Journal of Cellular Biochemistry 70:1–7 (1998)

VIEWPOINT

Cell Cycle: Molecular Targets for Diagnosis and Therapy: Tumor Suppressor Genes and Cell Cycle Progression in Cancer

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Abstract A significant portion of published literature is dedicated to describing the cloning and the characterization of proteins involved in the progression of the cell cycle, which govern cell growth both in cancer and normal ontogenesis. With this abundance of information, the cascading pathways of molecular events that occur in the cell cycle are proving to be exceedingly complicated. The purpose of this conference was to attract the leading clinical and basic science investigators in the growth control field with a final goal to determine how this current wealth of knowledge can be used to impact upon patient care and management by the design of novel adjuvant therapeutics specifically targeted at tumor cells and the identification of molecular diagnostic and/or prognostic markers in an efficient and cost effective manner. J. Cell. Biochem. 70:1–7, 1998. © 1998 Wiley-Liss, Inc.

Key words: apoptosis; p53; pRb2/p130; E2F; transcriptional control; leukemia; protein phosphatases; colon cancer; retinoblastoma

In biomedical research, the field of cell cycle has grown rapidly with the discovery of the check points involved as the cell progresses from the G1 phase. Scientists in this field have defined some of the molecular events that serve as the fundamental basis for the regulation of cell proliferation and cell death. A number of cellular processes have recently been defined which allow innovative approaches to cancer diagnosis or therapy. These processes include the roles of growth factors and cytokines in signal transduction and the regulation of cell cycle and transcription. Tumor-suppressor genes such as the retinoblastoma gene and p53 are currently being studied to a degree to which their function is now better understood. The purpose of this conference was to bring together investigators who are demonstrating the function of these genes, how they function within tumors, and how these genes may be used for therapy.

This symposium was held on November 7–8, 1997, at the Roswell Park Cancer Institute, Buffalo, New York.

Contract grant sponsors: Bristol-Myers Squibb, Hoescht Marion Roussel.

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Accepted 9 February 1998

The mechanism of action of chemotherapeutic drugs is of interest in relation to the relatively unknown signaling pathways leading to apoptosis. The summation of activities of multiple signaling pathways appears to be crucial to cell-fate determination. In this context, the contribution of a particular pathway versus that of another plays an important role that may be cell specific. The application of knowledge gained from these pioneering investigators is likely to lead to a better understanding for therapeutic approaches.

The conference was organized to attract both clinical and basic cancer investigators. It was considered that several scientific collaborations may result from bringing together scientists from these areas. The major concern of many of the researchers at the meeting was the lack of specificity of many of the current therapeutic options for the treatment of cancer. They emphasized the need to delineate further the signal transduction pathways leading to growth arrest, terminal differentiation, or apoptosis that are disrupted during oncogenesis. This knowledge could be used to design therapeutics specifically targeted at tumor cells harboring such mutations. Even though great advances have

been made in the fields of cell cycle and cancer research, there is still much to do. This does not mean, however, that we cannot start to take advantage of our current knowledge base to design novel cancer treatments.

CELL CYCLE AND ASSOCIATED GENES

Rb Family Proteins Role in Human Cancer

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Dr. Giordano reported on the direct regulatory role of the retinoblastoma-related protein, pRb2/p130, in the cell cycle through its association and inhibition of cdk2 kinase activity in vitro and in vivo. Immunohistochemical studies implicated the involvement of pRb2/p130 in the development and/or progression of several human cancers including lung, endometrial, and ovarian. He also disclosed the identification of mutations within the RB2/p130 gene in human tumor cell lines and patient tumor samples. The first studies to demonstrate the dramatic growth-suppressive activity of pRb2/p130 expression in vivo were presented. These data strongly supported the hypothesis that RB2/p130 is a tumor-suppressor gene. Dr. Giordano also reported on two cdc2-related kinases cloned in his laboratory, PITALRE and PISSLRE [Bullrich et al., 1995; De Luca et al., 1997]. He conferred data demonstrating that the function of PISSLRE is required in the G2/M phase of the cell cycle, the first cdc2-related kinase to do so since cdc2 itself [Li et al., 1995]. PITALRE has recently been identified by others as a major component of the HIV Tat-mediated transcription elongation complex.

Tissue-Specific Consequences of Loss of Retinoblastoma Gene

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Many lines of evidence suggest that the critical function of the retinoblastoma gene (RB) product (pRB) is in development, but the most important consequences of loss of pRB, uncontrolled proliferation and cancer, are rare events almost specific to the retina in humans. In other developing tissues, the loss of pRB leads to apoptosis or, in myotubes, endoreduplication. In murine retinal development, Dr. Gallie reported that RB is first expressed around the time that the proliferating retinoblasts exit the cell cycle, start to express differentiation markers, and migrate to their permanent retinal layer.

The benign lesion "retinoma" documented in $RB^{-/+}$ humans may arise when developing retinal cells lose the second RB allele and enter S phase inappropriately, but if the process of programmed cell death is intact, only linear growth would result. Retinoblastoma may arise when further mutation distrupts the death-signaling pathway, thereby permitting exponential proliferation. However, apoptosis is intact in retinoblastoma tumors and p53 is a wild type that is expressed at low normal levels and inducible by appropriate stimuli. Unlike most other human tumors in several respects, the cyclin/kinase inhibitor p16 is not upregulated in the absence of pRB, and despite many passages in vitro, retinoblastoma cells do not express telomerase activity and have normal length telomeres. Retinoblastoma tumor cells always contain additional mutations, specifically the i(6p) chromosome, resulting in low-level amplification of chromosome 6p, perhaps disrupting the programmed cell-death signals.

Involvement of Protein Phosphatases in Apoptosis

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Dr. Eastman began by discussing the many pathways of apoptosis that can be induced by numerous drugs and other agents. He emphasized that steps required for apoptosis induced by one insult are frequently not required for other agents. He also questioned the terminology of p53-dependent apoptosis, rather suggesting that the p53 tumor-suppressor protein may modulate the response to DNA-damaging agents but is not required for apoptosis from such insults. The goal of his presentation was to define the point where all these pathways are integrated into a final common execution phase. Starting from the point of DNA digestion, he compared the contribution of Ca^{2+}/Mg^{2+}-dependent endonucleases and deoxyribonuclease II. Calcium was clearly shown not to be required for DNA digestion, whereas apoptotic cells consistently undergo intracellular acidification consistent with the activation of DNase II. The

cloning and expression of DNase II has been achieved, and expression leads to chromatin condensation and DNA digestion characteristic of apoptosis. Upstream of DNA digestion is a cascade of proteases known as caspases, each activating another caspase and digesting numerous cellular proteins. However, the cause of the first activation event remains unknown. Dr. Eastman presented evidence for the role of protein phosphatases in activating this caspase cascade and focused particular attention on the role of PP1. Expression of specific peptide inhibitors of PP1 but not PP2A was able to protect cells from apoptosis induced by staurosporine, strongly suggesting that PP1 is a critical activator of apoptosis. As an aside, he demonstrated that the Rb protein was dephosphorylated and cleaved during apoptosis. He also demonstrated a p53-dependent DNA damage-inducible response whereby Rb was dephosphorylated as an early event in apoptosis (i.e., prior to activation of caspases). Suppression of p53 prevented Rb dephosphorylation but did not prevent apoptosis. Hence, in this model, p53 did not contribute to DNA damage-induced apoptosis, perhaps because the pro-apoptotic protein Bax was not induced. In his summary, Dr. Eastman discussed how this information could guide the development of therapeutic agents. He felt that enhancing the execution phase of apoptosis suffered from a lack of specificity for the tumor. In searching for a more tumor-specific target, he suggested that effective therapy could best be targeted to cells defective in p53 rather than attempting to replace a wild-type p53 function in every tumor cell. Such approaches may take advantage of recent evidence for the role of p53 in regulating DNA damage-dependent cell cycle checkpoints.

Novel Mechanism of Activation of E2F Transcriptional Activity by a Viral Immediate Early Protein

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The transcription factor E2F and its regulation by pRB and related tumor-suppressor proteins are central to cell cycle control in higher eukaryotes. E2F is a family of factors that are regulated by association with cell cycle regulatory molecules including members of the retinoblastoma and cyclin families. Much of our knowledge of this regulation has come from studies using immediate-early proteins of DNA tumor viruses. They found that the 72-kDa immediate-early gene product of the human cytomegalovirus IE72 transactivates the dihydrofolate reductase promoter through the E2F site and that it physically interacts with E2F1 in vitro, in cells stably expressing IE72, and in HCMV-infected cells [Wade et al., 1992; Margolis et al., 1995]. The significance of this interaction is supported by the finding that a mutated IE72 that abolishes its ability to interact with E2F1 also abolishes its transactivation activity. Moreover, a mutation in IE72 that abolishes its transactivation function but preserves the ability to interact with E2Fs prevents activation of E2F-dependent transcription by wild-type IE72 and decreases the ability of the virus to infect cells. In experiments designed to characterize further the mechanism by which IE72 modulates E2F-dependent transcription, they found that IE72 is a kinase that autophosphorylates and phosphorylates E2Fs 1, 2, and 3 (but not E2F4 or 5) and p130 and p107 (but not pRB) [Pajovic et al., 1997]. The region of IE72 spanning amino acids 173–197 shows a high level of homology to the ATP binding site in more than 500 kinases. The kinase-negative protein IE72 ATP, from which this region has been deleted, cannot activate E2F-dependent transcription. Although it can be phosphorylated, IE72 ATP cannot activate E2F-dependent transcription and prevents activation by IE72, indicating that kinase activity is required for transcriptional activation. To assess the role of the kinase activity of IE72 in activation of E2F-dependent transcription, they have determined that IE72 but not IE72 ATP can dissociate E2Fs 4 and 5 from p107 and p130. By mutating the sites in IE72 that are phosphorylated, they have now determined that the interaction of IE72 with E2Fs, the activation of E2F-dependent transcription, and phosphorylation of E2Fs and pocket proteins require that IE72 be phosphorylated. Taken together, these data indicate that the kinase activity of IE72 is essential for the effects of HCMV on E2F-dependent transcription and may be an essential mechanism whereby HCMV modulates host cell proliferation and differentiation.

Regulation and Linkage of Abrogated Nuclear Architecture to Transcriptional Control in Tumors

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This group of researchers has been investigating interrelationships between nuclear structure and gene expression. Emphasis is on consequential modifications in transcriptional control, which are linked to abrogated parameters of nuclear organization in tumor cells. Using the AML transcription factor, which is reorganized because of chromosomal translocations in leukemia cells, they have functionally mapped independent domains of AML that are responsible for nuclear import and subsequent association with sites within the nucleus where transcription occurs. They have shown that in transformed and tumor cells there are changes in the intranuclear distribution of transcription factors. In addition, they have observed cross-talk between regulatory proteins that influence subnuclear localization of factors that control cell growth and tissue-specific gene expression. This is the first identification of an intranuclear trafficking signal. Their findings indicate that association of a transcription factor with the nuclear matrix is obligatory for activity [Zeng et al., 1997]. Their results provide insights into relationships between rearrangements in nuclear organization and aberrations in gene expression with cancer.

Mechanism of p53-Induced Apoptosis

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The p53 tumor-suppressor gene is the most frequently mutated gene in human cancer. The most well-characterized property of p53 is to transactivate genes whose products will lead to growth arrest or apoptosis depending on the cell type and environmental conditions. In human colorectal cancer cell lines, the p53-induced G1 arrest is dependent on the p21 cyclin-dependent kinase inhibitor; the molecular basis of the apoptotic pathway is still largely unknown. To elucidate the mechanism of p53-induced apoptosis, we determined the global gene expression profile of a colon cancer cell line before apoptosis using serial analysis of gene expression. Of 7,202 transcripts analyzed, only 14 were found to be highly (more than tenfold) induced after p53 overexpression. Surprisingly, many of these genes (p53-induced genes; PIGs) seem to be involved in the regulation of the cellular redox status. This suggested further experiments, the results of which led to the following model of the p53-induced apoptosis: p53 induces the expression of genes whose products leads to the generation of reactive oxygen species (ROS) followed by mitochondrial damage culminating in rapid cell death. Inhibitors of transcription, ROS production, or mitochondrial permeability transition were able to block cell death, indicating that they are required for this apoptotic process. The molecular characterization of the PIGs will give further insight to the role of apoptosis in p53-mediated tumor suppression.

p53 and Colorectal Cancer

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Colorectal cancer represents the end result of approximately six essential genetic events. For even this minimal number to occur, some form of destabilization of the genome is imperative. For several years, the main route to genomic instability in sporadic colorectal cancer has been hypothesized to arise through defects in p53, as a lost "guardian of the genome." To test this idea, Dr. Anderson's laboratory developed inter-(simple sequence repeat) polymerase chain reaction (PCR) as a practical means of quantitating genomic damage in tumor biopsy specimens. The level of such genomic damage, representing the aggregate end result of genomic instability, was in turn compared with p53 status as determined by SSCP and DNA sequencing. For 58 consecutive cases of sporadic colorectal cancer, they found that the set with genomic instability below the median were predominantly mutant in p53 (19/29), whereas tumors with genomic instability above the median were predominantly wild-type p53 (18/29). They concluded that p53 mutation is not likely to be the key factor behind genomic instability in sporadic colorectal cancer. Similar studies have dissociated ras, Rb, and DCC from genomic instability.

In contrast, they found that expression of a specific nuclease did correlate with the degree of genomic instability. This nuclease has properties suggestive of DNase II, although its iden-

tity has yet to be confirmed. Expression of this nuclease is suppressible by NSAIDs, which may offer a novel therapeutic route of suppressing tumor progression.

Analysis of their inter-(simple sequence repeat) PCR data, comparing the number of genomic events in the sample with the fraction of the genome sampled, demonstrated that the number of genomic events that have occurred in the typical tumor cell is far larger than generally envisioned, with the number exceeding 10,000. Genomic instability begins very early in colorectal tumor progression, by the adenomatous polyp stage. These findings support a model in which cancer represents vastly accelerated somatic cell evolution, at the cell's interest alone, arising from the combined effects of genomic chaos and natural selection.

Alternately Spliced p53 and Its Clinical Relevance

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The gene p53 exists in cells in active and latent forms for sequence-specific DNA binding and transcriptional activation. The full-length latent p53 protein may specialize in functions such as binding to mismatched DNA or repair, whereas active forms, including the alternatively spliced form p53as in mouse cells, bind efficiently to consensus p53 DNA binding sequences. Active forms of p53 were also present in human primary breast cancers grown in SCID mice, although the activation mechanism is undetermined. Activation of p53 may occur by binding of a cellular protein to the p53 C-terminus. Several p53-binding proteins were detected in epidermal nuclear extracts, and crude fractions of these proteins were able to activate latent p53 for in vitro transcription. The activity of one of the p53-binding proteins, topoisomerase I, was stimulated by the latent form of p53, as demonstrated by equivalent activity among wild-type and several mutant p53 proteins deficient in sequence-specific DNA binding. These data suggest that distinct activities of active and latent sequence-specific binding forms of p53 can be differentially lost or retained in tumor cells and indicate the need for functional evaluation of p53 status rather than of p53 genotype alone in the prognosis and treatment plan of human cancer.

Mutants of Epstein-Barr Virus That Transform B Cells

John Yates, Ph.D., Department of Human Genetics, Roswell Park Cancer Institute, Buffalo, New York

Epstein-Barr virus (EBV) contributes to the malignancy of certain lymphoid and epithelial cancers. Two potential molecular targets for therapeutic control of EBV-associated malignancies are the oncogenic membrane protein LMP-1 and the nuclear protein EBNA1. Genetic studies indicate that EBNA1 performs an essential segregation function that is required to maintain the EBV chromosome in latently infected cells and that its additional role in directing initiation of replication on the viral chromosome is less important. Results from experimental infections of epithelial cells with EBV suggest that events that lead to abnormal expression of LMP-1 in epithelial cells could be critical to the development of the EBV-associated undifferentiated carcinomas.

Oncogenic Regulation of Topoisomerase 2a Expression and Toxicity

Dennis Stacey, Ph.D., and Guan Chen, Cleveland Clinic Foundation, Cleveland, Ohio

To help explain the selective toxicity of tumor drugs, the possibility that a tumor mutation (oncogenic ras) could alter the expression of a drug target (topoisomerase [topo] II alpha) was examined. Microinjected oncogenic ras induced topo II alpha expression, whereas anti-ras antibody blocked its expression. Moreover, in cotransfection experiments, oncogenic ras induced the topo II alpha promoter independently of the cell cycle. To extend these results, a novel time-lapse-based analytical procedure was used to assess the effects of microinjected ras on topo II alpha levels throughout the cell cycle in NIH3T3 cells. Although topo II alpha was degraded within 5 hours of mitosis in all cases, in the ras-injected cells topo II alpha levels were observed to increase thereafter. The time-lapse procedure also allowed a sensitive analysis of the cell cycle aspects of killing by the anti-topo II drug etoposide. In these experiments, the toxicity of the drug was related to cell cycle levels of topo II alpha. These results support the possibility that the oncogenic signaling constantly present within tumor cells may influence sensitivity to antitumor drugs by altering the expression levels of the drug target.

Novel Apoptosis Suppressor Drugs

Graham Goddard, Ph.D., Samuil R. Umansky, M.D., Ph.D., and L. David Tomei, Ph.D., LXR Biotechnology, Inc., Richmond, California

These investigators used serum deprivation-induced apoptosis of C3H 10T½ cells as a screening system to identify anti-apoptotic agents for use in inhibiting apoptotic cardiomyocyte death after cardiac ischemia and reperfusion. A group of phospholipids purified from soy flour were found to be cytoprotective, the most potent of which was lysophosphatidic acid (LPA). An LPA-based phospholipid dispersion (APM), which was capable of blocking up to 80% of serum deprivation-induced apoptosis, was also demonstrated to inhibit the death of primary neonatal rat cardiomyocytes exposed to a simulated ischemia and reperfusion regimen. Ischemia (O_2, serum, and glucose deprivation) alone resulted primarily in necrotic death of these cells, whereas reperfusion (addition of O_2, serum, and glucose) resulted in additional apoptotic death. APM added before the onset of ischemia or at the initiation of reperfusion equally blocked up to 100% of the apoptotic cell death in a protein synthesis-dependent manner. These data indicate that LPA-based formulations should be potent inhibitors of postischemic myocardial injury involved in the developing myocardial infarction after coronary occlusion.

Cytokines and Therapeutic Intervention

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Proliferation and differentiation of many cell types in addition to homeostatic functions in adult organisms are controlled in part by hematopoietic cytokines. The molecular signal transduction mechanisms, which are engaged by the principal hematopoietic cytokines, are elucidated to identify the potential contribution of abnormal cytokine signaling to the deregulated growth control in leukemic cells and to assess the possibility of modulating tumor proliferation by treatment with differentiation-inducing cytokines. These studies have characterized the activation of the JAK-STAT pathways by members of the interleukin (IL)-2, IL-3, and IL-6-type cytokines and the role of these pathways in the induction of gene expression through specific regulatory elements. The activation of alternatively processed STAT proteins, elevated activities of oncogenic protein tyrosine kinases of the Src, Fes, and Abl families, and constitutively active growth factor receptors have been recognized to interfere with the regulation of genes that encode differentiation and to contribute to the malignant phenotypes of myeloid and lymphoid leukemic cells. The current work evaluates to what extent the activities of the cellular protein kinases and phosphatase need to be modulated to enhance differentiation and to reduce proliferation of hematopoietic and nonhematopoietic cells.

FUTURE DIRECTIONS

Given the enormous multiplicity of functions a cell has to perform to ensure its survival and the different environments to which it is exposed, the integration of these activities into a functional whole requires precise regulation. Such a regulation needs not only to survey the growth of a cell, it also has to regulate the extent of differentiated cells that are produced to ensure the specialized properties required for the function of the organism and the extent of cell death. Given the thousands of genes that participate in regulating the expression of these cellular properties, the gathering of information on the control of these factors remains a primary interest. The objective of learning how the cell is able to regulate proliferation and differentiation formed the focus of this 2-day meeting. A major therapeutic challenge is understanding the tumor phenotype so that appropriate targeting of the faulty regulation of the tumor cell can be exploited. Current examples of such experimental therapies include the exploitation of attacking tumor cells with p53 mutations. The recent example of an E1B gene-attenuated adenovirus that causes tumor-specific cytolysis and augmented efficacy with standard chemotherapeutic agents may represent one avenue for potential success [Heise et al., 1997].

In addition, the hunt for new tumor-suppressor genes and cell cycle regulatory genes holds much promise as future targets for cancer therapeutics. Even though we are still plagued by the problems of targeting and specificity, we may nonetheless take advantage of the already vast amount of information already uncovered, which could have a potentially great impact on patient care. Many cell cycle regulatory proteins have been shown to be valuable as molecu-

lar markers for the diagnosis and/or prognosis for several types of cancer such as pRb2/p130 in lung and endometrial carcinoma [Baldi et al., 1996, 1997; Susini et al., 1998].

ACKNOWLEDGMENTS

The organizers wish to thank Dr. Alexander Bloch (RPCI) for his contributions to the meeting summary and Dr. Susan Leong (RPCI) for her help in coordinating meeting arrangements.

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