Meeting Report

Experimental Models of Prostate Cancer Research

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The Pathology B study section sponsored a one-day workshop focusing on new experimental models of prostate cancer research and preceded the International Conference on Prostate Cancer Research in Iowa City, Iowa. The workshop began with a discussion of the integrin-mediated modulation of prostate cancer proliferation and motility by Lucia R. Languino (Yale University, New Haven, CT). Interactions between (cancer) cells and the extracellular matrix are largely mediated by integrins, which have also emerged as key regulators of cell proliferation, migration, and intracellular signaling. β_{1C} and β_3 integrins may act as "growth or motility modulators" in prostate cells and play a role in modulating downstream intracellular signaling events. The β_{1C} integrin is expressed in nonproliferative, differentiated benign prostatic epithelium and is down-regulated in prostatic adenocarcinoma, as well as in hyperplastic prostate glands. The β_{1C} integrin is an alternatively spliced variant of the β_{1A} subunit that, in contrast to β_{1A} , inhibits fibroblast and epithelial cell proliferation. To investigate whether β_{1C} plays a role in prostate epithelial cell proliferation, the prostatic carcinoma cell line PC3 was transfected with an inducible system with either β_{1C} or β_{1A} cytoplasmic tails and normal epithelial cell transfectants expressing β_{1C} . In contrast to β_{1A} , expression of β_{1C} or its cytoplasmic domain completely inhibited thymidine incorporation by serum stimulation. Further results point to β_{1C} as an upstream regulator of the cell cycle inhibitor p27^{kip1} expression. Immunohistochemical and immunoblotting analysis of human prostate epithelial cells reveal that β_{1C} is co-expressed with p27^{kip1}, the loss of which correlates with poor prognosis in prostate cancer. *In vitro*, increased levels of p27^{kip1} and inhibition of cyclin A-dependent kinase activity were observed in normal prostate epithelial cells upon expression of β_{1C} . These data show that p27^{kip1} is a key downstream effector of β_{1C} , in that β_{1C} inhibitory activity on cell proliferation is completely pre-

vented by p27^{kip1} antisense (but not mismatch) oligonucleotides. In parallel studies, a role for the $\alpha v \beta 3$ integrin in the regulation of prostate cancer cell functions has been identified. Specifically, $\alpha v \beta 3$ is expressed in primary cultures of prostate cancer epithelial cells, whereas it is undetectable in normal prostate epithelial cells; and $\alpha v \beta 3$ mediates prostate cancer epithelial cell migration on β_3 integrin substrates, such as vitronectin, an $\alpha v \beta 3$ ligand expressed in mature bone where prostate cancer cells preferentially metastasize. Exogenous expression of $\alpha v \beta 3$ induces LNCaP cells to adhere to and migrate on vitronectin. In response to $\alpha v \beta 3$ engagement, increased tyrosine phosphorylation of focal adhesion kinase (FAK), a signaling molecule activated by integrins and able to modulate cell migration, is detected. Transfection of FAK-related non-kinase (FRNK), known to compete with FAK for its correct localization and phosphorylation, causes inhibition of β_3 -LNCaP cell migration, specifically on vitronectin. The study of the pathophysiological relevance of β_{1C} and β_3 integrin downstream effectors is likely to yield new insights into the mechanisms that contribute to prostate cancer progression and metastatic spread.

Jack Schalken (University of Nijmegen, Nijmegen, The Netherlands) then spoke about E-cadherin as a prognostic parameter. Prostate cancer, like many solid tumors, is characterized by an unpredictable biological behavior. Some tumors remain indolent for many years, whereas others progress rapidly to a life-threatening disease. Clearly, the acquisition of a metastatic phenotype is a hallmark of clinical aggressiveness that often leads to an incurable disease. It is now well recognized that in the maintenance of epithelial integrity the calcium-dependent adhesion molecule E-cadherin plays a crucial role and that it can function as a suppressor of invasive ability. An ongoing prospective clinical trial (Biomed II MPC project) is underway to establish the clinical usefulness of E-cadherin immunohistochemistry as molecular marker for prostate cancer prognosis. The mechanism by which E-cadherin function is impaired is not yet fully resolved, although transcriptional down-regulation and concomitant up-regulation of other cadherins, particularly N-cad-

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herin and cadherin-11, appear to be common steps in the malignant progression of prostate cancer. It is important to note that this implies that the mechanisms might be reversible and that cadherins can be considered not only as molecular markers for prostate cancer prognosis but also as targets for therapy. Considering these observations, up-regulation of E-cadherin would result in prevention of progression of metastatic disease. This in fact provides a rational basis for differentiation therapy and the use of E-cadherin to evaluate and/or target new therapeutic modalities.

Gary J. Miller (University of Colorado, Denver, CO) presented some of his work on Vitamin D in prostate cancer. Although it is usually thought of as an androgen-dependent disease, it has recently become clear that numerous other hormones including 1,25-dihydroxyvitamin D₃ (1,25D₃) can regulate its growth and differentiation. In addition to its role in calcium homeostasis, 1,25D₃ is also known to play pleiotropic roles in modulating the differentiation of various benign and malignant cells. Epidemiological data exist indicating that mortality from prostate cancer is inversely related to latitude and ultraviolet exposure. Prostate cancer incidence has also been tied to prediagnostic serum levels and vitamin D receptor polymorphisms. Specific receptors for 1,25D₃ exist in all of the prostatic carcinoma cell lines examined to date. Furthermore, these receptors regulate both the antiproliferative and differentiating effects of 1,25D₃ in various prostatic cancer cell lines. For example, 1,25D₃ increases the production of prostate-specific antigen (PSA) and prostate-specific acid phosphatase in LNCaP while inhibiting their growth. Numerous nonhypercalcemic analogues of 1,25D₃ have been synthesized that retain the differentiating effects (increasing the expression of prostate-specific antigen and prostate-specific acid phosphatase) of the parent compound. Those with hexafluorinated side chains have been found to enhance activity in prostatic carcinoma cells. Recent data also indicate that aging individuals, especially those with advanced stage prostate cancer, may have profound deficiencies of 1,25D₃ even in the presence of dietary supplementation. A hypothesis was presented which suggested that the lack of response to chemotherapeutic agents may be related to vitamin D deficiency. Isobologram analyses of *in vitro* studies have revealed that 1,25D₃ has a synergistic effect on the antiproliferative effects of cis- and carboplatin. A clinical trial is underway to determine whether such combined effects will also occur in patients. Finally, questions remain regarding the molecular signal transduction pathways that operate in prostatic cancer cells exposed to vitamin D. Of the cell cycle-related regulators, p21 is known to have a vitamin D response element. Ongoing studies indicate that the expression of p21 is up-regulated in prostatic cancer cells exposed to 1,25D₃ and that the degree of this change is correlated with the observed antiproliferative effects. Regarding differentiation, Miller has also examined the HOX transcription factors and found that selected members are inappropriately expressed in prostatic carcinoma cells. In addition, preliminary results indicate that the expression of individual HOX genes, such as HOXA10, is regulated by 1,25D₃ in

prostatic carcinoma cells. In summary, a substantial body of evidence exists indicating that 1,25D₃ is important in modulating the malignant phenotype of prostatic carcinoma cells. In view of this, we should no longer think of prostatic carcinoma solely in terms of its response to androgens.

The role of early growth response genes in prostate cancer was discussed by Donald J. Tindall, (Mayo Foundation, Rochester, MN). Using a differential display reverse transcriptase-polymerase chain reaction technique, a number of androgen-regulated genes that are differentially expressed in prostatic carcinoma cell lines were isolated. A novel gene, *EGR-α* (closely related to *TIEG*), is expressed to a higher extent in the androgen-independent cell lines DU-145 and PC3 but not in the androgen-sensitive cell line LNCaP. These observations led to a more detailed study of EGR-1 expression in prostate cancer. *In vitro*, EGR-1 expression increases concomitantly with an increase in the index of malignancy. These results were further corroborated with immunocytochemistry, which indicated that EGR-1 is expressed primarily in the basal epithelial cells and detected primarily in the cytoplasm of normal and benign epithelial cells, whereas it is predominantly nuclear in malignant cells. To further corroborate the subcellular localization of EGR-1 in cancer *versus* noncancerous prostate cells, immunocytochemistry on two human prostate cell lines from benign and cancerous origin was performed. EGR-1 protein is primarily localized in the cytoplasm of BPH-1 cells and in the nuclei (mostly in the nucleolus) of PC-3 cells. Interestingly, in all dividing cells EGR-1 is preferentially associated with the mitotic spindle. Further studies have focused on the effect of calcium on EGR-1 expression. LNCaP was maintained in DCC-FBS medium for 24 hours and then treated with 10⁻⁷ M thapsigargin and EGR-1 levels reached a maximum of fourfold induction between 40 and 60 minutes of treatment. This expression is blocked by staurosporine, suggesting a role of PKC in EGR-1 regulation in prostate cancer cells. In addition, the calmodulin inhibitor, trifluoroperazine, also suppressed the thapsigargin-inductive effect. These data suggest that a calmodulin-mediated pathway may be involved in the regulation of EGR-1 expression in prostate cancer cells. Moreover, since thapsigargin induces apoptosis in LNCaP, EGR-1 may also be involved in this process in prostate cancer cells. These findings have led to a working hypothesis that EGR-1, in an active nuclear form, is involved in the initiation and progression of prostate cancer.

In the afternoon there were several presentations about apoptosis in prostate cancer. Michael B. Cohen (The University of Iowa, Iowa City, IA) began with a review of his work on TNF receptor family-mediated apoptosis in prostate cancer. Of 6 human prostatic carcinoma cell lines examined by flow cytometric analysis, all were found to be positive for Fas (CD95) antigen. Furthermore, all of the prostate tissue specimens studied revealed Fas expression in benign and malignant epithelial cells. Agonistic anti-Fas monoclonal antibody induced apoptosis in only 2 of 6 cell lines investigated. Subsequent effort has focused on identifying the mechanism of resistance

to Fas-mediated apoptosis in prostate cancer, because treatment with the protein synthesis inhibitor cycloheximide (CHX) converted the phenotype of resistant cell lines from Fas resistant to Fas sensitive. Subsequently, Fas-mediated apoptosis in cell hybrids between resistant and sensitive cell lines was investigated. All three types of F1 hybrid cells investigated were found to be resistant to Fas-mediated apoptosis at the same level as the corresponding parental resistant cell lines. Furthermore, treatment with CHX converted the phenotype of the hybrids from resistant to sensitive. These results indicate that resistance to Fas-mediated apoptosis dominates over sensitivity in cell hybrids and suggest that an apoptosis suppressor factor or factors acting in resistant but not in sensitive cells may regulate resistance. Finally, they have also investigated the sequential activation of caspase family members, to gain insight into the likely site of action of the suppressor protein(s). In prostate cancer cell lines, caspase-8 activation is followed by caspase-7; caspase-3 does not appear to be involved. These results suggest that an inhibitory protein or proteins, which suppress apoptosis in Fas-resistant cell lines, presumably act at the apex of apoptotic cascade by preventing the activation of caspase-8. In addition, Fas ligation results in the release of cytochrome C and activation of caspase-9, and the timing suggests that this is an early event. Current studies are targeted at identifying the inhibitory protein(s) responsible for resistance to Fas-mediated apoptosis. Additional work has focused on the role of p53 in TNF- α -mediated apoptosis. LNCaP is a human prostatic carcinoma cell line that expresses wild-type p53 and is sensitive to TNF- α treatment. To analyze the role of p53 in TNF- α -mediated apoptosis, a LNCaP subline has been created, termed LN-56, that expresses GSE-56, a dominant-negative element of p53. p53 inactivation in LN-56 was associated with an increased resistance to apoptosis induced by TNF- α treatment. Caspase-7 activation and PARP proteolysis were delayed in LN-56, TNF- α treatment increased p53 in LNCaP, but not in LN-56, and resulted in up-regulation of p21/WAF1, which was accompanied by p21/WAF1 proteolysis in LNCaP, but not in LN-56; this proteolysis was inhibited by a pan-caspase inhibitor (Z-VAD-FMK). Interestingly, accumulation of p53 was decreased in the presence of Z-VAD-FMK, indicating a new role of activated caspases in acceleration of p53 response. Mdm2 as not found to be a target for caspase-mediated degradation. In summary, these results suggest that p53 plays an important role in TNF- α -mediated apoptosis.

Next, Martin Tenniswood (University of Notre Dame, South Bend, IN) spoke on the implications of the changes in biogenesis of clusterin during anti-androgen induced apoptosis, and put forth a very intriguing hypothesis. Glandular tissues, such as the prostate, regress when deprived of their trophic factors. Previous studies have shown that a number of genes involved in the destruction of the extracellular matrix and basement membrane are required for the apoptotic death of the epithelial cells that occurs during tissue regression. Metastatic cells express many of the same proteins. In cells that initiate the apoptotic pathway, extracellular matrix proteases are ex-

pressed and the DNA is fragmented. Metastatic cells, on the other hand, express elevated levels of the proteases but do not fragment their DNA. This has led to the hypothesis that the invasive phenotype might arise after the initiation of apoptosis by anticancer agents if the DNA in the affected cells was not fragmented appropriately. To test this hypothesis he has established the androgen-dependent LNCaP prostate cancer cell line in defined serum-free medium. Treatment of these cells with Casodex (bicalutimide), a pure nonsteroidal anti-androgen, or TNF- α , induced apoptosis. In addition, cognate mRNA levels and activity of several extracellular matrix proteases, including MMP-2 and MMP-9, are increased during Casodex induced apoptosis, and there is a corresponding decrease in the level of TIMP-1. Furthermore, Casodex induces a dose-dependent, statistically significant increase in the invasive potential of the surviving cells. He estimates that between 0.1 and 0.2% of the surviving cells acquire the ability to invade following anti-androgen treatment; this is not seen when the cells are treated with TNF- α . Although this represents a very small percentage of the surviving cells, these cells represent a clinically significant population, because it is these cells that have the potential to give rise to metastatic disease. Clonally selected invasive sublines, referred to as LNCaP^{Cas}inv sublines, have been developed and retain their invasive ability, suggesting that these cells may serve as a model of late stage, androgen-resistant metastatic disease.

Vivek M. Rangnekar (University of Kentucky, Lexington, KY) discussed the mechanism of prostate apoptosis response-4 (Par-4) gene-dependent apoptosis. The Par-4 gene was identified in a differential screen for genes induced during apoptosis of prostate cancer cells. Par-4 is widely expressed in the nucleus and evolutionarily conserved in vertebrates. Interestingly, Par-4 is exclusively induced in *in vitro* and *in vivo* paradigms of apoptosis, but not during necrosis, growth arrest, or growth stimulation. It is induced in the secretory epithelium of the involuting prostate in castrated rats, in myoblasts of the tadpole tail undergoing resorption during metamorphosis, and in the web area between the digits during limb bud development. It is also up-regulated in degenerating neurons of Alzheimer's disease patients. The deduced amino acid sequence of Par-4 predicts a protein with a leucine zipper sequence at its carboxy terminus. Functionally, Par-4 is necessary but not sufficient for apoptosis: inhibition of Par-4 with antisense oligomers or with a dominant-negative mutant results in abrogation of insult-driven apoptosis. Overexpression of Par-4 sensitizes cells to apoptosis. Interestingly, Par-4 expression is decreased in some but not all tumors. Moreover, oncogenes down-regulate Par-4, and replenishment of Par-4 prevents oncogene-induced cellular transformation. The mechanism of Par-4-mediated apoptosis is currently being studied with Bcl-2, NF- κ B, and ERK as potential downstream targets. For example, Par-4 and Bcl-2 expression are inversely correlated. Investigations are underway on Par-4 function in neuronal degeneration, ischemia/reperfusion-induced injury, and development, in addition to cancer paradigms.

Finally, Timothy C. Thompson (Baylor College of Medicine, Houston, TX) presented some of his work on caveolin-1, a gene involved in prostate cancer metastasis. The high level of mortality from prostate cancer results in large part from the inexorable growth of overt or occult metastasis present at the time of diagnosis. To better understand the metastatic phenotype in prostate cancer, a strategy to identify mRNAs that are expressed differentially in cell lines derived from primary *versus* metastatic mouse prostate cancer (MPR mouse prostate reconstitution model) using differential display-polymerase chain reaction has been developed. In using this system a number of metastasis-related sequences were identified, including a cDNA that encodes caveolin-1. Caveolin-1 was found to be overexpressed not only in metastatic mouse prostate cancer, but also in human metastatic disease. Recent studies have indicated that suppression of caveolin-1 expression induces androgen sensitivity in high caveolin, androgen-sensitive mouse prostate cancer cells derived from metastases. Conversely, overexpression of caveolin-1 leads to androgen insensitivity in low caveolin-1, androgen-sensitive mouse prostate cancer cells. Caveolin-1, therefore, is both a metastasis-related gene as well as a candidate androgen resistance gene for prostate cancer in man. Interestingly, recent studies also point to a potential role for caveolin-1 in the

resistance of various malignancies to multiple antineoplastic agents. The linkage of caveolin-1 expression with the androgen-resistant phenotype in prostate cancer and the multidrug resistance phenotype in various solid tumors establishes a novel paradigm for understanding these clinically important and now potentially related processes in malignant progression. Additional studies are ongoing to more broadly define the role of caveolin-1 as an apoptosis resistance gene in prostate cancer.

The studies presented at this workshop consisted of some of the contemporary lines of investigation being pursued to address the fundamental questions about prostate cancer biology. New information was presented regarding the role of integrins in modulating cell biology, studies focusing on Vitamin D and EGR α as agents in androgen-independent growth, several new lines of investigation in apoptosis (including receptor ligand-mediated cell death, PAR-4), and studies in the area of metastasis—a possible link between apoptosis and metastasis, the role of caveolin-1, and the use of selected prognostic markers (E-cadherin) in predicting metastases. The work presented highlights exciting avenues for further investigation. In addition, although many of the studies focused on basic molecular cancer biology, it is clear that translational components are at varying stages of development. This is indeed an exciting prospect.