MOLECULAR, BIOCHEMICAL AND CELLULAR BIOLOGY OF HUMAN BREAST CANCER

Organizers: Marc Lippman, Robert Dickson and Dennis Slamon

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Molecular, Biochemical and Cellular Biology of Human Breast Cancer

Oncogenes

CF 001 IDENTIFICATION AND CHARACTERIZATION OF MUTATIONS IN PRIMARY BREAST TUMORS, [1]Robert Callahan, [1]Bruno Blondel, [1]Craig Cropp, [1]Daniel Gallahan, [1] Giorgio Merlo, [1]Joan Robbins, and [2] Rosette Lidereau, National Cancer Institute, Bethesda, MD 20892 [2] Centre Rene Huguenin, St. Cloud, France.

The mouse mammary tumor virus (MMTV) has been shown to activate the expression of two previously silent cellular genes (int-1 and int-2) in mouse mammary tumors. We previously identified a third gene (designated int-3) in CZECHII V^+ mouse mammary tumors which is frequently altered by MMTV integration. We have found that the int-3 gene is unrelated to other known cellular genes by nucleotide sequence analysis. Two species of int-3 RNA (6.2 kb and 4.0 k6) are expressed at very low levels in many adult tissues. In tumors in which the gene is interupted by a viral genome two new species of int-3 RNA are abundantly expressed. The elevated levels of int-3 sequences 5' to the viral insertion site presumably occur as a result of the enhancer element of the MMTVLTR. The transcription of sequences 3' to the viral insertion site begin within the MMTVLTR. Sequences related to int-3 have been cloned from human cellular DNA. The gene is located on human chromosome 6p21. The organization and expression of the gene in primary human breast tumors is being studied.

We have embarked on a systematic study of primary human breast tumor DNAs for frequently occuring mutations to determine whether they have a significant association with clinical parameters of the disease. In addition to the previously reported mutations we have found loss of heterozygosity (LOH) on chromosome 1p (37%), 1q (20%), 3p (30%), 13q (30%), 17p (49%), 17q (29%) and 18(34%) in primary breast tumor DNA. Specific subsets of tumors containing LOH on multiple chromosomes has been observed. For example LOH on chromosomes 17p and 18q frequently occur in the same tumor. The regions of the human genome which have been found to be affected by LOH are similar to those observed in carcinomas of other tissues. Whether the same target genes are also affected is under study.

CF 002 INT-2, A PROTO-ONCOGENE OF THE FIBROBLAST GROWTH FACTOR FAMILY IMPLICATED IN MAMMARY TUMORIGENESIS, Clive Dickson, Piers Acland, Frances Fuller-Pace, Wolfgang Walther, Richard Deed, Mark Dixon and Gordon Peters, Departments of Viral Carcinogenesis and Molecular Oncology, Imperial Cancer Research Fund, Lincolns Inn Fields London WC2A 3PX, UK.

Int-2, a member of the fibroblast growth factor family, was discovered as a proto-oncogene implicated in virally mediated mammary carcinoma of mice. This gene is not detectably expressed in normal adult mouse tissues, except for low levels in brain and testis. In contrast, int-2 RNA is expressed at a variety of sites at specific times during embryonic development. A structural analysis of int-2 RNA's has revealed six classes of transcript originating from three promoters and terminating at either of two alternative polyadenylation sites. However, despite this complex pattern of expression, all transcripts appear to encode the same open reading frame. Using site directed mutagenesis in conjunction with a transient expression system based on an SV40 vector in COS-1 cells, we have uncovered several novel features affecting post-transcriptional expression of the int-2 products. The primary amino acid sequence predicts a short hydrophobic N-terminus which can act as a signal peptide for transmembrane synthesis, and an internal consensus site for asparagine linked carbohydrate addition. Four major products have been detected between 27.5kd to 31.5kd, consistent with glycosylated and nascent product with and without the signal peptide. However the efficiency of translation appears to be compromised by an upstream AUG codon in the +1 reading frame which has the potential to encode a pentapeptide which overlaps the predicted initiation codon. In vitro translation of cRNA with a similar structure to mRNA reveals the presence of int-2 products which are larger than the AUG initiated forms, and which appear to initiate at an upstream CUG codon. We are presently investigating the biochemical and functional consequences of the alternatively initiated sets of int-2 products.

CF 003 HORMONAL CONTROL OF GROWTH AND GENE EXPRESSION IN MAMMARY EPITHELIAL CELLS AND THE EFFECTS OF ONCOGENES, B.Groner, R.Ball, M.Schmitt-Ney, W.Doppler, W.Höck, and N.E.Hynes, Friedrich Miescher-Institut, P. O. Box 2543, CH-4002 Basel, Switzerland.

Cultured mouse mammary epithelial cells have been used to investigate the influence of growth factors, peptide and steroid hormones on the differentiation state and the potential to synthesize milk proteins. Oncogenes were introduced into these cells and their effects on the cellular phenotypes was monitored. We found that EGF was required to efficiently plate the cells and to grow them to a high cell density. After removal of EGF, beta casein gene transcription could be introduced in confluent cells by the synergistic action of glucocorticoid hormones and prolactin. Glucocorticoids act slowly, possibly in a indirect fashion by introduction or repression of cellular genes. Prolactin acts rapidly and can induce beta casein changes in the binding of nuclear proteins to the essential region of the promoter accompany the hormonal induction process. Introduction and expression of the activated Ha-ras oncogene or the TGF-alpha gene cause accumulation of TGF-alpha in the medium. TGF-alpha suppresses the lactogenic hormone action. Cells transfected with the Ha-ras and TGF-alpha genes become tumorigenic. Tumorigenicity was also observed with cells transfected with the c-erb-2 oncogene. However, these cells remained responsive to the lactogenic hormones. Receptor interactions could be observed between the EGF receptor and the c-erb-2 product. EGF stimulation of the cells led to a rapid increase in c-erb-2 phosphorylation and a subsequent downregulation. The half life of the c-erb-2 protein decreased from 12 to 4 hours in the presence of EGF. Although these receptor kinases are closely linked in their action, they seem not to be equivalent with respect to their influence on mammary cell differentiation.

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CF 004 **THE int and neu ONCOGENES IN MAMMARY CANCER. Roel Nusse, Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.**

In mice, the Mouse Mammary Tumor Virus is used to identify oncogenes activated in mammary tumors by insertional mutagenesis. By applying transposon tagging techniques, we have isolated two closely related cellular oncogenes, called int-1 and int-4. In human breast cancer, these genes do not seem to be involved, but another cellular oncogene, called neu, is relatively frequently amplified and overexpressed. We examine the properties of the int and neu proteins, and we study the role of the int genes in normal early development.

The int-1 gene encodes a cysteine-rich protein with a signal peptide. Normally, the int-1 gene is expressed in early embryogenesis of the mouse, in particular in the developing nervous system. The essential role of int-1 in embryogenesis was underscored by its high degree of homology with the Drosophila segment polarity gene wingless, a gene involved in pattern formation in segments of the developing fly. Also in Drosophila, the int-1/wingless gene appears to encode a secreted factor; by antibody staining techniques we have visualized the protein in whole mount embryos and in individual cells. The wingless protein is secreted from cells, accumulates in the intercellular space and is taken up in endosome-like particles suggesting signaling through binding to a receptor. We have examined interactions of the wingless protein with the products of other segmentation genes by staining for wingless expression in embryos mutant for other genes.

The int-4 gene is activated in a relatively small number of mammary tumors. The gene is highly related to int-1. Its expression pattern in mouse embryos is similar to int-1 as well: the gene is only expressed during specific time intervals in mouse embryos and it is highly conserved in evolution.

neu (or c-erbB-2) encodes a transmembrane protein with high similarity to the EGF receptor. A ligand for neu has not been found however. We have made monoclonal antibodies to various parts of the neu protein, with which we have established that overexpression, resulting from gene amplification, is an early step in the pathogenesis of human breast cancer. In particular ductal carcinomas in situ of the comedo type have a very high incidence of neu overexpression. The biological effects of these monoclonal antibodies on the growth of tumor cells expressing high levels of neu will be discussed.

Control of Mammary Epithelial Growth and Differentiation

CF 005 INVOLVEMENT OF THREE DISTINCT MEMBERS OF THE *erb*B/EGFR FAMILY IN HUMAN MAMMARY CARCINOMAS, Stuart A. Aaronson, Pier Paolo Di Fiore, Jacalyn H. Pierce and Matthias H. Kraus, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892

The homology of certain oncogene products with growth-factor receptors has implicated proto-oncogenes in normal cellular growth regulation. The aberrant expression of such genes appears to be fundamental to steps that convert normal cells along the pathway to malignancy. We have observed overexpression and amplification of the epidermal growth factor receptor (EGFR) gene and the closely related receptor-like molecule, *erb*B-2, in human malignancies. Furthermore, we have tested with *in vitro* model systems the conditions under which these receptor genes are able to convert the transformed phenotype onto NIH/3T3 fibroblasts. Overexpression of the EGFR was capable of inducing malignant transformation of NIH/3T3 cells only when the cells were grown in the presence of the physiological ligand (EGF), while transformation induced by the overexpression of *erb*B-2 occurred without addition of a specific ligand. The levels of EGFR or *erb*B-2 protein necessary to confer a transformed phenotype *in vitro* compare to those observed in mammary tumors and tumor cell lines with amplification of the EGFR or *erb*B-2 gene, respectively. Recently, we identified a DNA fragment related to but distinct from EGFR or *erb*B-2 in human genomic DNA by reduced stringency hybridization with v-*erb*B as a probe. Characterization of the cloned DNA fragment mapped the region of v-*erb*B homology to three exons with closest identity of 64% and 67% to a contiguous region within the tyrosine kinase domains of the EGFR and *erb*B-2 proteins, respectively. cDNA cloning revealed a predicted 148-kDa transmembrane polypeptide with structural features identifying it as a member of the *erb*B gene family, prompting us to designate the gene as *erb*B-3. It was mapped to human chromosome 12q13 and was shown to be expressed as a 6.2-kilobase transcript in a variety of normal tissues of epithelial origin. Markedly elevated *erb*B-3 mRNA levels were demonstrated in certain human mammary tumor cell lines. These findings suggest that increased *erb*B-3 expression, as in the case of EGFR and *erb*B-2, may play a role in some human malignancies.

CF 006 BASEMENT MEMBRANE IS NECESSARY FOR MAMMARY CELL ORGANIZATION & GENE EXPRESSION. Mina J. Bissell, Charles Streuli and Li-How Chen, Division of Cell & Molecular Biology, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720.

We have shown previously that a basement membrane preparation derived from EHS tumors allows mammary cells from mid-pregnant mice to form alveoli-like structures and to synthesize and secrete high levels of milk proteins vectorially (Barcellos-Hoff et al., 1989). We now show that formation of alveoli-like structures also allows expression of whey acidic protein (WAP) by inhibiting production and/or accessibility of a WAP-specific inhibitor (Chen and Bissell, 1989). We also show that on floating type I collagen gels, production of an endogenous basement membrane correlates with functional differentiation. Formation of the basement membrane itself may be dependent on cell-cell interactions and establishment of polarity. Cells on tissue culture plastic express high levels of mRNA for laminin and type IV collagen and secrete these and other matrix proteins. Interestingly, when a basement membrane is formed on collagen gels which are floated there appears to be a negative feedback on mRNA for extracellular matrix proteins. We conclude that tissue-like multicellular architecture and basement membrane, as well as hormones, are necessary for the establishment and maintenance of a fully lactational phenotype. We also propose that a disruption in cell-extracellular matrix interactions may play a role in etiology of breast cancer.

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Chen, L-H and Bissell, MJ (1989) Cell Reg. **1** (In Press).

CF 007 MOLECULAR GENETIC LESIONS ASSOCIATED WITH BREAST CANCER, Helene S. Smith[1], Ling-Chun Chen[1], Charles Dollbaum[2], Britt-Marie Ljung[3], Fred Waldman[3], Brian Mayall[3], Edison Liu[4], Christopher Benz[3] [1]Brush Cancer Research Institute S.F., CA, 94115, [2]Peralta Cancer Research Institute, Oak, CA, 94609, [3]University of California, San Francisco, S.F., CA, 94143, [4]University of North Carolina, Chapel Hill, NC, 27599

We are attempting to define the genetic aberrations occurring in breast cancer by determining 1) the frequency with which various genetic lesions occur, and 2) their association with various clinical and biological parameters. Unlike the incidence observed with other adenocarcinomas, activating *ras* mutations were seen in only 1 of 40 primary breast cancers. Amplifications of the c-*int2*, c-*myc*, and c-*erb*-B2 proto-oncogenes were seen in 33%, 22%, and 14% respectively. There was no correlation between increased oncogene dosage and hormone receptor status; however, there was a correlative trend between amplification, overall DNA aneuploidy, and positive nodal status. In 25% of primary breast cancers, there was an allelic loss defined by an overlapping set of deletions at chromosome 1q23-32. This lesion correlated with negative nodal status and estrogen receptor positivity. Allelic loss at chromosome 17p12-13 was seen in 38% of breast cancers. Among 19 cases informative at loci on both 17p and 1q, 2 tumors had only the 1q loss, 3 had only the 17p loss and 3 had losses at both loci. Examination of the latter 3 cases suggested that the loss at 17p occurred earlier in malignant progression than did the 1q loss or that cells with the 17p lesion selectively overgrew the tumors.

Short-term culture of primary mammary epithelial cells were used to further study the role of these genetic lesions in breast cancer. Transcript levels of proto-oncogenes, c-*erb*B-2, c-*myc*, and c-Ha-*ras*, increased in proliferating cultures of normal mammary epithelium to levels commonly found in proliferating malignant cells, suggesting that the variable expression of these proto-oncogenes observed in breast biopsy specimens needs to be controlled for cellular growth rate. With culture of 3 malignant effusions sequentially obtained from the same patient, only the last effusion could be reproducibly immortalized. Despite common cytogenetic abnormalities in all 3 effusion samples, this last effusion had an allelic loss at c-Ha-*ras* and an activating mutation of Ki-*ras*, suggesting that these oncogene abnormalities may be associated with the capacity for in vitro immortalization and are not necessarily associated with the initiation of the primary breast cancer or with the establishment of metastatic disease. Supported by NCI grant, PO1CA44768.

CF 008 EFFECTS OF EGF AND TGF-β ON GROWTH AND DIFFERENTIATION OF NORMAL AND TRANSFORMED HUMAN MAMMARY EPITHELIAL CELLS IN CULTURE, Martha Stampfer, Paul Yaswen, Myriam Alhadeff, and Junko Hosoda, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720.

Normal human mammary epithelial cells (HMEC) show long-term active growth (~45-80 population doublings) in the serum-free medium MCDB 170. We have characterized this cell population for its relationship to cell lineages *in vivo*, its differentiation potential, and its behavior after transformation *in vitro*. Based on the pattern of keratin and milk fat globule expression, the cell population exhibiting long-term growth in MCDB 170 has properties consistent with that of a stem cell population derived from the basal cell layer *in vivo*. Growth of these cells is acutely dependent upon EGF/TGF-α. Although this dependence can not be readily demonstrated in mass culture due to endogenous TGF-α production, addition of antibodies to the EGF-R rapidly, and reversibly, leads to cessation of growth. Growth inhibition is also induced by exposure to TGF-β; the extent of this inhibition varies among individual specimen donors and as a function of age in culture. TGF-β induced inhibition is less rapid, and not fully reversible compared to that seen with the antibodies to EGF-R. Normal cultured HMEC express mRNA and protein for several extracellular matrix (ECM) associated products (fibronectin, collagen IV, laminin, plasminogen activator inhibitor-1), and the intermediate filaments keratins 5/14, 8/18, and vimentin. We have also identified a gene product, NB-1, expressed in the normal HMEC but downregulated in transformed HMEC. TGF-β markedly increases the synthesis of the ECM associated products, as well as stimulates the mRNA levels for vimentin and NB-1. The levels of some of these gene products can also be modulated by placement of the HMEC on reconstituted basement membrane material. The cells assemble into tubular and globular structures, with changes in keratin expression consistent with the development of a more luminal cell phenotype. In general, TGF-β appears to reinforce migratory or wound healing characteristics while the basement membrane substrate appears to induce characteristics of a developmentally more mature phenotype. The response to different growth factors and substrate material has been compared in normal HMEC vs. HMEC cell lines derived from normal cells transformed to immortality after *in vitro* exposure to benzo(a)pyrene, and further transformed to malignancy using oncogenes/oncongenic viruses. Numerous differences in growth factor responses and gene expression suggest that the transformed cells may vary from the normal HMEC in their differentiated state and/or their cell-cell and cell-matrix interactions.

Prognostic Variables in Breast Cancer

CF 009 PROGNOSTIC FACTORS IN BREAST CANCER. William L. McGuire, Department of Medicine, University of Texas Health Science Center, San Antonio, TX 78284.

We need prognostic factors to determine which patients with primary breast cancer are cured by surgery and/or radiotherapy alone, and which patients will have a recurrence. The latter group might well benefit from adjuvant therapy.

DNA flow cytometry -- San Antonio data reveals that the S-phase fraction is a highly significant predictor of disease-free survival among axillary node-negative patients with diploid tumors, so that those patients whose tumors were diploid with low S-phase (28% of all node-negative patients) experienced a 5-year recurrence rate of only 10% compared to 27% for the remainder. Patients with aneuploid tumors had a significantly worse disease-free survival than patients with diploid tumors.

Cathepsin D -- Rochefort and colleagues from France have reported a measurement of the lysosomal enzyme cathepsin D in breast cancer cytosols. They found a small subset of patients with high levels of cathepsin D which correlated with a high percentage of positive axillary nodes. In San Antonio we have examined the level of cathepsin D in a pilot study of axillary node negatie breast cancer patients, and preliminary evidence reveals a high correlation between the level of cathepsin D and shortened relapse free survival.

Stress responsive proteins -- There is now a body of literature regarding stress responsive proteins which include drug resistance associated proteins and heat shock proteins. One such protein studied in San Antonio is SRP27 which is stimulated in the breast cancer cells by both estrogen and heat shock. Studies of the level of SRP27 in the cytosols of breast cancer patients reveal a significant correlation with relapse free survival.

Oncogenes -- Preliminary efforts with the oncogene HER-2 find a major correlation with the amount of HER-2 protein expression and both relapse free and overall survival but only in node positive breast cancer.

Conclusion: Measurement of traditional prognostic factors such as histopathology, steroid hormone receptors, and tumor size in addition to DNA ploidy, % S-phase, cathepsin D, and oncogenes provide the clinician with sufficient information about patient outcome to make rational decisions regarding adjuvant therapy of breast cancer.

CF 010 **HPR EPITOPE EXPRESSION AND RECURRENCE IN HUMAN BREAST CANCER**, Francis P. Kuhajda, M. Salah Shurbaji, and Gary R. Pasternack, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The ability to determine at diagnosis which breast cancer patients will have clinically aggressive disease and which will have indolent disease remains an important clinical goal. Some human breast cancers express a substance which is either identical to, or shares epitopes with haptoglobin-related protein (Hpr), the product of the haptoglobin-related protein gene (*Hpr*). This study examined the correlation between Hpr expression and disease recurrence in 70 cases of early (Stage I and II) breast cancer, using immunohistochemical analysis of routinely fixed, paraffin embedded tissue sections to detect Hpr epitopes. Among all of the variables examined, expression of Hpr epitopes correlated best with recurrence by life-table ($p<0.0002$) and chi-square ($p<0.0004$) analyses. Progesterone receptor (PR) status, tumor size, clinical stage, axillary lymph node status, mitotic index, and tumor necrosis also correlated with recurrence, but stepwise discriminant analysis showed that the association of Hpr expression was independent of these features. Since Hpr and PR status were both independent predictors of recurrence, they could be combined for greater stratification of patients into high, intermediate, and low risk groups: 11/12 (92%) of Hpr positive, PR negative patients recurred; 5/11 (45%) of Hpr positive, PR positive patients recurred; and 9/41 (22%) of Hpr negative patients recurred with PR status showing little effect.

In a separate longitudinal autopsy study, Hpr epitope expression was evaluated in 17 cases of fatal breast cancer, where tissue was available from the primary, and either the recurrences or the autopsy. Hpr epitope expression was found in 13/17 (76.5%) of the primary tumors, and in nearly all recurrences and metastases found at autopsy. These data suggest that Hpr epitope expression is acquired with increasing malignancy, and that expression, once acquired, remains a permanent characteristic. Interestingly, the frequency of Hpr epitope expression in the primary tumors of the autopsy group was twice that of the unselected early breast cancer patients, further supporting its role as a prognostic indicator.

A number of estrogen-receptor positive and negative human breast cancer cell lines have been found to express Hpr epitopes and are undergoing further evaluation at the protein and molecular genetic levels.

Growth Factors

CF 011 ROLE OF GROWTH FACTORS IN NORMAL HUMAN MAMMARY EPITHELIAL CELLS AND THEIR ONCOGENIC TRANSFORMANTS, Robert B. Dickson[1], Mozeena Bano[1], Susan E. Bates[2], Dorothy Walker-Jones[3], Martha R. Stampfer[4], Eva Valverius[1], Robin Clark[5], and Marc E. Lippman[1], Lombardi Cancer Research Center[1], Georgetown University Medical Center, Washington, DC; Medicine Branch[2], NCI, Bethesda, MD; Department of Biology[3], Howard University, Washington, DC; Lawrence Berkeley Laboratories[4], Berkeley, CA; and Cetus Corporation[5], Emeryville, CA.

Growth factors are important components of the secretions of the normal lactating breast epithelium. In addition, they may play fundamental roles in regulating cyclic growth, development and regression of the mammary gland. We have examined the roles of three growth factors found in milk, TGFα, MDGF-1, and TGFβ, on proliferation and differentiation of normal human mammary epithelial cells. In addition, the ability of stepwise malignant transformation by benzo[a]pyrene, a single activated oncogene (v-ras^H, v-mos, SV40T) or a combination of two of these oncogenes to modulate either growth factor production or response was studied. TGFα begins to be produced in high concentrations by normal mammary epithelial cells as they adapt from non-proliferating organoids to rapidly proliferating primary cultures. TGFα appears to be an autocrine growth factor in high density culture since an antibody directed against the EGF receptor reversibly blocks cellular proliferation. MDGF-1 is also produced by normal, proliferating human mammary epithelial cells. This 60 kDa growth factor does not seem to be structurally related to any other known growth factor; it binds to a 120-140 kDa high affinity binding site. MDGF-1 stimulates proliferation, collagen production and tyrosine phosphorylation when added to cultures of normal breast epithelial cells or some human breast cancer cell lines. Finally, TGFβ is also produced at high levels by normal human mammary epithelial cells. When added to these cells it causes reversible growth arrest, increased cellular adhesion, cell elongation, and induction of milk fat globule antigen and PDGF B chain mRNA. Benzo[a]pyrene immortalization of mammary epithelial cells does not markedly compromise function of any of these growth factors, nor does it modulate their production. SV40T compromises MDGF-1 function but sensitizes cells to TGFα and β. v-ras^H compromises growth modulatory functions of TGFα and MDGF-1. Finally, combinations of v-ras^H with v-mos or SV40T interfere with growth modulation of cells by all three growth factors; however, TGFβ can still induce differentiation unabated in these cells. Transformation of the cells with combinations of two oncogenes is associated with acquisition of tumorigenic potential in the nude mouse. These data suggest that growth factor production does not specifically characterize breast cancer; rather, changed growth factor responses mark the transformed phenotype.

CF 012 REGULATION OF HUMAN BREAST CANCER BY SECRETED GROWTH FACTORS. Marc E. Lippman. Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC 20007.

The mechanisms by which physiologic concentrations of estrogen control the growth of hormone dependent breast cancer are unclear as are the means by which hormone dependent breast cancer progresses to hormone independence. Our studies suggest that hormone dependent breast cancer cells secrete growth factors which are under estrogenic control. These include an insulin-like growth factor I activity, insulin-like growth factor II, transforming growth factor alpha, platelet derived growth factor, and an epithelial transforming growth factor which is a member of the fgf family. Secretion is stimulated by estradiol. Antiestrogens act by decreasing the secretion of these growth factors. A variety of strategies which block either the secretion or action of these growth factors in vitro can interfere with the growth of human breast cancer cells. These include anti-growth factor antibodies; anti-growth factor receptor antibodies; drugs which interfere with the ligand receptor interaction and stable transfection of breast cancer cells with antisense genes for specific growth factors. Hormone independent breast cancer secretes a similar array of growth factor activities. Conversion to the hormone independent phenotype is accompanied by constitutive growth factor production. Hormone dependent human breast cancer cells invariably require estrogen for in vivo tumorigenesis in athymic nude mice. We have been able to mimic the effects of estrogen supplementation by continuous infusion of concentrated conditioned medium from hormone dependent cells cultured in vitro. Taken together, these data support the pathophysiologic role of secreted trophic substances in neoplastic progression of human breast cancer.

CF 013 THE ROLE OF TRANSFORMING GROWTH FACTOR ALPHA (TGFα) IN THE ETIOLOGY AND PROGRESSION OF RODENT AND HUMAN BREAST CANCER, David S. Salomon, Fortunato Ciardiello, Toshiaki Saeki, Eva Valverius and Nancy Kim. Laboratory of Tumor Immunology & Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892. Expression of a specific 4.8 kb TGFα mRNA transcript and/or the presence of biologically active and immunoreactive TGFα protein can be detected in a number of spontaneous, MMTV-or carcinogen-induced (DMBA or NMU) mouse and rat mammary tumors. Ovariectomy results in a rapid decline in the level of TGFα mRNA expression in primary rat mammary adenocarcinomas which are estrogen-dependent for their growth in vivo. Conversely, estrogen is capable of inducing an increase in TGFα production and secretion in primary cultures of estrogen-responsive rat mammary tumor cells. Transformation of EGF responsive normal mouse (NOG-8) or human (MCF-10) mammary epithelial cell lines with a point-mutated c-Ha-*ras* proto-oncogene, but not with an activated c-*neu* (*erb*B-2) gene, results in a loss in or an attenuated response to EGF in these cells which may be due in part to an enhanced production of TGFα that is coordinately linked to the expression of the activated *ras* gene. Several human breast cancer cell lines also secrete TGFα and express major 4.8 kb and minor 1.6 kb TGFα mRNA transcripts. In general, estrogen receptor(ER)negative, estrogen-nonresponsive breast cancer cell lines (e.g. MDA-MB-231 and MDA-MB-468) produce higher basal levels of TGFα than ER positive, estrogen-responsive cell lines (e.g.MCF-7,T47-D and ZR-75-1). In MCF-7 cells, growth-stimulating concentrations (1-10nM) of 17-β estradiol can induce a rapid (within 6 to 12 hr.) 3 -to 5-fold increase in the expression of TGFα mRNA and in TGFα protein secretion. Elevated levels of TGFα mRNA can also be detected in approximately 60-70% of primary human breast tumors. The expression of TGFα mRNA observed in the tumors is not due to any gross amplifications or major rearrangements of the TGFα gene. In addition, significantly higher levels of biologically active and immunoreactive TGFα (approximately 2-to 3-fold)can also be detected in the pleural effusions from breast cancer patients as compared to the TGFα levels in the serous effusions from noncancer patients suggesting that this growth factor is secreted by metastatic tumor cells. Overexpression of a TGFα expression vector in mouse NOG-8 or human MCF-10 cells is capable of transforming these cells in vitro and in vivo. Collectively, these results suggest that the expression of TGFα in breast cancer cells can be controlled by estrogens and by activated proto-oncogens such as *ras* and that the overproduction of this growth factor can lead to the malignant transformation of immortalized rodent and human mammary epithelial cells which are also expressing a sufficient complement of functional EGF receptors.

CF 014 C-MYC ONCOGENE EXPRESSION IN ESTROGEN-RESPONSIVE AND -NONRESPONSIVE HUMAN BREAST CANCER CELLS, Don Dubik and Robert P.C. Shiu, Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3.
C-myc plays an important role as a progression factor in the regulation of cell growth. C-myc gene expression has been shown to be stimulated by a variety of peptide growth factors in many cell types. Elevated c-myc gene expression has been reported in a significant proportion of human breast cancer *in vivo*. Our study was to investigate if c-myc gene expression plays a role in the mitogenic action of estrogen in human breast cancer cells. Estradiol was shown to stimulate an early, rapid but transient accumulation of c-myc mRNA in estrogen-receptor-positive MCF-7 and T-47D cell lines, with a maximal level of c-myc mRNA (> 15-fold) occurring 90 minutes after estradiol treatment. In addition, c-myc expression was suppressed by treatment with an antiestrogen such as tamoxifen. The expression of several other oncogenes, such as c-fos, c-Ha-ras and c-sis, was not affected by estradiol. In estrogen-independent breast cancer cells such as MDA-MB-231, neither cell growth nor c-myc expression was affected by estradiol or tamoxifen. In these latter cells, however, there was a high level of constitutive expression of c-myc mRNA, suggesting that constitutive expression of c-myc may account for the cells' autonomous growth characteristics. To better understand the mechanism of estrogen regulation of c-myc expression, *in vitro* nuclear run-on transcription assays and mRNA stability study was carried out. These experiments revealed that, in MCF-7 cells, estradiol increased the rate of transcription of the c-myc gene within minutes, reaching a peak in 20 minutes, followed by a rapid decline. This increase in c-myc transcription could fully account for the increase in c-myc mRNA levels following estradiol treatment. Estradiol had no effect on the stability of c-myc mRNA (half-life: 18 minutes). In the estrogen-independent MDA-MB-231 cells, estradiol had no effect on c-myc transcription or mRNA stability; however, the c-myc mRNA was 3 times more stable (half-life: 50 minutes) as compared with those in the MCF-7 cells. These findings indicate that the c-myc gene in estrogen-responsive cells (MCF-7) is regulated solely at the transcriptional level, but in estrogen-independent cells (MDA-MB-231), an increase in c-myc mRNA stability can be a mechanism, distinct from gene amplification, to achieve high level of expression. Furthermore in MCF-7 cells, cycloheximide treatment did not abolish the estradiol-induced c-myc transcription, indicating that the induction of c-myc gene is a primary action of estradiol. This observation suggests that the c-myc gene may contain cis-acting elements (estrogen responsive elements, ERE) conferring estrogen activation. This possibility was pursued by transfecting into MCF-7 and Hela cells of a series chimeric gene constructs consisting of different lengths of the 5'-flanking human c-myc sequence upstream of the reporter gene chloramphenicol acetyltransferase (CAT), with and without co-transfecting with a estrogen receptor expression vector. Preliminary results of this study revealed a functional ERE(s) in the 5'-flanking region (-667 to +67) of the human c-myc gene. Future effort will include precise localization of the cis-acting ERE(s) and the identification of the transacting factor (if not the estrogen receptor itself) involved in the regulation of c-myc gene by estrogen.

Control of Malignancy: New Therapeutic Strategies and Immunology

CF 015 FROM BIOLOGY TO CLINICAL TESTING IN AN INTEGRATED BREAST CANCER RADIOIMMUNOTHERAPY PROGRAM, Roberto L. Ceriani, John Muir Cancer and Aging Research Institute, 2055 North Broadway, Walnut Creek, CA 94596. Serological immunotherapy has been the subject of much investigation for several decades. The advent of monoclonal antibodies (MoAbs) and valuable immunoconjugates has allowed the re-introduction of former approaches against the background of increased knowledge on both characteristics of tumor host and neoplastic tissue. Our molecular cloning of the mucin, the 70 and 45 kDa breast epithelial antigens and their subsequent recombinant production has given us insight into molecular structure that allows production of new and more desirable MoAbs. Moreover, having identified specific epitopes permits the synthesis of more effective immunogenic peptide sequences. At the cellular level, information on the interrelationship among expression and distribution of these 3 antigens, their corresponding MoAb radioconjugate access to the tumor and these MoAb conjugates' clearances have permitted developement of protocols for increasing maximal tolerated doses and reducing toxicity. Also, precise understanding of both the antigenic molecular characteristics and antigen expression levels, (the latter in terms of heterogeneity and cell location), clarifies our interpretation of our dosimetric data and therapeutic effectiveness results. Presently, we demonstrate effective experimental immunotherapy with radioconjugated anti-breast epithelial MoAbs (Mc5, BrE-1, BrE-2 and BrE-3 against the mucin, McR2 and Mc13 against 70 kDa and Mc3 against 45 kDa) derived from immunizations with human milk fat globule. Our flow cytometric analysis of breast tumor antigen expression and quantitation of circulating antigen levels suggest possible mechanisms modifying of MoAb radioconjugate attack. Correlation among these parameters, DNA replication in the target tumors and tumor dosimetric values gave a clue to the contribution of each of them to immunotherapy. We also obtained guidelines that made possible considerable prediction ability of the therapeutic effectiveness of a MoAb by the ordered study of host and tumor biological and immunochemical parameters in distribution and treatment studies. ^{131}I-labeled MoAbs against the breast epithelial mucin, the 70 and 45 kDa antigens are shown to be effective in experimental human breast tumor treatment by themselves but are even more effective in mixtures. This ability demonstrated in models is now being tested in a clinical trial. Our results could help predict which is the most effective target antigen(s) on breast tumors for radioimmunoconjugate therapy. This work was supported by NIH, NCI grants RO1-CA39932, RO1-CA39936 and PO1-42767.

CF 016 ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR MONOCLONAL ANTIBODIES AS POTENTIAL ANTI-CANCER AGENTS. John Mendelsohn, M.D., Memorial Sloan-Kettering Cancer Center and Cornell University Medical College, New York, N.Y., 10021. Receptors on cell surface membranes for polypeptide growth factors are often overexpressed on solid tumors and may be involved in autocrine/paracrine stimulation of tumor growth. We have produced monoclonal antibodies (mAbs) 225 IgG1 and 528 IgG2a against the receptor for epidermal growth factor (EGF), which can inhibit binding of EGF and transforming growth factor alpha to the receptor and prevent activation of receptor tyrosine protein kinase activity. These mAbs can inhibit growth factor-dependent proliferation of normal and malignant human cell lines, and this has been demonstrated with breast adenocarcinoma cells in collaborative studies with a number of laboratories. Furthermore, the growth of cell lines expressing elevated EGF receptors (including breast adenocarcinoma MDA 468) is directly inhibited by antireceptor mAb, both in culture and in xenografts. We believe the mechanism is related to blocking of an autocrine loop. Antibody labeled with ^{111}In can selectively concentrate in and image xenografts bearing high numbers of EGF receptors (A431 squamous carcinoma and MDA 468), but not MCF-7 breast adenocarcinoma which bears normal receptor numbers. A Phase I clinical trial with ^{111}In-225 IgG1 was carried out in collaboration with Hybritech, Inc., La Jolla, CA, in patients with advanced squamous lung cancer, which invariably expresses high levels of EGF receptors. Four mg ^{111}In-225 (5 mCi) was administered by itself or co-infused with 16, 36, 116 or 296 mg of unlabeled mAb in groups of 3 patients each. No toxicity occurred at any dose level. Tumor visualization was observed in all but one of 10 patients receiving >20 mg mAb, and was optimal 3 days after injection. Significant liver visualization was also observed. Serum clearance of mAb was dose related. At a dose of 120 mg, mean serum levels were maintained at >10% ID/L for >24 hrs, and the distribution of ^{111}In-225 to the tumor site reached 2% ID at 72 hrs, compared with 28% ID to the liver and 77% ID in the whole body. All patients produced human anti-mouse antibodies. We conclude that 225-IgG1 mAb against the EGF receptor can be administered safely in the doses and schedule studied, and that it can localize squamous lung cancer, a tumor known to express elevated levels of receptor. Applications to the therapy of breast cancer as well as other malignancies should be evaluated with antireceptor mAbs, or related agents with blocking capacity, given in multiple courses and in patients with low tumor burden. Supported in part by grants from the NCI.

CF 017 NEW GROWTH REGULATORY FACTORS: AMPHIREGULIN AND ONCOSTATIN M, G. J. Todaro, G. D. Plowman, P. S. Linsley, H. Marquardt, and M. Shoyab, Oncogen, Seattle, WA 98121

Amphiregulin (AR), a bifunctional growth regulatory peptide with tumor inhibitory activity, was isolated from human breast carcinoma cells (MCF-7) which had been treated with the phorbol ester TPA. The protein exhibits striking structural homology with the EGF/TGF-α family of growth factors, including the conserved spacing pattern of the six essential cysteine residues. In addition to its EGF-like domain, AR possesses an amino-terminal region that is extremely hydrophilic, containing high numbers of lysine, arginine, and asparagine residues. Also contained in this region are nuclear targeting motifs that enable AR to interact directly with DNA. Like EGF and TGF-α, AR is synthesized as a larger transmembrane precursor, which may be cleaved into either of two secreted forms, 78 or 84 amino acids in length. Both forms are biologically active. While AR promotes the growth of normal fibroblasts and keratinocytes, it inhibits various human tumor cell lines, including some breast adenocarcinomas. The pattern of tumor inhibition overlaps with, but is distinct from that of TGF-β and other known growth inhibitors. AR partially competes with EGF for binding to the EGF receptor; an amphiregulin-specific receptor has not yet been identified.

A second new growth regulatory protein, oncostatin M, was isolated from a human histiocytic lymphoma cell line (U937) after treatment with TPA; it is also produced by activated T lymphocytes. Like AR, oncostatin M stimulates the growth of normal human fibroblasts but inhibits the growth of several human carcinoma cell lines, including breast and lung carcinomas, some melanomas, but not colon carcinomas. Oncostatin M exhibits an inhibitory effect on MCF-7 breast carcinoma cells that is strongly synergistic with tumor necrosis factor (TNF). Similar synergistic activity has been demonstrated with TGF-β in a melanoma cell line (A375). Human oncostatin M, a single polypeptide chain of $M_r \sim 28{,}000$, demonstrates no significant homology with known proteins. Oncostatin M has been cloned and sequenced, and the recombinant product has the same biological properties as the native molecule. A high affinity cell surface receptor ($M_r \sim 150{,}000$-$160{,}000$) has been identified on cell lines whose growth is regulated by the factor. However, some cells express the receptor and bind significant levels of Oncostatin M but are not sensitive to the growth regulatory effects of the molecule.

Both oncostatin M and amphiregulin were discovered on the basis of their ability to inhibit neoplastic growth without inhibiting normal cells. The selective anti-tumor activities of these two growth regulators make them promising candidates for development as therapeutic agents.

Control of Malignancy: Anti-Oncogenes and Anti-Estrogens

CF 018 THE ROLE OF THE RETINOBLASTOMA GENE IN BREAST CANCER, Hong-Ji Xu, Shi-Xue Hu, and William F. Benedict, Center for Biotechnology, Baylor College of Medicine, The Woodlands, TX 77381. Structural changes of the retinoblastoma (Rb) gene including internal deletions have been reported in primary breast cancer as well as cell lines obtained from breast cancer specimens. Answers to critical questions regarding the role of the retinoblastoma gene in the initiation or progression of these tumors as well as an accurate measurement of the frequency of such changes, however, requires the ability to examine the presence or absence of the Rb protein at the single cell level in primary tumor as well as metastases. In order to investigate this issue, we have developed an immunohistochemical single cell assay to examine the presence or absence of the Rb gene. Using a high affinity polyclonal antiserum raised against a synthetic Rb peptide (1), it is now possible to identify the Rb protein present in single cells, including both cell culture and tissue specimens. We shall describe this system in detail and present our latest data regarding the role of the Rb gene in the initiation and/or progression of human breast cancer.

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CF 019 LOSS OF STEROID HORMONE SENSITIVITY IN HUMAN BREAST CANCER CELLS

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A major problem in the endocrine therapy of breast cancer concerns the progression of the tumor cells from a state of steroid hormone sensitivity to one of insensitivity. We have developed an in vitro model system for study of this problem in the hope of discovering underlying mechanisms and of being able to prevent the transition. This model involves study of the divergence of cloned steroid-sensitive breast cancer cells in tissue culture under conditions of steroid deprivation. Under these conditions, cells lose steroid sensitivity by a mechanism involving upregulation of the basal growth rate in the absence of steroid, not from any loss of the steroid-stimulated growth rate. The role of cell selection and the involvement of steroid receptors in this process will be discussed. The interaction of growth factors will also be discussed in relation to the current hypothesis that loss of steroid sensitivity could result from increased production of growth stimulatory factors/receptors or decreased production of growth inhibitory factors/ receptors.

CF 020 REGULATION OF BREAST AND ENDOMETRIAL TUMOR GROWTH BY ESTROGENS AND ANTIESTROGENS,

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Tamoxifen, a non-steroidal antiestrogen, has become the first line endocrine therapy for the treatment of breast cancer. Antiestrogens inhibit the estrogen stimulated growth of MCF-7 breast cancer cells. Stimulatory growth factor TGFα mRNA is decreased by antiestrogen whereas some members of the TGFβ family are increased. Tamoxifen is a reversible inhibitor of estrogen stimulated MCF-7 tumor growth in athymic mice. However the breast cancer cells can become sensitized to the stimulatory (weak estrogenic) effects of tamoxifen. Tamoxifen stimulated MCF-7 breast tumors can be grown in athymic mice and this may be a form of tamoxifen resistance [1]. Human endometrial tumors that are stimulated to grow in athymic mice have been reported [2] and recently a rise in endometrial carcinomas has been reported in at least one trial of long term adjuvant tamoxifen therapy for breast cancer [3]. A series of non-estrogenic antiestrogen is available that have shown promise to inhibit tamoxifen stimulated tumor growth in the laboratory [4]. These agents may find application in the clinics as a second line therapy after the failure of long-term adjuvant tamoxifen treatment.

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2. Gottardis MM, Robinson SP, Satyaswaroop PG and Jordan VC. Cancer Res. 48:812-815, 1988.
3. Fornander T, *et al*. Lancet i: 117-120, 1989.
4. Gottardis MM, Jiang SY, Jeng M-H, and Jordan VC. Cancer Res. 49:4090-4093, 1989.

CF 021 INHIBITION OF THE GROWTH OF HUMAN BREAST CANCER CELLS BY ANTIOESTROGENS: COMPARATIVE STUDIES WITH PARTIAL AGONISTS AND PURE ANTAGONISTS, Alan E. Wakeling, Bioscience I, ICI Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, England.

Pure (ICI 164,384) and partial agonist (tamoxifen:T and 4-hydroxytamoxifen:4-OHT) antioestrogens differ in their capacity to inhibit the growth of MCF-7 human breast cancer cells. Both types of compound block cell cycle progression in early G1. However, ICI 164,384 has a greater maximal inhibitory effect than T or 4-OHT. This is associated with the greater efficacy of ICI 164,384 in reducing the proportion of actively proliferating cells. Under basal conditions which maintain cell viability but do not support growth, 17β-oestradiol, phenol-red, insulin and 4-OHT, but not ICI 164,384, stimulate cell growth. ICI 164,384 inhibits the stimulatory action of oestradiol, phenol-red and 4-OHT. Oestradiol and insulin together increase cell growth rate synergistically; similarly 4-OHT, but not ICI 164,384, enhanced insulin-stimulated growth in the absence of oestradiol and this action of 4-OHT was also blocked by ICI 164,384. Growth stimulatory effects of TGF-α and IGFI were attenuated by both antioestrogens but ICI 164,384 was more effective than 4-OHT. The antiproliferative action of ICI 164,384 on oestradiol or growth factor stimulated cells was only weakly inhibited by TGF-β antibodies.

Malignant Progression and Metastases

CF 022 EPIDERMAL GROWTH FACTOR RECEPTOR: A MARKER OF EARLY RELAPSE IN BREAST CANCER: INTERACTIONS WITH NEU. Adrian L. Harris.
ICRF Clinical Oncology Unit, Churchill Hospital, Oxford OX3 7LJ, UK.

Epidermal growth factor receptors (EGFRs) were measured in 221 primary breast cancers by ligand binding with ^{125}I-labeled EGF, and high-affinity sites were quantitated. There was a highly significant inverse relationship between estrogen receptor (ER) and EGFR (15 EGFR-positive [$EGFR^+$] ER^+ and 92 EGFR-negative [$EGFR^-$] ER^+ : 54 $EGFR^-$ ER^- and 60 $EGFR^+$ ER^-). The relapse-free survival and overall survival were significantly shorter for $EGFR^+$ versus $EGFR^-$ tumors ($p<0.001$) by about 2 years in the case of relapse-free survival. When ER^- tumors were substratified by EGFR status, the $EGFR^-$ ER^- tumors had a prognosis almost as good as the ER^+ tumors. In 31 of 184 cases, high expression of neu, correlating with amplification, was found. Expression of neu conferred similar poor prognosis to EGFR expression in all prognostic subgroups. Coexpression of neu and EGFR had an additive adverse effect.

The role of EGF receptor expression on response to tamoxifen therapy in recurrent disease was also assessed. In ER +ve tumours, coexpression of EGFR lowered the response rate. In ER-ve tumours there was some response still to anti oestrogens and coexpression of EGFR further reduced this response. In contrast, in 25 patients who received first line single agent chemotherapy with Mitoxantrone, there is no correlation of EGFR status with response to therapy, time to tumour progression or survival. These results suggest that in EGFR +ve patients chemotherapy should be used as a first line treatment. Analysis of the interaction of EGFR with node status showed that it was particularly in the node -ve patients that EGF receptors predicted relapse free and overall survival. Thus it appears that in the node +ve patients neu is most important, as an independent prognostic variable, and in node -ve patients, EGFR. EGFR gene amplification was detected in a small sub-set of tumours which had the highest level of EGFR expression. However, there was a subgroup of tumours which had equally high receptor expression at the protein level, but which did not have amplification. This suggests that other factors, such as transcription control or RNA stabilisation are also important in high level expression of EGFR in breast cancer.

CF 023 NEGATIVE REGULATION OF CANCER METASTASIS BY PROTEINS INVOLVED IN MORPHOGENESIS AND METALLOPROTEINASE INHIBITION

Lance A. Liotta, Patricia S. Steeg, Allen Shearn+, C. Richter King*, H. Krutzsch, and W. Stetler-Stevenson, Laboratory of Pathology, National Cancer Institute, NIH, Bethesda Md. 20892,+Department of Biology, The Johns Hopkins University, Baltimore Md., *Molecular Oncology Inc., Gaithersburg, Maryland.

Invasion and Metastasis may be determined by loss of proteins which regulate morphogenesis and differentiation in normal cells, and loss of proteins which inhibit proteolysis of the extracellular matrix. We have identified the *nm23* gene, for which RNA levels are uniformly reduced in high metastatic potential rodent cell lines and human infiltrating breast duct carcinomas with lymph node metastasis. The 17 kDa human Nm23 protein is virtually identical over the entire translated region, to the developmentally regulated protein in *Drosophila*, encoded by the abnormal wing discs (*awd*) gene. Mutations in *awd* cause aberrant morphology and differentiation in *Drosophila*, analogous to changes in malignant progression. Loss of expression of functional Nm23/awd may lead to a disordered state favoring malignant progression. Metalloproteinases such as Type IV collagenase which degrade basement membranes may be blocked by specific inhibitors. We have identified a human 21kDa protein which binds latent Type IV collagenase with a 1:1 molar stochiometry, and abolishes the activity of the active enzyme. The protein has been purified and its complete primary structure has been determined by direct sequencing and confirmed by a full length cDNA clone. It has 41% amino acid identity and conservation of the position of all 12 cysteine residues with human TIMP. We propose the name TIMP-2 for this new member of the metalloproteinase inhibitor gene family.

CF 024 **ESTROGEN-INDUCED CATHEPSIN D IN BREAST CANCER.** Henri Rochefort, Patrick Augereau, Pierre Briozzo, Françoise Capony, Vincent Cavaillès, Gilles Freiss, Marcel Garcia, Marc Mathieu, Thierry Maudelonde, Philippe Montcourrier and Françoise Vignon. Unit Hormones and Cancer (U148) INSERM, and Faculty of Medicine, University of Montpellier 1, 60 Rue de Navacelles, 34090 Montpellier France.

The estrogen-induced 52,000-dalton (52K) glycoprotein secreted by estrogen receptor positive breast cancer cell lines has been identified as the secreted precursor of cathepsin D bearing mannose-6-phosphate signals, and normally routed to lysosomes via mannose-6-phosphate-IGF-II receptors. The amino-acid sequence of this pro-cathepsin D following the cloning and complete sequencing of its cDNA is identical, except one amino acid to that of normal cathepsin D. Its gene is localized on chromosome 11 close to H-ras. In MCF7 cells, cathepsin D is specifically and directly induced by estrogens at the transcriptional level. It is also induced by growth factors (EGF, IGF-I and bFGF), but this induction is dependent upon de novo protein synthesis. In MDA-MB231 and BT20 cells, cathepsin D is over-expressed but not regulated by estrogens. In hormone dependent and independent breast cancer cells, the processing of its pro-cathepsin D is altered compared to normal mammary epithelial cells in culture leading to its increased secretion. Using an IRMA, the total cathepsin D concentration can be assayed in the breast cancer cytosol routinely prepared for receptor assays. Two retrospective clinical studies indicate a significant correlation between high cathepsin D concentrations in the cytosol of primary breast cancer and development of metastasis (S. Thorpe et al. Cancer Res. and F. Spyratos et al. The Lancet, in press, 1989). This marker is independent of other classical prognostic factors, including lymph-node invasion and appears to be particularly useful in node-negative tumors. High cathepsin D concentration in the primary tumor may be either a consequence of, or, as is more likely a cause of the metastatic process. Pro-cathepsin D is an autocrine mitogen on cultured breast cancer cells and can be auto-activated to degrade extracellular matrix and to activate other pro-proteinases in acidic compartments. Transfection experiments using cDNA-cathepsin D expression vectors are in progress to demonstrate whether or not cathepsin D play a role in mammary carcinogenesis.

Oncogenes and Growth Factors

CF 100 HPV TYPES 16 AND 18 IMMORTALIZE NORMAL HUMAN MAMMARY EPITHELIAL CELLS AND REDUCE THEIR GROWTH FACTOR REQUIREMENTS, Vimla Band, Deborah Zajchowski, Victoria Kulesa and Ruth Sager, Division of Cancer Genetics, Dana-Farber Cancer Institute, Boston, MA 02115
Human papilloma virus (HPV) types 16 and 18 are most commonly associated with cervical carcinoma in patients and induce immortalization of human keratinocytes in culture. Here we describe the immortalization of normal human mammary epithelial cells (76N) by plasmids pHPV18 or pHPV16, each containing the entire viral genome. Transfectants have grown continuously for more than 60 passages whereas 76N cells senesce after 18-20 passages. The transfectants also differ from 76N cells in cloning in a completely defined medium (DD = DFCI-1 minus fetal calf serum and bovine pituitary extract), and growing in a minimally supplemented defined medium (DD_1) containing EGF. All transfectants tested contain HPV DNA, and express its RNA but do not form tumors in a nude mouse assay. HPV 16 and 18 transfectants were shown to express E7 protein. It is concluded that products of the HPV genome induce immortalization of human breast epithelial cells and simplify their growth factor requirements. This result raises the possibility that HPV might be involved in breast cancer. Furthermore, other tissue specific primary epithelial cells which are presently difficult to grow and investigate may also be immortalized by HPV.

CF 101 ONCOGENES AND GROWTH FACTORS IN BREAST CANCER,
Nandita J. Barnabas, J-C Kouyoumdjian, Gérard Dupré, Frank Feuilhade, Amu Therwath, Laboratoire d'Oncologie Clinique et Fondamentale,Service de Biochimie, Hôpital Henri Mondor, 94010 Creteil, France.
Oncogenes are expressed in breast cancers. There is no evidence concerning their putative role in benign mastopathies and during the metastatic evolution of the disease.We have determined the level of expression of different oncogenes in four groups of tissues1)normal breast2)benign mastopathies 3) other cancers 4) and metastatic cells ,by blot analysis, on total RNA and correlated the results with clinical histopronostic parameters. On the other hand the Epidermal Growth Factor receptor (EGF-r) is a glycoprotein of 170 KDa molecular weight whose gene is frequently amplified in human malignancies. In human mammary carcinomas its expression is associated with poor prognosis and metastatic extension. The EGF-r gene structure exhibited a high degree of homology with two other related oncogenes : v-erb-B and c-erb-B2/neu. We have examined the EGF-r gene in human metastatic mammary carcinomas samples by Blot analysis and compared it to the patterns obtained with the two related genes . Our data shows that 2 distinct types of modification are exhibited by metastatic cells : aberrant fragment size and sequencial amplifications. For some patients the duplicate alterations observed with 2 genes are discussed.

CF 102 LOCALIZATION OF HER-2/neu PROTO-ONCOGENE EXPRESSION IN THE HUMAN MAMMARY GLAND, Sue Bartow, Richard Hildebrandt, Marie Boyd and Michael Press, Departments of Pathology, University of New Mexico School of Medicine, Albuquerque, NM 87131 and University of Southern California School of Medicine, Los Angeles, CA 90033.
Amplification and overexpression of the HER-2/neu (c-erb-b2) proto-oncogene have been found in some cases of breast cancer. The protein product of the c-erb b2 proto-oncogene appears to be a cell membrane associated growth factor receptor. However, the ligand and function of the gene in normal cell growth and differentiation remains unclear. Elucidation of the role of the normal gene may help to explain its possible involvement in malignant transformation or progression. We have studied the expression of this gene by Northern analysis and immunohistochemistry in breast tissue from various stages of development and physiologic states from infancy through post-menopausal atrophy. Although the "stem-cell" of the terminal duct-lobular unit is currently implicated as the cell of origin of mammary carcinoma, we have found c-erb b2 expression to be more strongly and consistently present in the epithelium of the extra-lobular ducts. Expression of the gene was more variable in the epithelium of the lobular unit. No staining was present in stroma or myoepithelial cells. Staining was less in the non-malignant cases than in overexpressing breast cancer cell controls, but in the same epithelial membrane location. The duct epithelium of infants and elderly women stained as intensely as that of child-bearing age women. Lobular epithelium in pregnant women notably underexpressed c-erb-b2 by both histochemistry and Northern analysis.

CF 103 CHARACTERIZATION OF TWO TYPES OF NORMAL HUMAN BREAST EPITHELIAL CELLS THAT ARE EITHER DEFICIENT OR PROFICIENT IN GAP-JUNCTIONAL INTERCELLULAR COMMUNICATION

Chia-Cheng Chang, Shigeharu Nakatsuka, Ghulam Kalimi, James E. Trosko and Clifford W. Welsch, Departments of Pediatrics/Human Development and Pharmacology/Toxicology, Michigan State University, East Lansing, MI 48824

We have previously reported that normal human breast primary epithelial cell cultures can be established from tissues derived from reduction mammoplasty using a serum-free, low calcium (0.15 mM) defined medium (MCDB 153 supplemented with EGF, insulin, hydrocortisone and human transferin) (Proc. AACR 30:151, 1989). Recently, the culture originally developed in this medium was found to contain two morphologically distinguishable epithelial colonies when grown in a mixture of this medium and a modified Eagle's MEM supplemented with 10% fetal bovine serum (1:1 v/v). One type of the colonies was found to express low level of keratin antigen measured by the immunofluorescent method and to be completely deficient in gap-junctional intercellular communication measured by the scrape loading/dye transfer or the fluorescent return after photobleaching (FRAP) technique. The other cell type, in contrast, expressed high level of keratin and was proficient in gap-junctional intercellular communication. Both cell types express little or no epithelial membrane antigen (EMA). Other characterizations of these cells including the expression of different oncogenes and other antigen markers will be undertaken. (Research supported by a NCI grant CA 50430).

CF 104 TRANSFORMATION OF A HUMAN MAMMARY EPITHELIAL CELL LINE BY A POINT-MUTATED Ha-RAS PROTO-ONCOGENE OR BY OVEREXPRESSION OF THE HUMAN TRANSFORMING GROWTH FACTOR ALPHA

GENE, Fortunato Ciardiello[1], MaryLou McGeady[1], Jose Russo[2], Marc Lippman[3], Nancy Kim[1], Beatrice Langton[4], Hideo Masui[5], Toshiaki Saeki[1], John Mendelsonn[5], and David S. Salomon[1]. [1]Laboratory of Tumor Immunology & Biology, NCI, NIH, Bethesda, MD 20892, [2]Michigan Cancer Foundation, Detroit, MI 48201, [3]Lombardi Cancer Research Center, Washington, DC 20007, [4]Triton Biosciences, Inc., Alameda, CA 94501 and [5]Sloan-Kettering Memorial Cancer Center, New York, NY 10021.

The human point-mutated c-Ha-ras gene or the human TGFα gene along with the neo gene were introduced into the normal human mammary epithelial cell line, MCF-10. A clone of MCF-10 cells, MCF-10ras, was isolated following G418 selection. These cells grew aggresively as colonies in soft agar, expressed elevated levels of $p21^{ras}$ and c-Ha-ras mRNA and formed tumors in nude mice. MCF-10ras cells also exhibited a 3-fold increase in growth rate and showed a markedly diminished growth response to exogenous EGF or TGFα as compared to MCF-10 cells transfected with only the neo gene, MCF-10neo. MCF-10ras cells secreted 5-fold more TGFα than MCF-10 neo cells. Monoclonal anti-EGF receptor blocking or anti-TGFα neutralizing antibodies were able to significantly inhibit the growth of these cells in soft agar. Fifteen G418 resistant MCF-10 clones were isolated following infection of MCF-10 cells with an expression vector containing the TGFα and neo genes. All of the clones secreted approximately 6-to17-fold more TGFα than the uninfected MCF-10 cells. Nine clones which were secreting the highest levels of TGFα formed colonies in soft agar, exhibited a 3-fold increase in growth rate in serum-free medium as compared to MCF-10neo cells, and could be blocked by anti-EGF receptor blocking or anti-TGFα neutralizing antibodies.

CF 105 PROGESTERONE RECEPTOR REGULATION BY RETINOIC ACID IN THE HUMAN BREAST CANCER CELL LINE T-47D. C.L. Clarke, S.D. Roman, J. Graham, M. Koga and R.L. Sutherland, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, N.S.W. 2010, AUSTRALIA.

Progestins inhibit growth and regulate the concentration of their own receptor in the progesterone receptor (PR) positive human breast cancer cell line T-47D. The morphogen retinoic acid (RA) also inhibited growth and decreased progestin binding capacity. We asked whether RA was able to regulate PR expression and therefore investigated PR protein and mRNA concentrations following RA treatment of T-47D cells. T-47D cells were grown in RPMI 1640 + 5% fetal calf serum (FCS) for 5 days and changed to RPMI 1640 + 1% charcoal-stripped FCS 24h before treatment with all-*trans*-retinoic acid or vehicle. PR mRNA was quantitated by Northern analysis of total RNA and immunoreactive PR was measured by protein blot analysis. Treatment of T-47D cells with RA for 48h resulted in a marked concentration-dependent decrease in the level of PR mRNA and immunoreactive protein. RA did not bind to PR nor did it cause the increase in PR molecular weight observed after progestin exposure. Interestingly, when T-47D cells were treated with RA for 6h rather than 48h, no reduction in the level of PR protein was noted at any RA concentration, whereas the effects of RA on PR mRNA at 6h and 48h were identical. This discordance suggested that the timing of RA action on PR mRNA and protein differed, and therefore the time course of the effect of RA was examined. A rapid decrease in PR mRNA levels was detectable 1h after and was maximal 6h after treatment of T-47D cells with RA. This contrasted with previously demonstrated progestin effects on PR mRNA which were not apparent until 3h after and were not maximal until 12h after treatment. As expected PR protein concentration was unaffected for at least 6h, but was maximally decreased 34h after RA treatment. In summary, RA mimicked some of the known effects of progestins on PR, but the regulation by RA followed a different time course, suggesting that RA acts via a different, probably converging, mechanism. These data document the first known effect of RA on PR gene expression, and suggest that RA is capable of modulating sensitivity to progestins in human breast cancer cells.

CF 106 GROWTH FACTOR PRODUCTION BY HUMAN BREAST FIBROBLASTS FROM BENIGN AND MALIGNANT LESIONS. Kevin J. Cullen, Suzanne Hill, Helene Smith*, Marc Lippman and Neal Rosen. Vincent T. Lombardi Cancer Research Center, Georgetown University Washington, DC 20007, and *Geraldine Brush Cancer Institute, San Francisco, CA. Breast tumors are a complex mix of epithelial, stromal, vascular and fibrous elements. The overall growth of a breast malignancy requires the coordinated growth of these individual elements. Laboratory studies of growth factor production in breast cancer have almost exclusively focused on breast cancer epithelial cell lines. We examined a number of breast fibroblast lines derived from benign and malignant lesions as well as reduction mammoplasties for expression of various growth factors including TGF-α, TGF-β1, PDGF A Chain, PDGF B chain, Insulin-like growth factor I and Insulin-like growth factor II. 10/10 cell lines examined expressed PDGF A chain and TGF-β1 mRNA. None of the cell lines expressed PDGF B chain or TGF-α mRNA. There was no difference seen in the expression of these growth factors from between fibroblasts derived from benign or malignant lesions. However, examination of mRNA expression for the insulin-like growth factors revealed 5/6 fibroblasts derived from benign lesions expressed IGF-I mRNA, while only 1/8 fibroblasts derived from malignancies expressed IGF-I mRNA. The opposite picture was seen for IGF-II mRNA expression, in which 1/7 benign derived fibroblasts expressed IGF-II mRNA, while 5/9 malignant derived fibroblasts expressed IGF-II. Thus there was an apparant transition from IGF-I mRNA expression in the majority of the fibroblasts derived from benign lesions to IGF-II mRNA expression in the majority of the tumor derived fibroblasts. Since the insulin-like growth factors are potent mitogens for breast tumor epithelial cells, this further supports the notion of a paracrine growth promoting role for the insulin-like growth factors in breast lesions, and suggests that IGF-II may be the more important growth promoter in malignant lesions.

CF 107 HORMONAL REGULATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGF-BPs) IN HUMAN BREAST CANCER CELLS, Daisy D. De Leon, Darrell M. Wilson, Michael Powers and Ron G. Rosenfeld, Pediatric Endocrinology, Stanford University, Stanford, CA 94305 Although human breast cancer cells (HBCC) do not secrete IGF-I, they secrete at least three distinct IGF-BPs and contain mRNA for hIGF-BP-1, -2 and -3. In addition, they secrete a 24 kd yet unidentified IGF-BP. Utilizing two HBCC, MCF-7 and Hs578T, we studied the effect of IGF-II, E2, GH, and insulin on the secretion of IGF-BPs. HBCC were grown without insulin in serum-free and phenol red-free DMEM for 72 hrs. Conditioned media was collected and assayed by ligand blot. Summary of hormonal regulation of IGF-BPs in HBCC:

	MCF-7		Hs578T			
	hIGF-BP-2	BP-24	hIGF-BP-1	BP-24	hIGF-BP-3	
E2	+	+	-	-	-	
Ins	+	-	-	-	-	(- = no effect)
GH	-	-	-	-	-	(+ = increase)
IGF-II	-	+	-	-	+	

When cells were grown in 1 ug/ml insulin, the secretion of all IGF-BPs was significantly reduced and the response to the above hormonal treatments was altered. Interestingly, when MCF-7 HBCC were grown in insulin, the 24 kd BP was stimulated by E2 while progesterone, testosterone and dexamethasone inhibited hIGF-BP-2. Since HBCC are sensitive to IGF stimulation, and IGF-BPs can modulate IGF action, hormonal regulation of the IGF-BPs secreted by HBCC may play an important role in the proliferative response of these cells to IGF-I and IGF-II.

CF 108 RETROVIRAL INTEGRATION-INDUCED MUTAGENESIS: SEARCH FOR GENES INVOLVED IN THE DEVELOPMENT OF HORMONE INDEPENDENCE IN HUMAN BREAST CANCER. Lambert C.J. Dorssers, Marijke Mostert, Thecla L.A. van Agthoven, Ton van Agthoven and John A. Foekens. Dr. Daniel den Hoed Cancer Center, P.O.Box 5201, 3008 AE ROTTERDAM, THE NETHERLANDS.

Growth of breast cancer cells is regulated by steroid hormones and can be blocked by hormone antagonist in one-third of the clinical cases. A major problem of endocrine therapy is that eventually all tumors become hormone-insensitive. Our recent experiments show that hormone-dependence in human breast cancer cells (ZR-75-1) can be completely bypassed by introduction of the human Epidermal Growth Factor-Receptor (EGF-R) cDNA. Cells carrying EGF-R acquired the capability to grow on exogenous EGF, even in the presence of high doses of anti-estrogens, whereas the parental cells were unable to proliferate under these conditions. These experiments indicate that single genetic perturbations may induce hormone-independent growth. Therefore we have initiated a random search for genes capable of sustaining growth in the absence of estradiol. Retroviral DNA integration in the host genome is considered to be a random process and may result in (in)activation of gene expression. Large numbers of ZR-75-1 cells have been infected with a defective, amphotropic retrovirus (N2) and subsequently cultured in the presence of hydroxy-tamoxifen. Proliferating cell clones have been isolated from these cultures and are currently being investigated for retroviral integration patterns. This involves Southern analysis, in vitro DNA amplification using the Polymerase Chain Reaction (PCR) and molecular cloning of viral DNA containing chromosomal DNA fragments. This novel approach may lead to the identification of regulators of breast cancer cell growth.

CF 109 TYROSINE PHOSPHORYLATION OF A 175-kDa *neu* PROTO-ONCOGENE PRODUCT IS INDUCIBLE BY GROWTH FACTOR WITHDRAWAL IN MOUSE FIBROBLASTS BUT NOT IN HUMAN BREAST CANCER CELLS. Richard J. Epstein, Brian J. Druker, Thomas M. Roberts, Charles D. Stiles, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

The *neu* proto-oncogene product has been found to exist in two interconvertible forms in non-transformed G8/DHFR mouse fibroblasts, a 185-kilodalton form (p185) present in growing cells and a 175-kilodalton form (p175) present in serum-starved cells. The low molecular weight form of the receptor is down-regulated and characterised by increased tyrosine kinase activity, while conversion of p175 to p185 reflects phosphorylation of serine and threonine. Tyrosine phosphorylation of p175 is reversed within two minutes of serum replenishment, preceding conversion to the 185-kilodalton form, and similar effects are seen following addition of platelet-derived growth factor or phorbol ester; removal of serum growth factors is followed by replacement of p185 with p175 over several hours. Unlike G8/DHFR cells, the human breast cancer cell line SK-Br-3 expresses a high molecular weight *neu*/HER2 receptor with negligible phosphotyrosine content in both serum-starved and serum-stimulated cultures. These findings indicate that activation of the *neu* proto-oncogene product in non-transformed cells may be regulated in part by protein kinase C-mediated receptor transmodulation rather than by ligand availability alone, and raise the possibility that the activated 175-kilodalton c-*neu* receptor isoform may play a role in cell quiescence.

CF 110 EFFECT OF TAMOXIFEN ON ONCOGENES EXPRESSION IN BREAST CANCER. Chantal Escot*, Xavier Le Roy*, Thierry Maudelonde*, Françoise Soussaline§, Jacques Domergue°, Henri Pujol°° and Henri Rochefort* - *INSERM U 148, Unite Hormones et Cancer, 60 Rue de Navacelles, 34090 Montpellier France. §IMSTAR, 60 rue Notre Dame des Champs 75006 Paris France. °Chirurgie digestive, CHR, 34059 Montpellier cedex France. °°Centre Paul Lamarque, BP5054, 34033 Montpellier cedex France.

Using the RNA in situ hybridization technique, we screened 50 malignant breast tumors for c-myc, c-erbB-2, hst and int-2 expression. RNA expression of each oncogene was measured in three independent experiments and RNA levels were evaluated by quantitating silver grains with the STARWISE GRAINS computer program that we developped. The variability of the quantitation between experiments being estimated to 10-20%, it was possible to assess differences between tumor samples. Half of the patients with breast cancer received a three weeks treatment with tamoxifen prior surgery. All four oncogenes were expressed in both treated and untreated patients. Tamoxifen had no effect on hst and int-2 expression. 45% of the tumors co-expressed hst and int-2 and 38% showed either int-2 or hst RNA. c-erbB-2 expression in the untreated population was significantly correlated with the absence of estrogen receptor. Moreover statistical analysis performed on the primary ductal carcinomas showed a two to three fold decrease of c-myc and c-erbB-2 RNA levels in the treated patients. In order to investigate the mechanism of this inhibitory effect of anti-estrogen adjuvant therapy on the expression of these two oncogenes in vitro studies on breast cancer cell lines are underway.

CF 111 THE ROLE OF DIFFERENT MECHANISMS OF GROWTH FACTOR INDEPENDENCE ON IN VIVO GROWTH POTENTIAL OF RAT MAMMARY CARCINOMA CELLS. Stephen P. Ethier, Ramesh Moorthy and Caterina Chiodino, Department of Radiation Oncology, The University of Michigan Medical School, Ann Arbor, MI, and Department of Pathology, Wayne State University, Detroit, MI.

In previous studies we examined the mechanisms by which rat mammary carcinoma cells become independent of growth factors strictly required by normal mammary epithelial cells and have found no evidence to support a role for autocrine factors in mediating this phenotype. The present studies were aimed at examining this phenotype further by comparing the in vivo and in vitro growth potential of tumor-derived growth factor independent cells and growth factor dependent cells rendered IGF-I independent by transfection with an IGF-I expression vector. Cells from a transplantable mammary carcinoma were seeded into culture in serum-free medium in the absence of either IN, EGF or cholera toxin (CT). Growth factor independent populations were established and the cells were transplanted into the interscapular fat pads of syngeneic female recipients. Cells from all three growth factor independent populations were tumorigenic in vivo. Next, the three growth factor independent populations were tested for their ability to grow in media devoid of multiple growth factors. All three populations grew best in the absence of IN, EGF and CT. Thus, tumor-derived growth factor independent cells are independent of multiple growth factors and these cells have high neoplastic potential in vivo. Next, a growth factor dependent cell strain was transfected with an IGF-I expression vector. The transfected cells were independent of IGF-I for growth in serum-free medium and IGF-I like biological activity was readily detectable in the conditioned medium of these cells. Transplantation of these cells into syngeneic recipients did not result in tumor development after six months in vivo. Thus, tumor-derived growth factor independent cells in which autocrine loops appear not to be operative have high neoplastic potential in vivo, whereas cells rendered growth factor independent by imposition of an autocrine loop do not acquire in vivo tumor potential.

CF 112 GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH, EC, 1.2.1.12) GENE EXPRESSION IN TWO MALIGNANT HUMAN MAMMARY EPITHELIAL CELL LINES: BT-20 and MCF-7. REGULATION OF GENE EXPRESSION BY 1,25 DIHYDROXYVITAMIN D3 (1,25(OH)2D3).

Frappart L, Desprez PY, Leguelec D, Denier JF, Poujol D, Saez S. Laboratoire de Biologie - Centre de lutte contre le cancer Léon Bérard. 69373 Lyon cedex 08 France

GAPDH is a key enzyme in the control of glycolysis. It is known to be functionnal in man, mouse, rat, and chicken. Its activity has been previously investigated in tumor tissue. In the present study, on 2 breast cancer cell lines from human origin (BT-20 and MCF-7), we used a rat cDNA probe of 1.3 Kb for Northern blot and in situ hybridization (ISH) coupled with semi-quantitative analysis.

It is found that GAPDH mRNA is overexpressed in the poorly differentiated (ER- PGR-) BT-20 cell line while it is not in the well differentiated (ER+ PGR+) MCF-7. Treatment of the cell by 1,25(OH)2D3 overstimulated GAPDH mRNA in a dose dependent manner only in BT-20 cells. Data from ISH are in agreement with Northern blot analysis. In addition, it discloves that the effect is located in the most mature cells of the population. The increased expression of GAPDH in BT-20 cells treated by 1,25(OH)2D3 is associated to a reduced proliferation rate, while it is usually observed in rapidly growing cells like lung cancers, hepato and kidney carcinomas.

CF 113 SENSITIVE DETECTION AND CLONING OF VARIANT ESTROGEN RECEPTOR (ER) RNA TRANSCRIPTS FROM HUMAN BREAST TUMORS, Suzanne A.W. Fuqua, Nicole F. Falette, William L. McGuire, Department of Medicine/Oncology, University of Texas Health Science Center, San Antonio, TX 78284.

Breast cancer is one of the few human malignancies responsive to endocrine manipulation with the presence of ER identifying patients most likely to respond to endocrine manipulation. Several groups have now reported variant ER RNAs in breast tumors. We have addressed how frequently breast tumors express variant ER RNAs and whether these variant ERs are responsible for the lack of ER protein in some tumors.

To detect variant ER RNA transcripts we have developed a simplified polymerase chain reaction (PCR) technique that utilizes only 1 ug of total RNA from breast tumor biopsies. For semiquantitation of ER RNA, we have developed a simultaneous RNA PCR assay using primers to both ER and ß-actin as an internal control; there is a good correlation between the expression of ER as determined by RNA PCR assay and ligand-binding in ER-positive tumors. We have cloned RNA PCR products from ER-negative, PR-positive tumors, and have identified an RNA species lacking exon 5 within the hormone binding domain. This variant RNA species may be an alternatively spliced RNA which is differentially expressed in breast tumors.

Conclusion: 1. We have developed a simplified PCR technique to detect and clone rare ER transcripts. 2. Alternative RNA splicing may occur within the hormone binding domain of ER. 3. Variant ER RNAs may be differentially expressed in breast tumors.

CF 114 AMPLIFICATION OF THE HER - 2/NEU PROTO - ONCOGENE IN BREAST CANCER DETECTED BY A RAPID METHOD. **Elena Giulotto, Livia Bertoni and Dino Amadori*. Dipartimento di Genetica e Microbiologia " A. Buzzati-Traverso ", Università di Pavia, Via S. Epifanio 14, 27100 Pavia, Italy.*Divisione di Oncologia, Ospedale Pierantoni, 47100 Forlì, Italy.**The methods to screen gene copy number in human tumors used so far in research laboratories, mainly Southern blotting and dot-blotting of purified DNA, are extremely laborious and time consuming. Since detection of oncogene copy number may soon became an important clinical test, a quick and easy assay is needed for routine analysis of surgical samples.We present here an alternative method to determine gene copy number from tumor samples. This technique is a modification of a recently described method for analysing specific DNA sequences from cultured cells (McIntyre and Stark, Analyt. Biochem. 174,209; 1988). Crude extracts from as little as 10 mg of tumor tissue were obtained by alkaline hydrolysis and directly transferred on a Nylon membrane with a dot-blot apparatus. The DNA on the membrane, more resistant then RNA and proteins to alkali, was hybridized with a ^{32}P labelled probe and autoradiographed. The efficiency of hybridization is dependent on amount of extract, time and temperature of hydrolysis and amount of radioactivity. After optimizing these conditions, we were able to quantify the 20 fold amplification of the HER-2-neu oncogene previously detected by Southern blotting in a sample of gastric carcinoma, and the 3 to 5 fold amplification of the same gene in two mammary carcinomas. As it does not require DNA extraction, the method is rapid and simple. Moreover, it can be used even when very small samples are available for the analysis.

This work was supported by a grant from Istituto Oncologico Romagnolo and by P.F. Oncologia No. 87.01308 from C.N.R. L.B. is the recipient of a fellowship from Istituto Oncologico Romagnolo.

CF 115 A HEAT SHOCK PROTEIN GENE IS CONSTITUVELY EXPRESSED IN THE HUMAN MAMMARY CELL LINE HBL100 TRANSFORMED BY THE EJ/T24 *ras* ONCOGENE. Gérard Goubin, Jérome Lebeau, Catherine Le Chalony and Marie-Thérèse Prosperi. Molecular Oncogenesis, Institut Curie, 26, rue d'Ulm, 75005 Paris. France. We have employed the human mammary cell line HBL100 to identify genes involved in the progression of multistage carcinogenesis. This SV40 immortalized cell line is unable to induce tumors when injected subcutaneously into athymic mice. The introduction and expression of the EJ/T24 bladder carcinoma H-*ras* oncogene in the HBL100 cell confers the ability to induce tumors in athymic mice to this cell line. We used this model system to isolate genes whose expression is modified after *ras* tumorigenic conversion. A cDNA clone coding for a member of the 90 kDa heat shock protein family (HSP89α) was found transcribed at a higher level in *ras* transformed HBL100 cells compared to untransformed HBL100 cells. A higher level of expression of the mouse homolog of the HSP89α gene was also found in mouse NIH3T3 cells transformed by the H-, K-, N- activated *ras* oncogenes. Serum induction studies revealed a constitutive expression of the HSP89α gene in *ras* transformed HBL100 cells. Work is in progress to determine whether the altered expression of the HSP89α gene is limited to transformation by the *ras* oncogenes or whether it can be also found following transformation by other oncogenes.

CF 116 ABILITY OF TAMOXIFEN TO INHIBIT MCF7 CELL GROWTH MEDIATED BY IGF-1 ALONE OR BY IGF-1 AND ESTRADIOL IN SYNERGISM. Manjula K. Gupta and Rodrigo Garcia. Department of Immunopathology, The Cleveland Clinic Foundation, Cleveland, OH 44195
Tamoxifen has been used effectively in treatment of hormone-dependent breast cancer. It is known to inhibit estrogen-mediated growth by blocking estradiol binding to its receptor. However, alternative mechanisms by which tamofixen causes tumor regression has been long sought for. IGF-1 is the most potent growth factor recognized under serum-free conditions for MCF-7 cells. We studied the effect of tamoxifen on IGF-1 mediated cell proliferation in these cells. IGF-1 causes cell proliferation in a dose-dependent manner starting at concentrations of 100 pg/ml (2x increase) with a maximum response at 10 ng/ml (3x increase) as measured by tritiated thymidine uptake or by total DNA measurement at 72 hr. Estradiol, in contrast, has a minimal effect (1.5 fold increase in total DNA at 10^{-8} M concentrations). However, at lower concentrations of IGF-1 (100 pg/ml) addition of estradiol (10^{-10} M) further enhanced cell growth significantly ($p < 0.5$). Interestingly, tamoxifen inhibited IGF-1 mediated growth even at high concentration (10 ng/ml) in a dose dependent manner. Complete inhibition is caused at 5×10^{-6} M concentrations of tamoxifen. Also this dose of tamoxifen inhibited synergistic cell proliferation caused by IGF-1 and estradiol. Cooperative mitogenic activity of IGF-1 and estradiol and its inhibition by tamoxifen, seen in our experiments, favors the hypothesis that estradiol may cause cell proliferation by increased production of IGF-1 or alternatively by making these cells more responsive to IGF-1. Our findings also indicate that possibly one additional mechanism by which tamoxifen inhibits estrogen-dependent or even estrogen-independent cell growth is via its ability to inhibit IGF-1 mitogenic activity.

CF 117 STUDIES OF RAT BREAST TUMORS INDUCED BY HUMAN ADENOVIRUS TYPE 9, Ronald Javier[1], Karel Raska[2], and Thomas Shenk[1], [1]Howard Hughes Medical Institute, Department of Biology, Princeton University, Princeton, New Jersey, 08544. [2]Department of Pathology, Robert Wood Johnson Medical School, Piscataway, New Jersey.
It was discovered in 1974 that human adenovirus type 9 (Ad9) induces with near 100% efficiency benign fibroadenomas of the breast in female Wistar-Furth rats. These tumors appear 14-25 weeks post infection of newborn female rats, transform to malignancy at a low frequency (~14%), and are estrogen-dependent in vivo. We have now reproduced and extended these original studies using a plaque purified isolate of the Ad9 "Hicks" strain. With our Ad9 isolate, we also found that 100% of virus infected female rats developed tumors, while male rats did not (0%). Tumor latency periods were as short as eight weeks, and the frequency of malignant tumors (exclusively fibrosarcomas) was approximately 25% after about seven months. Southern blot analysis of high molecular weight DNA derived from benign fibroadenomas indicated that between one and one-tenth of the cells within the tumor contained a single integrated copy of the entire Ad9 genome. In contrast, cells from malignant fibrosarcomas contained multiple integrated copies (between one and ten) of the entire Ad9 genome. Similarly, using the RNAse protection assay, we detected much lower E1a mRNA levels in benign fibroadenomas than in malignant fibrosarcomas. In summary, we now report for the first time that viral DNA and RNA are present in Ad9 induced breast tumors, and that malignant tumors contain more viral DNA and E1a mRNA than benign tumors.

CF 118 CSF-1, A LYMPHOHEMATOPOIETIC GROWTH FACTOR WHICH IS AN AUTOCRINE CYTOKINE IN BREAST, OVARIAN, AND LUNG CARCINOMAS, CAN ALSO BE EMPLOYED AS A TUMOR MARKER IN PATIENTS WITH ADENOCARCINOMAS. Kacinski, B.M.[1], King, B.L.[1], Chambers, S.K.[1], Scata, K.[1], Carter, D.C.[1], and Stanley, E.R.[2] Yale University School of Medicine[1], New Haven, CT 06510 and Albert Einstein College of Medicine[2], Bronx, NY 10461.

Initial studies on expression of 20 different oncogenes growth factors and receptors in breast, ovarian, endometrial and lung adenocarcinomas revealed that expression of transcripts complementary to the fms gene correlated with grade and stage in ovarian carcinoma, grade, stage and depth of myometrial penetration in endometrial carcinoma, and with the expression of genes generally associated with control of cell proliferation (e.g., c-fos, c-myc) in breast, ovarian and endometrial carcinomas. This led us to seek for and to find (via ISH and IHC) localization of fms-complementary transcripts and antigen to the malignant epithelial cells of these neoplasms, and to demonstrate in cultured cell lines the expression of fms-complementary transcript and a CSF-1 responsive protein very similar--if not identical--to that encoded by the c-fms proto-oncogene. About half of the adenocarcinoma specimens showed evidence of co-expression in vivo of both fms and CSF-1. Clinical studies carried out on the sera of such adenocarcinoma patients showed clear elevation of plasma CSF-1 levels in patients with active or recurrent disease, and changes in circulating CSF-1 level paralleled changes in clinical disease activity.

How malignant epithelial cells come to express CSF-1 and a CSF-1 receptor remains unclear. However, co-expression of these genes in vivo in at least half of these neoplasms and the elevated circulating levels which were seen in patients with active neoplastic disease suggest that a lymphohematopoietic cytokine (CSF-1) and its receptor may play an important role in the development, progression and dissemination of these nonhematopoietic neoplasms.

This work was supported by NIH Award CA47292 and a Bristol-Myers Cancer Research Award 100-R063 to BMK.

CF 119 THE SEARCH FOR A RETROVIRUS ASSOCIATED WITH HUMAN BREAST CANCER, L.P. Kahl*, A.C. Carroll*, N.G. Read** and M. Baum+.
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Human peripheral blood mononuclear cells from breast cancer patients and normal donors were cultivated in vitro and the following determined: Quantification of numbers of multinucleate giant cells using light microscopy; the presence or absence of reverse transcriptase (RT) activity in culture supernatant fluid; RNA hybridization using retroviral probes and e.m. analysis of selected cultures. Data obtained will be discussed and compared with that described by others who have reported syncytium formation, RT activity and identification of viral particles in either peripheral blood mononuclear cells or cell lines derived from the pleural effusion of breast cancer patients.

CF 120 CLONING AND EXPRESSION OF THE POLYMORPHIC GENE FOR A BREAST- ASSOCIATED EPITHELIAL MUCIN, PEM. C. Lancaster, J. Burchell, T. Duhig, N. Peat, E-N Lalani, D. Wilson, L. Pemberton, J. Taylor-Papadimitriou, S. Gendler, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Human mammary cells secrete and express on the cell surface a polymorphic epithelial mucin (PEM) which is developmentally regulated and aberrently expressed in tumors. Previous isolation of partial cDNA clones revealed that the core protein contains a large domain consisting of 20 amino acid (aa) repeat units. The number of repeats varies amongst individuals, giving rise to the polymorphism seen at the DNA and protein level. We now report the full protein sequence for PEM, as deduced from full-length cDNA sequence. The molecule contains 454 aa, in addition to tandem repeat domains of 650 to 1500 aa, with putative signal and transmembrane sequences followed by 72 cytoplasmic residues. The presence of 30 to 80 tandem repeats among individuals predicts a peptide backbone of 120 to 200 kD which is in good accord with a protein of apparent Mr of 240 to 400 kD that contains 50% by weight carbohydrate. Analysis of cosmid clones for PEM revealed that the gene spans approximately 7.5 to 10 kb of genomic DNA (depending on the number of tandem repeats present) and contains six exons. A full-length cDNA has been constructed and expressed in COS cells. The tandem repeat appears to be immunodominant as many of the Mabs reactive with this molecule (HMFG-1, -2 and SM-3) recognise epitopes present within this area. Using overlapping peptide octamers, we have precisely mapped the epitopes of 4 Mabs in this area including one, SM-3, which shows enhanced tumor specificity. The core of the SM-3 epitope corresponds to the continuous aa sequence PDTRP.

CF 121 RECEPTOR DOWNREGULATION AND DNA SYNTHESIS ARE MODULATED BY EGF AND TPA IN CELLS EXPRESSING AN EGFR/*neu* CHIMERA, Laura Lehtola, Heikki Lehväslaiho,Lea Sistonen, Laura Beguinot# and Kari Alitalo, Department of Virology and Pathology,University of Helsinki, 00290 Finland,# Department of Microbiology, University of Copenhagen,1353 Denmark

EGF was used to stimulate a chimeric receptor consisting of the epidermal growth factor receptor (EGFR) extracellular, transmembrane and protein kinase C-substrate domains linked to the intracellular tyrosine kinase and carboxyl terminal domains of the rat *neu* protein in NIH/3T3 cells. EGF induced rapid and delayed morphological changes in the cells. EGF bound to the cells was rapidly internalized in a complex with the EGFR/*neu* protein, as shown by loss of EGF binding and EGFR antigens from the cell surface. Metabolic labeling and pulse-chase experiments indicated that the receptor was degraded soon after its internalization. EGF treatment also induced the *jun*B transcription factor mRNA and a dose-dependent stimulation of DNA synthesis in cultures expressing the chimeric receptor. The tumor promoter TPA led to a transient loss of cell surface receptors and prevented EGF-stimulation of DNA synthesis, but did not completely abolish *jun*B mRNA induction or increase degradation of the chimeric receptor. These results show that the chimeric EGFR/*neu* receptor undergoes typical downregulation upon ligand binding and TPA pretreatment and is capable of transducing an EGF-induced mitogenic signal.

CF 122 MOUSE MAMMARY CELL SPECIFIC NUCLEAR PROTEINS BIND TO THE 5' DISTAL REGION OF THE LTR-MMTV.Lelong, J.C., Prévost, G., Foehrle E and Crépin M. Université de Paris XIII, I.O.C.M.H., 129 route de Stalingrad, 93000 - Bobigny, France.

Protein-DNA interactions within the regulatory sequences of Mouse Mammary Tumor Virus (MMTV) promoter were investigated to study the cell specificity of MMTV expression. Using two different approaches (electrophoretic mobility assay of protein-DNA complexes and South Western blot assays) we have identified four mammary cell specific nuclear proteins (55, 40, 30, 25 Kd) specifically bound to sequences upstream of the hormone responsive elements. The native DNA-protein complexes formation and the DNA binding activity of these proteins are not modified by an *in vivo* treatment of GR mammary cells with a glucocorticoid (dexamethasone). These mammary cell specific interactions are located in one MMTV-LTR region which has previously been described to be involved in the *in vivo* tissue specificity and the transcriptional regulation of the MMTV.

CF 123 UPREGULATION OF BREAST CANCER ASSOCIATED ANTIGENS AND OF DIFFERENTIATION MARKERS WITH INTERFERONS AND PKC STIMULATORS. Jorge A Leon+, Alison Estabrook+, Ricardo Mesa-Tejada'*, Shrishailam Yemul+, M. Caroline Gutierrez^, Paul B. Fisher",Departments of Surgery+, Pathology^, and Neurosurgery", Columbia University, New York, NY 10032, and *MetPath, Teterboro, NJ 07608

We have evaluated the effects of recombinant human interferons, TNF, TPA and several differentiation inducers on the expression and shedding of BCA 225 (a molecule belonging to the family of human milk fat globulin membrane (HMFGM) tumor associated glycoproteins), CEA, c-erb-2, EGF receptor, and HLA class I and II antigens on the human breast cancer cell lines T47D and MCF7.

Bio. Response Modulator	Expression BCA 225	Shedding BCA 225	CEA	erb 2	EGFr	HLA Class I	HLA Class II
INF-α	+	-	+	-	-	+	+
INF-β	+	-	+	-	-	+	+
INF-δ	+	+	+	-	-	+	+
TNF	+	+	-	-	+	+	+
TPA	+	+	+	+	+	-	+

No effect on BCA 225 was observed with calcium ionophores, retinoic acid, Vitamin D, demethylating agents, and steroid hormones. This investagation indicates that an HMFGM molecule can be upregulated by INFs, TNF, and PKC stimulators. The association of these efects with known differentiation markers may clarify the physiology of the regulation of membrane antigens.

This work was supported in part by PHS/NIH Grant CA 32984 & The Hanson Memorial Foundation.

CF 124 THE BREAST CANCER ASSOCIATED MUCIN MAM-6 IS GENERATED BY A POLYMORPHIC GENE ENCODING SPLICE VARIANTS WITH TWO ALTERNATIVE AMINO TERMINI, Marjolijn J.L. Ligtenberg, Annemiek M.C. Gennissen, Hans L. Vos and John Hilkens, Department of Tumor Biology, The Netherlands Cancer Institute (Antoni van Leeuwenhoekhuis), Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
The MAM-6 antigen is a polymorphic glycoprotein of the mucin type, which is present at the luminal side of most glandular epithelial cells. The expression of MAM-6 is increased in most carcinoma cells, where it is detected both intracellularly and on the entire cell surface. We have isolated cDNA clones encoding MAM-6 and determined the structure of the gene. Most of the cDNA clones encode a transmembrane protein, which consists mainly of tandem repeats of twenty amino acids that are located on a single exon. We show that the number of these repeats varies among different alleles. The repeats and most of the remainder of the protein are very rich in potential O-linked glycosylation sites. In addition, five N-linked glycosylation sites are present. The protein products encoded by the two most abundant splice variants only differ in their signal sequences and the extreme N-terminal part of the mature proteins, suggesting alternative processing pathways of these two species. A third type of cDNA clone is a splice variant, which encodes a highly divergent protein, since it contains neither the tandem repeat region nor the transmembrane part of the molecule.

CF125 A NEW MONOCLONAL ANTIBODY TO STUDY THE BIOLOGICAL FUNCTION OF THE HER-2/Neu GENE PRODUCT. Serenella M. Pupa, Elda Tagliabue, Antonio Mastroianni, Rita Pellegrini, Sylvie Ménard and Maria I. Colnaghi, Division of Experimental Oncology E, Istituto Nazionale Tumori, Via G. Venezian 1, 20133 Milano, Italy.

In order to investigate the possible receptor function for the unknown ligand of the HER-2/Neu gene product (p185), we produced MAbs against the extracellular domain of p185. To immunize the mice and screen the hybridoma supernatants, we selected a cell line (CaLu-3), which demonstrated an overexpression of p185, measured as the reactivity with polyclonal rabbit serum anti-HER-2/Neu synthetic peptide (a gift from Dr. D.J. Slamon UCLA - USA). A MAb designated MGR2, of IgG1 isotype, was found to immunoprecipitate a 185 KDa molecule. Immunodepletion experiments with the polyclonal anti-serum and MGR2 indicated that the two reagents recognized the same molecule. Moreover, immunohistochemistry performed on different carcinomas showed an 84% correlation between both antibody reactivities. When MGR2 was administered "in vivo" in nude mice bearing subcutaneous CaLu-3 transplants, a significant inhibition of the tumor growth was observed. In conclusion, "in vivo" CaLu-3 growth seems to depend on a growth factor whose receptor could be the Neu gene product.

CF 126 STUDY OF PROTO-ONCOGENE EXPRESSION IN A BREAST CANCER CELL LINE RESISTANT TO TOPOISOMERASE II INHIBITORS. Guy Riou, Dominique Lefevre, Jean-François Riou, Danyi Zhou and Zong-Mei Sheng. Laboratoire de Pharmacologie Clinique et Moléculaire, Institut Gustave Roussy, 94800 Villejuif, (France).
The development of simultaneous resistance to multiple structurally unrelated drugs is a major obstacle to breast cancer chemotherapy. Therefore a breast cancer cell line resistant to drugs widely used in breast cancer therapy is a suitable model to study the genes involved in the resistance process as well as in mammary cell growth and tumorigenesis. In the present study, we analyzed the expression of such genes in the CALc18 breast cancer cell line and in the derived line CALc18/R resistant to the topoisomerase II inhibitors mAMSA, ellipticine, adriamycin and VM26. As expected, growth properties of both cell lines were not significantly affected by estradiol since they did not contain either estrogen or progesterone receptors. Steady state mRNA levels of *c-myb*, *c-erb*B2 and EGFR genes were found to be respectively 5-4- and 3-fold lower in resistant cells than in the parental cell line, whereas c-Ha-*ras* and c-Ki-*ras* mRNA levels were unchanged. By contrast, the *c-myc* mRNA levels were found to be 5 to 6-times more elevated in CALc18/R than in CALc18. Differences in the transcript levels of some of these genes were also observed between MCF7 lines sensitive and resistant to adriamycin. These data indicate that, in addition to the resistance phenotype other genetic alterations are induced by drugs which select biologically distinct subsets of breast cancer cells.

CF 127 ONCOGENE COMPLEMENTATION IN HUMAN BREAST CANCER: AMPLIFICATION OF C-MYC, ERBB-2 AND INT-2 GENES, Magali Roux-Dosseto, Christine Desideri, Nadege Dussault, Sylvie Romain and Pierre M. Martin, URA CNRS 1175, Faculte de Medecine-Nord, 13326 MARSEILLE Cedex 15, FRANCE. Amplification of c-myc, erbB-2 and int-2 oncogenes has been investigated in tumor DNAs of 170 patients with primary breast cancer and concerns 27%, 28% and 17% of tumors, respectively. We further looked for any synergistic action of these genomic alterations on disease-free and overall survival. Using uni- and multivariate statistical analysis we showed that c-myc amplification was associated with early recurrence and shorter overall survival, in contrast erbB-2 and int-2 extra-copies resulted in later relapse events specially in patients whose tumors showed a normal copy number of c-myc genes. Co-amplified c-myc and erbB-2 genes showed positive cooperation with respect to disease reccurence and shortening of the overall survival. Finally, the harmful effects of amplified c-myc and erbB-2 oncogenes were dramatically increased in patient subgroups showing normal copy number of int-2 gene. A multivariate analysis was used to test for potential interactions of oncogene covariates. We pointed out multiple independant combinations which defined complementation groups with respect to clinical patient behaviour. As the large amount of DNA coamplified with the target genes may well contain other active transcription units, we are currently investigating the size and the organization of amplified regions in tumor DNAs.

CF 128 REGULATION OF ESTROGEN RECEPTOR GENE EXPRESSION IN MCF-7 CELLS BY TUMOR PROMOTING AGENTS, Saceda M. Dickson R. Lippman M.E. Knabbe C. and Martin M.B. Lombardi Cancer Research Center, Georgetown University, Washington, DC 20007 and UK Eppendorf, 2 Hamburg, IRG

We have studied the effects of phorbol esters on the morphology, cell cycle, and anchorage-independent growth of MCF-7 cells. In addition, we have studied the effects of phorbol esters on the level of estrogen receptor protein and binding activity, on ER mRNA, and on the induction of estrogen-responsive genes. Phorbol ester treatment blocks anchorage-independent growth of MCF-7 in the presence of estradiol; cells treated with TPA loss their typical epithelioid characteristics and become rounded. Cells appear to accumulate in both G_1 and G_{2+M} Treatment with TPA results in the down regulation of ER protein and binding. No apparent change in the estrogen receptor affinity for estradiol was found, however 24 hours after treatment competed binding is undetectable. Phorbol esters also induce the phosphorylation of ER in serine and threonine, however phosphorylation does not appear to be associated with down regulation of ER. The decrease in ER parallels a similar decrease in ER mRNA suggesting that TPA regulates the level of ER gene expression by a transcriptional/post-transcriptional mechanism. The addition of TPA prior to or following estradiol treatment blocks the induction of progesterone receptor suggesting that TPA blocks induction not only at the initiation of induction but at later times.

CF 129 ONCOGENE AMPLIFICATION CORRELATES WITH LYMPHOCYTEINFILTRATION IN HUMAN BREAST CANCERS. A ROLE FOR HEMATOPOIETIC GROWTH FACTOR RELEASE BY TUMOR CELLS? S.M.Scholl, P.Validire, X.Sastre, P.Benoît, A.de la Rochefordière, V.Mosseri, B.Kacinski(*), P.Pouillart. Département de Médecine Oncologique, Institut Curie 25, Rue d'Ulm 75231 Paris France and(*) Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, CT 06510

106 primary breast cancer samples were analysed for c-erbB2, int-2 and c-myc gene amplification. Surgically confirmed nodal involvment was observed in 43%. Level of gene amplification was studied by Southern and/or slot blot techniques. Amplified c-erbB2 gene sequences were present in 21,5% of all samples. Int-2 was amplified in 13,1% and c-myc was amplified in 10,3% .In a non parametric test (Kruskal-Wallis) a strong negative correlation was found between high levels of c-erbB2 amplification and absence of estrogen receptor (ER) (P=0.0009) or progesterone receptor (PR) (P=0.011) expression. No correlations were found between all levels or high levels of amplification of each oncogene separately or combined with T, N, grade, multifocality of tumor or associated carcinoma in situ. There was a trend approaching statistical significance for patients with erbB2 amplification to have positive lymph nodes at surgery (P=0.09). A somewhat surprising finding however was a very strong correlation between oncogene amplification and intense lymphocyte infiltration of the tumor. In a double blind analysis we detect a significant correlation between the amplification of the three oncogenes combined with intense lymphocyte infiltration of the tumor (P=0.05). This correlation is even stronger when only high levels of amplification are considered, either for each oncogene separately (P=0.0048) or in combination (P=0.0007). We propose that malignant cell cytokine (e.g. CSF-1) production may help explain this observation

CF 130 c-erbB-2/c-erbA CO-AMPLIFICATION IN HUMAN BREAST CARCINOMA

Mahvash Tavassoli, Farzin Farzaneh & Nigel Kirkham. Sussex Centre for Medical Research, University of Sussex, Brighton, UK

We have analysed a panel of 72 primary breast carcinomas, for the presence of amplifications and gross structural alterations, in the oncogenes c-erbB-2, c-erbA, c-myc, N-myc c-mos and c-Ha- ras. The tumours were also classified, graded and staged histopathologically and their DNA ploidy was determined by flow cytometry.

Amplification of c-erbB-2 was detected in 28% of the tumours, of which 91% had an increased steady-state level of c-erbB-2 mRNA. Amplification of c-erbA was found in 23% of tumours and was always associated with the amplification of c-erbB-2, 83% tumours which had c-erbB-2 and c-erbA co- amplification had metastasised to axillary lymph nodes ($P< 0.005$).

Amplification of c-myc was detected in 21% of the tumours studied: of which 82% ($P< 0.005$) were of histopathological grade 3 and none were of grade 1. Flow cytometry showed that 90% ($P< 0.01$) of the analysed tumours with c-erbB-2 and c-erbA co-amplification, and 70% ($P<0.1$) of those with c-myc amplification were DNA aneuploid. A number of these tumours have now been analysed for the possible mutations in the transmembrane domain of the c-erbB-2 gene by PCR mediated direct sequencing.

CF 131 PROTO-ONCOGENE AMPLIFICATION IN HUMAN BREAST CANCER C. Theillet[1], J. Adnane[2], X. Leroy[3], M.P. Simon[2], P. Szepetowski[2], D. Birnbaum[4], C. Escot[3], P. Gaudray[2]. [1] Biochimie, Centre Paul Lamarque, 34094 Montpellier cédex. [2] INSERM U 148, 34060 Montpellier. [3] LGMCH, Faculté de Médecine, 06034 NICE cedex. [4] INSERM U 119, 27 blv Leï Roure 13008 Marseille. FRANCE.

We screened a serie of human tumors from different anatomical origins, including bladder cancer, melanoma, various haemapoietic disorders and 293 human primary breast tumors for genetic alterations involving proto-oncogenes. Among these different tumor types breast cancer seemed particularly prone to gene amplification. Out of the 12 genes tested 7 showed amplification at variable incidence. The most frequently amplified were, in order of magnitude, *c-erbB-2* (21,3%), *c-myc* (17,6%) and a gene cluster (*hst,int-2* and *bcl-1)* located on chromosome 11q13 (16,5%). Our study developped along two lines of interest; (1) was there a relation to be found between proto-oncogene amplification and tumor phenotype? (2) the large size of the 11q13 amplicon (over 500 kb) raises the question of what selective mechanism could be responsible of its emergence.

Statistical analysis revealed that amplification of each *c-erbB-2*, *c-myc* and *hst/int-2* was representative of a different phenotypical subset of tumors, therefore suggesting that this type of data could be helpful in characterizing new biological subclasses in human breast cancer.

Due to *int-2* 's involvment in mouse mammary tumorigenesis and its close proximity (20 kb) with *hst* we analyzed their RNA expression levels using the RNA/RNA *in situ* hybridization technique. Both genes showed low levels of expression in human breast cancer, with *hst* being preferentially expressed in amplified tumors. In addition we checked the amplification status of 3 other molecular markers of 11q13 (*bcl-1, sea, GST- 1*). Only bcl-1 showed systematic co-amplification with *int-2/hst* and in a minority of cases *bcl-1* was the only 11q13 amplified marker. This set of data raises the question of which, between *int-2/hst* and *bcl-1*, is the driving force in the 11q13 amplicon. Statistical and molecular data will be presented and discussed.

CF 132 HUMAN EPITHELIAL MUCIN TUMOR ANTIGEN cDNA SEQUENCES - DIFFERENTIAL SPLICING GENERATES SECRETED AND MEMBRANE BOUND FORMS. Daniel H. Wreschner[1], Mara Hareuveni[1], Judith Horev, Ilan Tsarfaty, Richard Lathe[1], Arnold S. Dion[2] and Iafa Keydar, Department of Microbiology, Tel Aviv University, Ramat Aviv 69978, Israel; [1]CNRS, Faculte de Medecine, 11 rue Human, 67085 Strasbourg-Cedex, France; and [2]CMMI, 1 Bruce St. Newark, N.J. 07103, USA.

The serum concentration of epithelial mucin tumor antigen is becoming an increasingly important parameter for monitoring the progression of breast cancer. Till recently, the sole protein data on this antigen has been a 20 amino acid tandem repeat motif. We now report the complete amino acid sequences of different forms of the human epithelial mucin tumor antigen as deduced from the nucleotide sequence of isolated non-repeat cDNAs. Isolated cDNAs coding for the region upstream to a central tandem 60 bp (20 aa) repeat array show that differential usage of alternative splice acceptor sites may generate 2 protein forms containing putative signal peptides of varying hygrophobicities. The isolated cDNAs 3' to the repeats indicate that whereas one mRNA transcript is colinear with the gene and has an ORF of 160 a.a., a second cDNA correlates with a mRNA that is generated by a series of splicing events and contains an ORF of 328 a.a. A highly hydrophobic 28 a.a. peptide is located towards the carboxyl terminus and may correspond to a transmembrane region. These data demonstrate the existence of multiple protein forms that probably localize to different cellular and extracellular compartments.

CF 133 EXTINCTION OF ESTROGEN RESPONSIVENESS IN SOMATIC CELL HYBRIDS OF ESTROGEN RECEPTOR POSITIVE (ER+) AND ER- BREAST EPITHELIAL CELLS

Deborah A. Zajchowski and Ruth Sager, Dana Farber Cancer Institute, Boston, MA 02115

Although it has been established that estrogens play a role in the etiology and continued growth of the majority of human breast cancers, the mechanism by which estrogen-dependent breast tumors arise is still unknown. We have compared cultured normal breast epithelial cells with ER+ breast cancer cells with respect to the estrogen response pathway and found no detectable expression of the estrogen receptor protein in the normal cells. In order to determine whether ER expression and estrogen responsiveness are dominant traits in ER+ breast cancer cells, we produced somatic cell hybrids by polyethylene glycol fusion of benzopyrene-immortalized breast epithelial cells (ER-; 184B5, M.Stampfer and J. Bartley, PNAS 82, 2394, 1985) and MCF-7 breast cancer cells (ER+). None of the hybrid cell populations produce ER protein or ER mRNA, thereby showing that the ER+ phenotype is recessive. The hybrid cell lines also fail to express the estrogen-inducible pS2 gene. This is not unexpected, since control of pS2 gene transcription has been shown to be dependent upon one essential transcription factor: the ER. However, preliminary data show that transient introduction of the cloned human ER into the hybrid cells fails to induce endogenous pS2 gene expression. Active ER synthesis was ascertained by demonstrating estrogen-inducible CAT activity of a transfected pS2cat reporter gene. These results imply that the extinction of estrogen-responsive pS2 gene expression in these hybrid cells is effected by factors in addition to those which prevent production of a positive-acting essential transcription factor (the ER).

Prognosis; Control of Malignancy

CF 200 SELECTIVE TUMORICIDAL ACTION OF CIS-UNSATURATED FATTY ACIDS IN VITRO

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The incidence of cancer is rare among Eskimos on traditional diet. Tumor cells are known to be deficient in delta-6-desaturase activity, which is necessary for the conversion of dietary cis-linoleic acid to gamma-linolenic acid (GLA). It is also known that the invasive capacity of the tumor cells is inversely proportional to their arachidonic acid (AA) content. These evidences led me to investigate the affect of essential fatty acids and their metabolites on human breast cancer cells in vitro. It was observed that GLA, AA, and eicosapentaenoic acid (EPA) and to a limited extent linoleic acid (LA) and alpha-linolenic acid (ALA) can selectively kill the breast cancer cells without harming the normal cells under similar experimental conditions. Of all the fatty acids tested, GLA, AA, and EPA were found to be the most potent. In mixed co-culture experiments where in normal and breast cancer cells were cultured together GLA, AA and EPA were found to be selectively toxic to the tumor cells but not to the normal cells. It was also observed that anti-oxidants such as vitamin E, butylated hydroxyanisole, and butylated hydroxytoluene can completely block the tumoricidal action of GLA, AA, and EPA where as both cyclo-oxygenase and lipoxygenase inhibitors were without any action. GLA, AA, and EPA could augment free radical generation and lipid peroxidation process in the tumor cells but not in the normal cells. These results suggest that GLA, AA, and EPA have selective tumoricidal actions and that their actions are mediated by free radicals and increased formation of lipid peroxides.

CF 201 2,3,7,8 TETRACHLORODIBENZODIOXIN (TCDD) - A MODEL COMPOUND IN AN ALTERNATIVE TREATMENT OF ESTROGEN-DEPENDENT HUMAN BREAST CARCINOMAS, Herbert W. Dickerman, David C. Spink, David W. Lincoln, II, and John F. Gierthy, Wadsworth Labs., NYSDOH, Albany, NY 12201-0509.

In general, antiestrogen therapy is based on two strategies: receptor blockade and inhibition of 17β-estradiol (E_2) synthesis. A third strategy is enhancement of E_2 metabolism to less active compounds. TCDD, a ligand of the aryl hydrocarbon (Ah) receptor, provides a model compound for delineating effects of increased metabolism on the functions of E_2-regulated breast cancer cells. TCDD was recognized as an antiestrogen in MCF-7 cells by inducing an E_2-reversible suppression of tissue plasminogen activator activity without affecting E_2 receptors. Multicellular focus formation is the dominant proliferative effect in the postconfluent phase of MCF-7 growth and is inhibited by TCDD. Focus formation is an estrogen-dependent phenomenon blocked by more classical inhibitors, Tamoxifen and LY156758, as well as TCDD. TCDD induces aryl hydrocarbon hydroxylase (AHH) in MCF-7 cells but not in estrogen-independent MDA-MB231 cells, a result expanded to other ER positive and negative cell lines (Vickers et al., Mol. Endocrinol. 3:157, 1989). The involvement of increased E_2 metabolism was suggested by release of ^3H from 2^3H-E_2 and $16\alpha^3H$-E_2 to media after TCDD treatment of MCF-7 cells. Now, in a direct measurement of the effect of TCDD on E_2 metabolism of MCF-7 cells, we find: 1) after a 24 hr exposure to untreated cells, more than 60% of E_2 initially present at 10^{-8}M was recovered from the medium as free E_2, free estrone (E_1) and conjugates of E_2 and E_1. Less than 4% of the E_2 initially present could be recovered as free or conjugated E_2 or E_1 if the cells were treated with TCDD; 2) Microsomal preparations from TCDD-treated cells showed a marked elevation of AHH and concomitant elevation of multisite hydroxylation of E_2; 3) In TCDD-treated cells, hydroxylations of E_2 occurred at the C2, 4, 6α and 15α positions with a total velocity of 23 pmol/min/mg microsomal protein. No hydroxylated derivatives were found in untreated cells. These results suggest that TCDD, by induction of endogenous microsomal hydroxylases, causes an extensive alteration in E_2 metabolism within breast epithelial cells such that E_2 is converted to less active products, relaxing estrogen control of the treated cells.

CF 202 PRESENCE OF ESTROGEN-REGULATED pS2-PROTEIN IN PRIMARY BREAST TUMORS STRONGLY PREDICTS LATE RECURRENCE AND LONG SURVIVAL. J.A. Foekens[1], M-C. Rio[2], P. Seguin[3], W.L.J. van Putten[1], J. Fauque[3], M. Nap[4], J.G.M. Klijn[1] and P. Chambon[2]. Dr. Daniel den Hoed Cancer Center[1], Rotterdam, and Laboratory for Public Health[4], Leeuwarden, The Netherlands, and Institut de Chimie Biologique[2], Faculté de Médecine, Strasbourg, and CIS Bioindustries[3], Bagnols/Cèze Cédex, France.

We have quantitatively assessed the cytosolic levels of estrogen-regulated pS2 protein in a series of 205 breast cancer patients (median follow up: 47 months). With isotonic regression analysis and using length of relapse free survival (RFS) and overall survival (OS) as endpoints 11 ng pS2/mg protein was found as the best cut-off level to discriminate between $pS2^+$ and $pS2^-$. Patients with $pS2^-$ tumors showed significantly shorter RFS and OS ($p<0.0001$) than patients with $pS2^+$ tumors. Also after adjustment for tumor size, lymph node status and ER status, pS2-negativity was associated with earlier recurrence and death. Tumors positive for pS2 (22%) were almost exclusively confined to the subclass of ER^+ tumors (53/55, 96%). The death rate for patients with $pS2^+$ tumors was one tenth of the death rate for patients with $pS2^-/ER^-$ tumors. In the patients with ER^+ tumors, the prognostic power of the pS2 status was especially present in patients whose tumors were also positive for PgR (5-year RFS and OS: 85% and 97% for $ER^+/PgR^+/pS2^+$ tumors, compared to 50% and 54% for the patients with $ER^+/PgR^+/pS2^-$ tumors). In patients with axillary lymph node involvement (N^+), pS2 status could discriminate strongly between between a good and bad prognosis group (5-year RFS and OS: 65% and 88% for $N^+/pS2^+$, compared to 32% and 34% for $N^+/pS2^-$). A similar phenomenon was observed in patients without axillary lymph node involvement (5-year RFS and OS: 89% and 95% for $N^0/pS2^+$, compared to 58% and 82% for $N^0/pS2^-$).

We conclude that the pS2 status of primary breast tumors can identify patients at high risk for recurrence and death. Knowlegde of the pS2 status appeared of particular importance to identify patients at high risk in the ER^+/PgR^+ subclass of tumors, and in both the N^0 and N^+ subclasses of patients.

CF 203 MONOCLONAL ANTIBODIES TO c-erbB-2 ENHANCE THE CYTOTOXICITY OF CISPLATIN AGAINST HUMAN BREAST AND OVARIAN TUMOR CELL LINES, Miriam C. Hancock, Anthony W. Chan, Ronald P. Mischak, Pamela L. Toy, Sarah I. Fried, Beatrice C. Langton and John J. Monahan, Triton Biosciences Inc., Alameda, CA 94501.

Monoclonal antibodies specific to extracellular epitopes of the c-erbB-2 protein (gp185) were tested for the ability to inhibit in vitro proliferation of human tumor cell lines overexpressing gp185. Several antibodies inhibited cellular growth in a dose-dependent manner, ranging from no effect at 200 pg/ml to 50% inhibition at 100 μg/ml. The effects were reversed when antibody was removed. Cell lines not expressing gp185 were unaffected by the monoclonals. Treatment of cells with combinations of cisplatin and one of the monoclonal antibodies (TAb 250) resulted in a significantly enhanced cytotoxic effect that was irreversible. This synergistic cytotoxicity was apparent over the full range of antibody concentrations (200 pg/ml - 100 μg/ml), and was optimal when cisplatin alone gave 30-50% inhibition. Under these conditions, the combined treatment resulted in 80-100% cytotoxicity. Time course experiments suggested that effects of the combined treatment occurred within the first 24 hours of antibody/drug exposure. An analogous cytotoxic effect was observed with tumor xenografts in vivo. These data suggest that combinations of c-erbB-2 antibodies and cisplatin may have significant therapeutic potential.

CF 204 DETECTION AND QUANTITATION OF AN ANTIGEN SHED IN VIVO AND IN VITRO BY CELLS AND HUMAN BREAST AND OVARIAN TUMORS OVEREXPRESSING c-erbB-2, Beatrice C. Langton[1], James E. Jackson[1], Mary C. Crenshaw[1], Lorrine A. Chao[1], Susan G. Stuart[1], Robert W. Akita[1], Allison W. Gannon[1], Miriam C. Hancock[1], Avrum Z. Bluming[2] and Dennis J. Slamon[3]. [1]Triton Biosciences Inc., Alameda, CA 94501, [2]Encino, CA 91436, [3]UCLA, Los Angeles, CA 90024.

A panel of monoclonal antibodies (TAbs 250-265) reactive with the extracelluar domain of c-erbB-2 (HER-2/neu) was used to develop immunoradiometric assays (IRMA) specific for the c-erbB-2 protein (gp185) as well as a purified recombinant extracellular domain (gp75). The IRMA assays detected positive signals from lysates and supernatants of NIH3T3 cells transfected with c-erbB-2 cDNA ($NIH3T3_t$) and human breast and ovarian tumor cell lines overexpressing c-erbB-2. Lysates and supernatants from untransfected NIH3T3 cells, breast cell lines not expressing c-erbB-2, and EGF-receptor-overexpressing cell lines were negative in the assay. When grown as tumors in nude mice, c-erbB-2 overexpressing cell lines shed an antigen into serum which could be quantified in the TAb 259/256 IRMA. The levels ranged from 8->1000 ng/ml gp75 equivalents. Antigen was not detected in sera from animals with c-erbB-2 negative tumors. Treatment of $NIH3T3_t$-tumors with TAbs 250 or 252 resulted in up to 60% inhibition of tumor growth and depressed levels of shed antigen. A panel of sera from breast and ovarian cancer patients was also tested and 6-20% of the samples were positive. One breast cancer patient from whom both tissue and serum was available, gave strong positive tissue membrane staining with TAb 250 and had a serum shed antigen level of 490 ng/ml gp75 equivalents. Several human ovarian samples were also positive for both tissue staining and levels of shed antigen in matched sera.

CF 205 MOLECULAR CLONING, SEQUENCING AND HIGH LEVEL EXPRESSION IN E. COLI OF cDNAs ENCODING DIFFERENTATION ANTIGENS OF BREAST EPITHELIAL CELLS AND HUMAN MILK FAT GLOBULE, David Larocca, Roberto L. Ceriani, Gary Walkup, Ramon Urrea, Antonio M. Bistrain and Jerry A. Peterson, John Muir Cancer and Aging Research Institute, 2055 No. Broadway, Walnut Creek, CA 94596. The human milk fat globule (HMFG) membrane contains several glycoproteins, referred to as breast differentation antigens, that are found in normal breast, breast carcinomas, and breast cancer patient serum. We have used MoAbs which bind the high M.W. mucin and several smaller components of HMFG including 150 kDa, 70 kDa and 46 kDa glycoproteins, to select cDNA clones from 2 human breast λgt11 libraries. Northern blot analysis showed an epithelial specificity for the 70 kDa and 46 kDa protein mRNAs (1.8 and 2.2 kb, respectively) and breast specificity for a mucin specific mRNA. The relative abundance of each mRNA, varied among the cell lines examined by as much as 50-100 fold. Partial sequencing of the cDNAs revealed no extensive homology with published sequences. High level expression in E. Coli using pEX2 resulted in recombinant proteins that are being used to develop assays for breast membrane components in breast cancer patients and for generating additional polyclonal and monoclonal sera for improved breast cancer therapy and diagnosis. Supported by NIH Grants CA39932, CA42767 and RR05929.

CF 206 THE PROGNOSTIC SIGNIFICANCE OF TRANSFORMING GROWTH FACTOR EXPRESSION IN HUMAN BREAST CANCER, Philip A. Murray, Peter Barrett-Lee, Maureen Travers, Yunus Luqmani, Nicki Payne, Trevor Powles, R. Charles Coombes, Medical Oncology Department, St George's Hospital Medical School, Cranmer Terrace, London SW17 ORE and The Medical Breast Unit, The Royal Marsden Hospital, Sutton, Surrey, U.K.

There is a need for better prognostic indicators in breast cancer to facilitate the selection of patients who require more aggressive primary therapy. Both TGF-alpha and TGF-beta are thought to be involved in the control of breast cancer proliferation *in vivo*. We have been investigating whether the levels of TGF-alpha and TGF-beta mRNA transcripts in biopsies from patients with non-metastatic primary breast cancer correlate with subsequent relapse and survival. A preliminary analysis of 66 patients after a median follow up of 42 months revealed no relationship between TGF-alpha or EGF-R transcripts and either relapse free survival or survival. The level of TGF-beta transcript was inversely related to the presence of regional node metastases at primary surgery ($p = 0.01$) and high levels of TGF-beta were associated with a longer relapse free survival although this did not reach statistical significance. This study is currently being extended to a total of 140 patients with longer follow up (median 75 months) and the data will be presented.

CF 207 CHARACTERIZATION OF MULTIPLE FORMS OF PROTEIN TYROSINE KINASE FROM BREAST CANCER USING A NON-RADIOACTIVE DOT-BLOT ASSAY.

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Cytosolic protein tyrosine kinase (PTK) activity may be used as a diagnostic and prognostic factor in breast cancer: in cancers that caused systemic disease PTK activity was significantly increased compared to non-disseminating breast cancer (A. Hennipman et al., Cancer Res. 49,1989,516-521). The cytosolic PTK activity of malignant tumors appeared to originate from the presence of at least two different forms as was shown by fast protein liquid chromatography (FPLC). In normal breast only one of these activity peaks could be detected. The "tumor-specific" activity increased in correlation with the total cytosolic PTK activity and thus may play a role in the discrimination of carcinomas that cause systemic disease and those that do not. Kinetical characterization revealed differences in affinities both for the peptide- and nucleotide substrate. Immunoblot experiments showed the copurification of material crossreacting with anti-EGFreceptor and anti-neu polyclonal antibodies with the two respective PTK activity peaks. However, in experiments using the EGFreceptor kinase inhibitor tyrphostin RG 50863-2 (P. Yaish et al., Science 242,1988,933-935) no inhibition of either form of PTK could be demonstrated.

Determination of PTK activity was performed using a new dot-blot assay based on the detection of phosphorylated tyrosyl residues with monoclonal antibodies to phosphotyrosine.

CF 208 THE EFFECT OF FRACTIONATED IRRADIATION ON THE EXPRESSION OF ESTROGEN RECEPTOR OF MCF-7 CELLS, R. Schmidt-Ullrich, W. Chan, K. Valerie, D. Wazer, and P. S. Lin, Department of Radiation Oncology, Medical College of Virginia/VCU, Richmond, VA 23298.

MCF-7 human breast carcinoma cells, passage 50, were exposed in vitro to daily 2 Gy fractions to total doses of 60 Gy. Control MCF-7 cells were compared to cells exposed to 20 Gy, MCF-7-IR, with respect to their proliferate rates, radiosensitivities in the absence or presence of estrogens/antiestrogens. MCF-7-IR exhibit a significantly increased proliferative rate but do not differ from MCF-7 in their radiosensitivity. As we find a range of proliferative rates in cloned MCF-7 cells we may select for survival of rapidly proliferating clones. The growth inhibitory response to tamoxifen, 0.1 - 5 uM, is markely reduced in MCF-7-IR relative to control MCF-7 cells. This correlates with the reduced expression of estrogen receptor (ER). Our data indicate that fractionated irradiation of MCF-7 reduces the expression of ER or selectively kills ER-positive cells. Based on our results on cloned MCF-7 the first possibility is more likely and is being confirmed by the quantitation of ER mRNA levels from MCF-7 clones of different proliferative rates and from MCF-7-IR.

CF 209 PROTEIN TYROSINE KINASE ACTIVITIES IN BENIGN AND MALIGNANT BREAST TISSUES, A POSSIBLE ROLE AS PROGNOSTIC MARKER.

G.E.J. Staal, A. Hennipman, J.Smits, B.A. van Oirschot, G. Rijksen, Dept. Haematology, Lab. Medical Enzymology, Surgical Department, Dept. Pathology, University Hospital Utrecht, P.O. Box 3508 GA Utrecht, The Netherlands.

A number of oncogene products and growth factor receptors with protein tyrosine kinase activity (PTK) have been identified in human breast cancers, some of which have possible relevance for diagnosis and prognosis. We studied the total PTK activity of cytosolic and membrane fractions of the homogenates of cancers of the breast, benign breast diseases and normal breast tissues. PTK activity was determined using poly (glutaminic acid:tyrosine=4:1) as an artificial substrate. Breast cancers demonstrated considerable higher PTK activities in cytosol and in membrane fractions compared to benign disease and normal tissues ($p = <0.001$).

Cytosolic PTK activity may have prognostic significance. Of a consecutive series of 18 patients with operable breast cancer, 6 relapsed during a median follow-up of 17.5 months, 1/6 showed PTK activity in cytosol less than or equal to the mean activity for the total number of carcinomas, while 5/6 had activities higher than the mean. In a second series of 86 patients we studied PTK-activities in cytosolic and membrane fractions of cancers of the breast in relation to accepted clinical parameters of prognosis. We observed weak, positive correlations of cytosolic PTK activity with the number of tumor positive axillary nodes and histological grade. A weak negative correlation was noted of cytosolic PTK activity with the estrogen receptor content.

CF 210 S-PHASE ANALYSIS USING A BROMODEOXYURIDINE (BRDURD) LABELING INDEX (LI) METHOD: COMPARISON WITH %S-PHASE DETERMINED BY FLOW CYTOMETRY (FC), Thomas E. Witzig, Nick J. Gonchoroff, Mary J. Stenson, Jerry A Katzmann, Clive S. Grant, Les E. Wold, James N. Ingle, Departments of Hematology,Lab Medicine and Oncology, Mayo Clinic, Rochester, MN 55905. We developed a BrdUrd LI method and used the LI to validate the %S determined by FC. Fresh tissue samples from 28 cases of breast cancer were incubated in BrdUrd, then paraffin-embedded. The slides were stained with anti-BrdUrd monoclonal antibody (IU-4) and bound IU-4 was detected by an anti-mouse gold-conjugated second antibody with silver enhancement. The BrdUrd LI was determined by counting the number of BrdUrd + cells/1000 tumor cells. In addition, a parallel, non-pulsed, paraffin-embedded tissue sample was analyzed for DNA FC. Percentage S-phase was determined from the histograms by use of both manual and rectangular models. Correlations of the BrdUrd LI and models of FC %S analysis with respect to high (>8.0% FC; >5.5% LI) vs low proliferative rate are presented below.

PLOIDY	LI vs Manual (% Agreement)	LI vs Rectangular (% Agreement)
DNA Diploid n= 9	78%	78%
DNA Tetraploid n= 8	63%	50%
DNA Aneuploid n= 11	82%	55%

We found the BrdUrd method to be relative simple from a technical standpoint. We conclude that in this small series the level of agreement of both FC models was similar in the DNA diploid cases; however, in the DNA aneuploid cases the manual method appeared better than the rectangular. Further work is needed to optimize methods of obtaining %S from FC histograms. (Supported in part by Mayo Clinic/Foundation and NCI CA 15083.)

CF 211 REGULATION BY EXTRACELLULAR MATRIX AND TGF-β OF A GENE PRODUCT (NB-1) WHICH IS LOST DURING IMMORTAL AND TUMORIGENIC TRANSFORMATION OF HUMAN MAMMARY EPITHELIAL CELLS. Paul Yaswen, Donna Peehl* and Martha Stampfer, Division of Cell and Molecular Biology, Lawrence Berkeley Laboratory, Berkeley, CA 94720 and Dept. of Urology*, Stanford University School of Medicine, Palo Alto, CA 94305

NB-1 was originally identified by subtractive hybridization as a gene whose transcripts are significantly downregulated in tumorigenic human mammary epithilial cells (HMEC) with respect to closely related normal HMEC. Expression of NB-1 has only been detected, thus far, in epithelial cells cultured from breast and prostate. Total RNA samples from normal and primary tumor tissues fail to demonstrate the same high level of NB-1 expression exhibited in primary cultures and successive passages of normal HMEC. The organ specificity observed, combined with the lack of detectable expression of NB-1 in primary tissue, suggest that this gene might be involved in differentiation or growth control of particular glandular organs. In order to understand better the observed differences in NB-1 expression in vivo and in vitro, we have begun to try to identify both soluble and insoluble factors which may modulate the expression of this gene. We have found that TGF-β treatment is capable of increasing the accumulation of NB-1 mRNA in normal HMEC and in some transformed HMEC. In contrast, exposure to reconstituted extracellular matrix from the EHS tumor causes downmodulation of NB-1 mRNA in a cell density dependent manner in normal HMEC and in immortalized HMEC which express it. The downregulation in transformed HMEC grown on plastic of the NB-1 gene could reflect variations in the differentiated states of the normal and transformed cells. The differences in expression could also represent a divorce of transformed cells from constraints normally imposed by cellular shape and contacts.

Progression and Metastases

CF 300 MULTIPLE SERUM MARKERS IN MONITORING RESPONSE TO HIGH-DOSE INTENSITY DOXORUBICIN THERAPY OF ADVANCED BREAST CANCER. E Anderson, *MH Bronchud, JE Turnbull, L Jones and *A Howell, Depts of Clinical Research and *Medical Oncology, Christie Hospital, Manchester M20 9BX, UK.

Carcinoembryonic antigen (CEA), mucin-like carcinoma-associated antigen (MCA) and CA 15.3 were measured in serum samples taken serially from 14 patients before, during and after high dose intensity doxorubicin therapy for advanced breast cancer (Bronchud et al, Br J Cancer, 1989,60,121). Of the 11 patients who responded to treatment, 9 had raised pre-treatment levels of at least one marker and in 8/9, these levels fell to normal or by >50%. Two of the five complete responders had increased levels of only one marker. In the partial responders, 4/6 had raised levels of only 2 markers which fell to normal in 3 patients. In one patient, who had a good partial response, pre-treatment marker levels were not raised but increased upon progression. The rate of fall in marker levels was slower in the partial responders (median times (days) taken for a 50% decrease were CA15.3:48d; MCA:47d; CEA:70d) than in the complete responders (21d, 26d and 13d respectively). These results indicate that not all markers are raised in all patients with advanced breast cancer and a panel of markers may have to be assayed in order to follow response to treatment. The results also indicate that raised pre-treatment marker levels may not be necessary for monitoring the course of the disease. Finally, since the complete responses were seen in the patients receiving the higher doses of doxorubicin, the faster rates of fall in marker levels may reflect a higher cell kill per unit time at the higher dose intensities.

CF 301 A NOVEL GROWTH INHIBITOR SECRETED BY HORMONE-INDEPENDENT HUMAN BREAST CANCER CELLS AFFECTING HORMONE-DEPENDENT CELLS, Willy Kung, Max Hasmann, Roland Loser and Urs Eppenberger, Department of Research, Kantonsspital Basel, CH-4031 Basel, Switzerland and Klinge Pharma, D-8000 Munich, FRG.

Mutual testing of conditioned serum free media and coculturing of hormone-dependent (estradiol-responsive) human breast tumor cell lines with hormone-independent (nonresponsive to estradiol) cell lines reveiled that fast growing hormone-independent cells release a factor which strongly affects the morphology and growth of hormone-dependent cells such as MCF-7, ZR-75-1, ZR-75-30, T-47D and MDA-MB-361. Induction of membrane ruffling is already observed after a few minutes and the cells, which grow in tightly clustered groups, start to dissociate and migrate within an hour. Cell cycle analysis shows that the factor is a G_1-specific growth inhibitor which neutralizes the stimulatory effects of mitogens such as estradiol, EGF or IGF-I. This factor has an estimated molecular ratio of 55,000, is relatively heat and acid stable but very sensitive to trypsin and dithiothreitol treatment. This growth inhibitor is not identical with already described scatter and motility factors. Because its presence can also be shown in human mammary tumor tissue, this factor could be involved in late tumor development through elimination of hormone-dependent tumor cells by the more aggressive hormone-independent cells. Thus high tissue levels may correlate with poor prognosis. Supported by the Swiss Cancer League.

CF 302 DESIGN OF SYNTHETIC PEPTIDE FOR BREAST CANCER, Ian F C McKenzie, Pei-Xiang Xing, Kerry Reynolds, Geoff Pietersz and P. Mark Hogarth, Research Centre for Cancer and Transplantation, Department of Pathology, University of Melbourne, Grattan Street, Parkville, Victoria, 3052, Australia.

Three mAbs BC1 (IgG3), BC2 (IgG1) and BC3 (IgM) to human milk fat globules (HMFG) are known to recognize breast carcinoma associated antigens. The antibodies react with the synthetic peptide p1-24 PDTRPAPGSTAPPAHGVTSAPDTR representing amino acid sequence correspondence to partial cDNA sequence of the protein core of HMFG mucin. We have recently shown that these antibodies all react with the sequence APDTR. Several variations of this peptide were examined to determine the optimum reactivity. Firstly, several peptides were synthesised with single amino acid variations in APDTR; APDTR was found to be the major reacting sequence. Secondly, Polyethylene rods were used to make all 20 of the amino acid substitutions in each position (= 100 peptides); there were some "permissible" substitutions in A, D and T but not in P or R for BC1 and BC2; there were more "permissible" substitutions in all of the 5 amino acids for BC3. It was also noted that different substitution patterns occurred with the 3 antibodies which, therefore, can be considered to bind to different epitopes. Thirdly, the position of APDTR in the 20-mer was also examined - greater reactivity was obtained when APDTR was in the "middle" of the 20-mer peptide than at the end, and furthermore, the addition of cysteine (C) gave greater reactivity both observations suggesting that conformation of the peptide was important for antibody reactivity. The studies will be of value for the rational design of peptides for use in immunising against breast cancer, although thus far, we find that the peptides (as opposed to HMFG) have only a limited capacity to induce antibodies or T cell immunity.

CF 303 FLOW CYTOMETRY ANALYSIS OF SURFACE AND CYTOPLASMIC EXPRESSION OF DIFFERENT EPITOPES OF A POLYMORPHIC MUCIN IN BREAST CARCINOMA CELLS IN VITRO AND IN VIVO. Jerry A. Peterson, Edward W. Blank, Michael Wong, and Roberto L. Ceriani. John Muir Cancer and Aging Research Institute, 2055 N. Broadway, Walnut Creek, CA 94596.
The expression of four different epitopes, identified by different MoAbs (Mc5, BrE1, BrE2, BrE3), on a large molecular weight mucin was analyzed by flow cytometry in 10 different breast carcinomas grown in culture and as tumors in nude mice. Mc5 recognizes an epitope usually on the cell surface, while the other three MoAbs identify epitopes predominantly in the cytoplasm. There was considerable heterogeneity among the different tumors and within each tumor, both in antigen content and in the intracellular distribution. Some tumors exhibited similar epitope distributions in vitro and in vivo, some showed increased antigen expression in vivo, while still for others there was inhibition of expression in vivo. Estrogen and progesterone receptor expression was correlated with greater antigen expression and an epitope distribution closer to that of normal breast epithelial cells cultured from a reduction mammoplasties. These results will be discussed with regard to the polymorphic nature of this mucin complex and to its altered processing associated with in vitro culture and with malignancy.
Supported by NIH Grant No. CA39932, CA42767 and RR05929.

CF 304 CLONAL SELECTION IN THE GROWTH AND METASTASIS OF A HUMAN BREAST CARCINOMA CELL LINE (MDA-MB-435) IN NUDE MICE. Janet E. Price, Barbara Hunt, and Philip Frost. Department of Cell Biology, U.T. M. D. Anderson Cancer Center, Houston, Tx. 77030.
The human breast carcinoma cell line MDA-MB-435 is tumorigenic in the mammary fatpad (m.f.p.) of nude mice and metastasizes to distant organs (primarily lungs and lymphnodes). The cell line was transfected with pSV2neo using a scrape-loading procedure, and 25 G418-resistant clones isolated. Eleven of these clones were identified as having a unique insertion site of the pSV2neo gene. A mixture of equal proportions of the eleven clones was injected into the m.f.p.s of nude mice. DNA extracted from the resulting tumors was subjected to Southern blot analysis and probed for pSV2neo sequences. In tumors removed after 8 to 12 weeks, the banding pattern characteristic of only one of the pSV2neo-labelled clone, clone 24, was identified. DNA from metastases in lungs and lymph nodes from several animals also showed the banding pattern of clone 24. Measurements of growth rates of the eleven individual clones showed no differences in vitro, and clonal selection was not found in the mixed population of pSV2neo-labelled clones grown in tissue culture for 12 weeks. All of the clones were tumorigenic and metastatic to the lungs of the nude mice, thus confirming that the transfection procedure had not altered the malignant phenotype of the MDA-MB-435 cells. However, clone 24 cells produced the largest tumors in the m.f.p. of nude mice compared with the 10 other pSV2neo-labelled clones. Thus one explanation for the observed clonal dominance or selection in this experiment could be the more rapid growth of the clone 24 cells in vivo. This study illustrates how an introduced selectable gene can be used in lineage studies of human tumor cell populations.

CF 305 ROLE AND SUBCELLULAR LOCALIZATION OF PROTEINASE CATHEPSIN B IN INVASIVE AND NON-INVASIVE HUMAN BLADDER TUMORS: A POTENTIAL MODEL FOR TUMOR INVASION. S. Mark Redwood, Robert E. Weiss, and Brian C.-S. Liu. Dept. of Urology, Mount Sinai School of Medicine, New York, NY 10029.

To study the biochemical mechanisms of human tumor invasion, we analyzed specimens of invasive transitional cell carcinoma cell line EJ and non-invasive transitional cell carcinoma cell line RT4 which had been implanted into the bladders of nude mice.

Subcellular fractionation followed by immunoblot analysis and enzymatic analysis demonstrated that invasive EJ cells had active cathepsin B localized to its plasma membrane, while non-invasive RT4 cells had cathepsin B confined to lysosomes. Furthermore, in vitro degradation assays with plasma membrane fractions isolated from invasive EJ cells and non-invasive RT4 cells domonstrated that the plasma membrane of EJ cells but not that of RT4 cells had the ability to degrade purified laminin, and that the degradative products were similar to those obtained with purified cathepsin B.

Using cysteine proteinase inhibitors, we demonstrated that we can abolish the degradation of purified human laminin by plasma membrane fraction of invasive EJ cells. If so, inhibitors to cysteine proteinases could potentially limit the invasive potential of human bladder cancer and other human cancers which require the invasive phenotype prior to the development of metastatic disease.