems to be an "unfriendly" environment for development of metastases. In an animal model, metastatic outgrowth within the liver could only be observed if a large number of tumor cells ($>10^6$ tumor cells) were injected into the portal vein.[35]

17-1A is strongly and extensively expressed on liver metastases of patients with colorectal carcinoma. Because of this strong expression and wide distribution, 17-1A seems to be highly conserved on metastatic tumor cells in this tumor entity and might therefore be an ideal target antigen for immunotherapy against small tumor loads. Only a few of these patients displayed expression of the membrane-bound complement resistance factors CD55 and CD59 on their metastases rendering these cells resistant to monoclonal antibody therapy. Therefore adjuvant antibody therapy with anti–17-1A monoclonal antibody 17-1A in properly selected patients undergoing resection of liver metastases of colorectal cancer with curative intent warrants a trial. Prior to therapeutic antibody application, immunohistochemical assessment of 17-1A, CD55, and CD59 on liver metastases will identify patients who are potential candidates for antibody therapy. Because all of the patients analyzed in this study expressed 17-1A on at least two thirds of their tumor cells and the few patients with heterogeneous 17-1A expression (4 of 71) showed no immunoreactivity for both CD55 or CD59, one may speculate that most patients with resectable liver metastases would probably benefit from 17-1A antibody therapy even more because in breast cancer strong immunostaining of more than only 10% of tumor cells determines inclusion into anti-HER2-neu antibody therapy, which results in a prognostic benefit in these patients.

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Book Review

Minimally Invasive Abdominal Surgery
Kare Kremer, W. Platzer, Hans W. Schreiber, and F.M. Steichen, eds. New York: Thieme Medical Publishers, 2001. Pages: 465. Price: $249.

This 465-page book by 82 contributors covers most of the important aspects of minimally invasive abdominal surgery. There are 1088 illustrations, which make this book a joy to read and help in the elucidation of concepts and procedures. The topics range from the basic to the most complicated and unusual in minimally invasive abdominal surgery. The index section is good, which makes this a very useful reference book.

The chapters are written by experts on instrumentation and technique, basic and advanced surgery of the biliary tract, surgery of the spleen and liver, endoluminal gastric operations, vagotomy, antireflux procedures, operations for morbid obesity, appendectomy, small and large bowel resection, and inguinal hernia repair. The chapters all follow the same format, which allows for easy reading. They are organized according to the following format: goals and methods, indications, techniques, setup, step-by-step flow of each procedure, complications, and a bibliography. In most sections there is a highlighted summary of the steps of each procedure.

The book is beautifully illustrated with clear color drawings. These have been expertly drawn and make the steps of the procedure easy to understand. The subjects are presented in a clear and scholarly fashion. However, there are some omissions, such as the absence of chapters dealing with ventral hernia repair, congenital diaphragmatic hernia, and adrenal and renal procedures.

This book adequately covers the subject of minimally invasive abdominal surgery making it useful for both the beginner and those with more experience. It offers useful and comprehensive information on the latest in minimally invasive abdominal surgery in Europe and the United States.

Ronald A. Hinder, M.D.

Letters to the Editors

Resected Adenocarcinoma of the Pancreas—616 Patients: Results, Outcomes, and Prognostic Indicators

To the Editors:

The Johns Hopkins Pancreatic Cancer Group is to be congratulated for presenting much-needed prognostic data on adenocarcinoma of the pancreas.[1]

I have one question, and this concerns the issue of tumor involvement of the portal vein/superior mesenteric vein. In the Methods section, there is mention of the fact that a small proportion of patients did, in fact, have a venous resection. I would be very interested to know if a subset analysis has been done on this group of patients. Also, in the analysis of positive and negative margin status, I wonder where the patients who underwent venous resection were placed.

The answer to the above question is critical. In recent times, reports have established that resection of the portal vein/superior mesenteric vein can be performed relatively safely and is therefore justifiable from the technical point of view. The question that remains is whether or not it is justifiable oncologically. With thin-slice CT scanning and endoscopic ultrasound interrogation of the tumor/portal vein/superior mesenteric vein interface, the surgeon can predict with reasonably high confidence the likelihood of venous involvement before the operation. Is it justifiable to operate on these patients with a view toward resection, which is very likely to involve a venous resection? Will a radical surgical approach in these patients improve their survival over those who are simply treated either by surgical or endoscopic palliation?

I look forward to the authors' comments with interest. It is only from a large-volume experience such as that documented at Johns Hopkins that will obtain an answer to this question.

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Reply

Dr. Jorgensen notes that the use of thin-slice CT scanning and endoscopic ultrasound interrogation of the tumor/portal vein/superior mesenteric vein interface can often raise the suspicion of venous involvement. Of course, such suspicion is not always correct, as tumor proximity to the major venous structures does not routinely correlate with infiltration of the venous structures by tumor ingrowth. It has been our practice to perform an exploratory operation in those patients whose superior mesenteric vein and portal vein are patent, avoiding exploratory surgery in those patients who have evidence of occlusion of the superior mesenteric vein or portal vein.

In the group of 15 patients who underwent venous resection in our series, all resections were partial resections, involving resection of a portion of the vein, followed by either primary closure or patch repair. Of the 15 patients undergoing venous resection, 6 (40%) of the 15 were left with positive margins elsewhere, and 14 (93%) of the 15 had lymph node involvement in the specimen. Contrast these data with those from the 601 patients who did not undergo venous resection, where only 30% of the patients underwent a margin-positive resection, and only 73% of the patients had positive lymph node involvement in the resection specimen. Thus patients undergoing venous resection in our series had more advanced tumors. A subset analysis was performed of the 15 patients undergoing venous resection, compared to those not undergoing such resection. The 1-year survival rate was 43% for those undergoing venous resection compared to 62% for those not undergoing venous resection ($P = 0.14$).

Based on our data, partial venous resection appears safe. However, because it is usually used in patients with more advanced tumors, the impact of venous resection on survival remains uncertain.

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