The role of stroma in prostatic carcinogenesis

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Abstract
Most human prostate tumors are adenocarcinomas which arise from the epithelial cells that line the glands and ducts of the prostate. Consequently, the malignant epithelial cell, or more specifically genetic damage suffered by that malignant epithelial cell, has been the major focus of prostate cancer research to date. There is, however, increasing evidence to suggest that alterations in the stromal microenvironment associated with a malignant epithelium may be necessary for progression of carcinogenesis.

We have recently hypothesized that interactions between the stroma and epithelium become altered as a result of genetic damage to the prostatic epithelial cell. During prostatic carcinogenesis, this abnormal signaling may lead to changes in both the prostatic epithelium and smooth muscle with concomitant loss of growth control. In this way, both a malignant epithelium and an abnormal or ‘tumor stroma’ evolve.

The purpose of this article is to describe interactions between the stroma and epithelium of the normal prostate, and then to summarize evidence suggesting that stromal cells derived from benign versus malignant sources may exert differential effects on epithelial cell growth and differentiation.

Introduction
Cancers of the prostate, lung and colon are among the most common malignancies diagnosed in men living in the Western world. In fact, recent estimates have placed prostate cancer as the most commonly diagnosed malignancy, and the second leading cause of cancer-related death among men in the United States (Landis et al. 1998). Prostate cancer is comparatively less common in other parts of the world, where the incidence and/or mortality rates of other tumors, such as those that arise from the mouth, pharynx and stomach exceed those of prostate cancer.

Prostate cancer is unique among carcinomas in that it is a slow growing malignancy that is diagnosed almost exclusively in men over 50 years of age. Consequently, when determining survival benefits associated with any of the currently available forms of local treatment, at least ten to fifteen years of follow-up is required before a true survival benefit can be confirmed. Thus, for an individual patient with competing co-morbidities, overall life expectancy must be taken into consideration before definitive local treatment can be recommended. It is for this reason that some patients may require no treatment whatsoever. This group may include patients who are elderly with significant co-morbid conditions as well as those patients with low grade, low volume disease. In this regard, ‘watchful waiting’ has become a treatment option that is unique to prostate cancer (Johansson et al. 1992, Chodak et al. 1994).

For most men diagnosed with prostate cancer, however, there is a high risk of disease progression if no treatment is delivered. In these patients, the benefits derived from local treatment with respect to disease-free and overall survival must be balanced against potential side-effects associated with such treatment. For patients with favorable disease characteristics, such as a low pretreatment serum prostate specific antigen (PSA), a low clinical stage, and a well or moderately differentiated tumor, disease-free and overall survival five years following treatment is excellent utilizing either radical prostatectomy or radiotherapy as definitive local treatment. However, these results have not been as encouraging for patients with higher risk disease
characteristics (Catalona & Smith 1994, Walsh et al. 1994, Zietman et al. 1994, Hanks et al. 1995, Zagars & Pollack 1995). These results, in combination with the potential side-effects that have been associated with both surgery and conventional external beam radiotherapy (including urinary incontinence, rectal complaints and impotence), have led to several newer treatment options aimed at providing better results with fewer side-effects. However, any benefit of these newer treatment modalities with respect to disease-free survival or improved quality of life remains unproven.

Our laboratory has begun to examine some of the unique biological characteristics associated with prostate cancer with the ultimate goal of formulating novel, non-invasive treatment strategies. More than 95% of human prostatic cancers are adenocarcinomas which arise from the epithelial cells that line the glands and ducts of the prostate (Stamey & McNeal 1992). Consequently, most research to date on prostate cancer has examined changes occurring in the prostatic epithelial cell as it progresses from a normal to a frankly malignant carcinoma. There is, however, a growing body of evidence to suggest that, as a carcinoma evolves, changes also occur in the stromal compartment associated with the tumor. In many instances these changes may serve to enhance the invasive and/or malignant potential of the nascent epithelial tumor. With this in mind, we have hypothesized that epigenetic influences originating from stromal cells in the immediate vicinity of a prostatic tumor may be critical in determining whether a particular tumor

Figure 1 Schematic representation of interactions between the prostatic stroma and prostatic epithelium during normal development, adulthood and carcinogenesis. (a) Testosterone acts through androgen receptors (AR) in the urogenital sinus mesenchyme to induce epithelial development and differentiation. In a reciprocal fashion, epithelium signals to mesenchyme to induce smooth muscle differentiation. (b) In the growth-quiescent adult prostate, testosterone acts through both smooth muscle and epithelial AR. Androgens act through prostatic smooth muscle AR to maintain prostatic epithelium in a fully differentiated, growth-quiescent state and through epithelial AR to stimulate secretory function of the fully differentiated epithelium. It is hypothesized that the epithelium also acts to maintain smooth muscle differentiation through paracrine acting factors. (c) Prostatic carcinogenesis appears to be initiated by genetic insult to the epithelium. This leads to a change in epithelial phenotype and aberrant local signaling between the epithelium and smooth muscle. (d) The aberrant local signaling described in (c) leads to phenotypic changes in both the stromal and epithelial compartments of the tumor. The stroma becomes a fibroblastic 'tumor stroma' which promotes epithelial mitogenesis. This would predictably lead to increased epithelial proliferation, migration and, ultimately, an invasive epithelial phenotype.
assumes a slowly growing or an invasive phenotype (Cunha et al. 1996, Hayward et al. 1996b, 1997b). It is possible that, following genetic alteration to the prostatic epithelium, signaling from the epithelium to the surrounding smooth muscle becomes aberrant. This may result in stromal dedifferentiation towards a fibroblastic phenotype. One of the consequences of such a transformation may be that the local microenvironment changes from promoting epithelial homeostasis to promoting epithelial mitogenesis. These changes would be predicted to lead to increased epithelial proliferation, migration and, ultimately, could enhance the invasive potential of the genetically altered epithelial cell (Figs 1 and 2).

The purpose of this article will be (1) to describe the stroma of the normal (non-malignant) prostate, (2) to summarize genetic changes that are known to occur in the prostatic epithelium during carcinogenesis, (3) to present evidence suggesting that stroma derived from non-malignant sources may be able to alter the malignant phenotype of prostatic carcinoma cells, (4) to summarize the evidence in support of a ‘tumor stroma’, and (5) to summarize data describing the role of tumor stroma in prostatic carcinogenesis.

The stroma of the normal prostate

The human prostate is composed of two compartments: (1) an epithelial compartment, which includes the exocrine glands with their associated ductal structures, and (2) a surrounding connective tissue stroma. The stroma of the human prostate consists of a number of different cell types. The most abundant cell type in this compartment is the smooth muscle cell, which is derived

Figure 2 Immunohistochemical expression of smooth muscle α-actin and vimentin in the stroma of normal prostate and prostate cancer. (a) Smooth muscle α-actin expression in normal prostate. Note that the stroma is comprised predominantly of smooth muscle. (b) Smooth muscle α-actin expression in the stroma associated with prostate cancer. Note the paucity of immunostaining in this high power field. (c) Vimentin expression in normal prostate. Other than in blood vessels (which serve as positive internal controls), there is virtually no vimentin staining in the stroma of the normal prostate. (d) Vimentin expression in prostate cancer. Note the increased expression of this fibroblastic marker in the stroma associated with prostate cancer compared with the stroma of the normal prostate. The findings summarized in panels a-d suggest that the predominantly smooth muscle stroma of the normal prostate is altered in prostatic carcinogenesis to include vimentin-positive fibroblastic cells.
from the mesenchyme of the embryonic urogenital sinus (UGS). Other cell types located in the stroma of the normal adult prostate include fibroblasts, nerves, endothelial cells and vascular smooth muscle cells. In addition to being the most abundant stromal cell type, the smooth muscle cell appears to be the most important cell type with respect to prostatic development and maintenance of homeostasis. In this regard, changes in the smooth muscle cell may be important in the evolution of prostatic carcinogenesis (see below).

Tissue recombination experiments utilizing the androgen insensitive testicular feminized (tfm) mouse have established that an androgen responsive stroma is necessary for the development of normal prostatic epithelial architecture in the rodent (Cunha et al. 1987). In adulthood, prostatic smooth muscle cells, which are known to express androgen receptors (AR), interact with epithelial cells and under androgenic conditions maintain the epithelium in a fully differentiated, growth-quiescent state (Cunha et al. 1996). This occurs both in the presence and in the absence of epithelial AR expression which emphasizes the paracrine nature of smooth muscle-epithelial interactions. Human prostatic smooth muscle cells, which also express AR, are believed to play a similar role in maintaining prostatic homeostasis. However, definitive experiments to confirm this role for human prostatic smooth muscle cells are not possible given our inability to access AR-deficient human fetal urogenital sinuses.

Fibroblasts, which make up a large proportion of the stroma of the rodent prostate, are found sporadically in the normal human prostate. It has been suggested that these cells are important in mediating epithelial proliferation in the rodent prostate (Nemeth & Lee 1996). Their function in the human prostate remains unclear; they may serve as a scaffolding to keep smooth muscle bundles together, or they may play an active role in organ homeostasis.

Prostatic development occurs as a direct result of androgenic stimulation of the fetal UGS. Analysis of tissue recombinants composed of AR-positive wild type urogenital sinus mesenchyme (UGM) plus AR-negative tfm epithelium indicates that androgens act through AR in the mesenchymal cells of the UGS to stimulate epithelial proliferation, ductal branching morphogenesis, and columnar cytodifferentiation. In rats and mice, prostatic tissue can develop from the UGS derived from either a male or a female embryo if appropriately stimulated by androgens at critical developmental stages. In laboratory rodents the initial phases of prostatic budding require exposure to androgens prenatally. In contrast, ductal branching morphogenesis, canalization and epithelial cytodifferentiation all require postnatal androgenic stimulation. These early developmental events occur between birth and the onset of puberty, during which time circulating androgen levels are very low. These observations indicate that the developing prostate is extremely sensitive to low levels of circulating androgen (Donjacour & Cunha 1988).

Interactions between stroma and epithelium appear to be reciprocal in nature. Not only does the developing stroma induce epithelial development, but the developing epithelium also induces primitive mesenchymal cells to differentiate into smooth muscle (Cunha et al. 1992b). Urogenital sinus mesenchyme grown in the absence of epithelium will not form smooth muscle. Thus, the development of prostatic smooth muscle in vivo requires the presence of both epithelium and appropriate androgenic stimulation. This ability of epithelium to induce the formation of visceral smooth muscle is not restricted to the prostate, but it appears to be a common feature of many organs including the gut, uterus and bladder (Haffen et al. 1982, Cunha et al. 1989, Baskin et al. 1996).

The differentiation of prostatic smooth muscle occurs in an orderly manner with the sequential expression of a number of characteristic markers, including vimentin, actin, myosin, desmin and vinculin. The adult prostate, in which the stroma contains fully differentiated smooth muscle cells, is essentially growth-quiescent and maintains very low and balanced levels of cellular proliferation and cell death. It should be emphasized that this growth-quiescent, homeostatic state exists in the presence of high levels of circulating androgens. In the adult rodent, androgens act directly on the prostatic smooth muscle cells to maintain this fully differentiated growth-quiescent state (Hayward et al. 1996b). We have postulated that androgens act in a similar fashion in the adult human prostate to maintain growth-quiescence (Hayward et al. 1996b). Evidence supporting this hypothesis is as follows. After castration, the well recognized rapid regression of prostatic epithelium is associated with an ordered loss of expression of the various smooth muscle differentiation markers. This appears to reflect a ’dedifferentiation’ of the smooth muscle cells. The loss of smooth muscle markers following castration occurs in the order opposite to that to which these markers were expressed during normal development (Hayward et al. 1996a). In its final form, the prostatic stroma of a long-term castrated animal reverts to a stroma which contains fibroblasts or mesenchymal cells that coexpress AR and vimentin. Very little expression of the characteristic smooth muscle markers is evident in the fully regressed prostate.

If exogenous androgens are subsequently administered to a long-term castrated animal, prostatic tissue will respond in a highly coordinated manner, with the differentiation of both stromal smooth muscle (again expressing its characteristic markers) and a secretory
epithelium (Bruchovsky et al. 1975). Thus, the relatively undifferentiated fibroblastic cells in the prostatic stroma of a castrated animal can respond to androgens by inducing epithelial proliferation and columnar cyto-differentiation while they revert back to highly differen-tiated smooth muscle cells.

In summary, low levels of circulating androgens act upon the mesenchymal cells of the developing prostate to induce prostatic epithelial proliferation and differentia-tion. In contrast, high circulating levels of androgen in the adult act through the prostatic smooth muscle to maintain a fully differentiated, growth-qui-escent epithelium. Proliferative effects of stroma on epithelium are mediated through the stromal AR, while the epithelial AR appears to be required only for the expression of prostatic secretory proteins (Cunha & Young 1991, Donjacour & Cunha 1993). In long-term castrated animals, exogenous androgens initially promote prostatic epithelial proliferation and cytodifferentiation as well as the re-emergence of a smooth muscle stroma. Ultimately, androgen replacement leads to regeneration of a fully differentiated, growth-qui-escent gland. These data suggest that the local control of prostatic epithelial proliferation and differentia-tion occurs through androgenic stimulation of the prostatic stroma, and that the nature of the epithelial response to such a stimulation is predominantly determined by the nature of the stromal cells which are stimulated. Thus, AR-expressing prostatic smooth muscle cells appear to respond to androgenic stimulation by inhibiting epithelial proliferation and maintaining epithelial differentia-tion, while an AR-expressing fibroblastic stroma (either the urogenital sinus mesenchyme or the stroma from an androgen-deprived adult prostate) may respond to androgens by stimulating epithelial proliferation and eliciting columnar cyto-differentiation.

Genetic changes in human prostatic cancer

It appears that human prostate cancer begins with genetic alteration to the prostatic epithelium. In this regard, a variety of techniques have been useful in demonstrating genetic abnormalities in the prostatic epithelium, both in the primary tumor and at metastatic sites. However, none of these techniques has successfully defined a specific mutation that is characteristic for human prostate cancer. Although no study to date has specifically addressed genetic changes to the prostatic stroma during carcinogenesis, preliminary data from our laboratory using karyotypic analysis and comparative genomic hybridization demonstrate the absence of gross genetic alterations in the stromal cells surrounding a prostate cancer.

The familial concentration of some prostate cancers suggests that risk for this tumor may be inherited in an autosomal dominant fashion. Risk factors for the inherited form of this disease, including early age of onset and multiple affected family members, has allowed for the identification of some putative genetic abnormalities in these patients (Carter et al. 1990, 1991, 1992, Smith et al. 1996). Loss of heterozygosity has been reported frequently on chromosomes 10q, 7q and 16q in prostate cancer specimens (Isaacs et al. 1995). This has resulted in the recent description of a candidate tumor suppressor gene, PTEN/MMAC1, for patients with inherited prostate cancer (Suzuki et al. 1998). It must be emphasized, however, that patients with hereditary prostate cancer represent only a minority of prostate cancer patients. Aside from these rare families, prostate cancer appears to be a common but spontaneously arising disease with no consistent pattern of genetic alteration.

Previous studies have described aberrant expression of oncogenes, metastasis suppressor genes and tumor suppressor genes in prostate tumor specimens. While oncogenes such as ras, c-myc, c-sis and neu have been shown to be overexpressed in prostate cancer samples, no single oncogene is consistently overexpressed across a wide range of prostate tumors (Peehl 1993, Wang & Wong 1997). As a result, neither the initiation nor progression of prostate cancer can be linked to a specific oncogene. While experimental overexpression of various oncogenes has demonstrated the potential to initiate and/or promote the disease (Thompson et al. 1989, 1993a,b, Bello et al. 1997, Webber et al. 1997), the relevance of these interesting findings to clinical disease remains to be established.

A second class of genes which may prove to be important in human prostate cancer progression are the so called ‘metastasis suppressor’ genes. Examples of such genes include the KAI1 gene and thymosin-beta 15 (a gene which appears to be related to cell motility). KAI1 has been mapped to human chromosome 11p11.2. Introduction of this gene into the metastatic rat AT6.1 prostate cancer cells was shown to suppress prostate cancer metastases (Dong et al. 1995). In the human, expression of this gene appears to be reduced in cell lines derived from metastatic prostate tumors (Dong et al. 1995, 1996a,b). Thymosin-beta 15, a 5.3 kDa protein, binds actin monomers, thereby inhibiting actin polymerization (Gold et al. 1997). In the rat Dunning tumor model, cell motility has been shown to correlate well with metastatic phenotype. Transfection of these tumor cells with anti-sense thymosin-beta 15 has been shown to cause a decrease in cell motility (Bao et al. 1996). Thymosin-beta 15 levels appear to be elevated in human prostate cancer, and levels of this protein have been correlated with Gleason grade (Bao et al. 1996). Additional studies have
suggested that there may be other metastasis suppressor genes important in human prostate cancer located on chromosomes 8 and 10 (Ichikawa et al. 1996).

The tumor suppressor genes represent a third class of genes that may demonstrate alterations in human prostate cancer specimens. Mutations in the retinoblastoma (Rb) gene have been reported to occur in prostate tumor samples; however, such mutations are present in only a minority of cases (Isaacs 1995, Kubota et al. 1995). p53 alterations are also inconsistently found in prostate cancer, appearing in only 20% of cases. In contrast, alterations in E-cadherin may be the most commonly detected defect in human prostate cancers, with up to 50% of cases demonstrating altered expression of this protein.

E-cadherin is a 120 kDa transmembrane glycoprotein which is involved in epithelial cell adhesion (Birchmeier & Behrens 1994, Birchmeier et al. 1995, Jiang 1996, Shiozaki et al. 1996). The extracellular domain of E-cadherin undergoes calcium-dependent homophilic interactions in adherent junctions and along lateral epithelial membranes. The cytoplasmic domain of E-cadherin is associated with a group of proteins known as catenins that link the intracellular domain of E-cadherin to the actin microfilament network (Jiang 1996). A functional E-cadherin system is required to maintain normal epithelial morphology.

Regulation of E-cadherin is poorly understood. The molecule is developmentally regulated in the embryo where it is initially expressed coincident with the appearance of epithelial tissue at the time of compaction of the embryo. Epithelial to mesenchymal transformation (i.e. the generation of mesoderm through the primitive streak) is associated with loss of E-cadherin, while mesenchymal to epithelial transformation (i.e. formation of the nephron) is associated with increased expression of E-cadherin (Hay & Zuk 1995). A critical role for E-cadherin in development (Takeichi 1988) is underscored by the observation that 'knockout' of the E-cadherin gene in transgenic mice is embryonic lethal (Larue et al. 1994). While regulatory elements have been identified in the E-cadherin promoter (Behrens et al. 1992), the relevance of these regulatory elements to the biology of E-cadherin remains unclear.

An important role for E-cadherin expression in cellular adhesion is demonstrated by the fact that cells lacking E-cadherin are unable to aggregate or adhere. Furthermore, neutralizing antibodies to E-cadherin cause adherent cells to dissociate. Mutation or deletion of genes encoding E-cadherin or the cadherin-associated proteins (catenins) also render cells non-adherent. Transfection of E-cadherin-negative cells with E-cadherin cDNA leads to reversal of the non-adherent phenotype.

These concepts are of obvious importance to tumor cell invasion and metastasis in the prostate and many other organs. Experimental impairment of E-cadherin with either antibodies or anti-sense cDNA converts non-invasive cells into invasive ones (Vlemincx et al. 1991). Moreover, loss of E-cadherin expression has been shown to coincide with the transformation of a well differentiated adenoma into a carcinoma using a transgenic mouse model of pancreatic carcinogenesis (Perl et al. 1998). In the prostate, decreased expression of E-cadherin in invasive and metastatic cancers is associated with poor prognosis (Giroldi et al. 1994, Umbas et al. 1994, Shiozaki et al. 1996). While E-cadherin is consistently expressed in normal epithelial cells (including prostatic epithelium) and in various highly differentiated ‘non-invasive’ carcinoma cells (including prostatic carcinomas), E-cadherin expression is considerably lower (or entirely lacking) in poorly differentiated invasive carcinoma cells (including prostatic carcinomas) (Umbas et al. 1992, Giroldi & Schalken 1993, Birchmeier & Behrens 1994, Giroldi et al. 1994, Jiang 1996). Bussemakers et al. (1993) demonstrated that coincident with the loss of E-cadherin expression, a variant of the rat Dunning prostatic tumor spontaneously changed from a well differentiated, androgen-responsive, slow growing non-metastatic line into an anaplastic, androgen-independent, rapidly growing metastatic line. Similarly, patients with prostatic tumors that do not express E-cadherin, or that have abnormal patterns of E-cadherin expression, appear to have a poorer prognosis than patients with prostatic tumors that express E-cadherin normally (Umbas et al. 1994). The relatively common loss of portions of chromosome 16 in human prostatic carcinoma may result in deletion of all or part of the E-cadherin gene which maps to 16q21 (Joos et al. 1995). Other mechanisms that may cause dysfunction of cadherin-mediated interactions include increased tyrosine phosphorylation of β-catenin and mutational inactivation of either the extracellular or intracellular domains of the E-cadherin gene (Behrens 1993, 1994). This type of mutational inactivation of the E-cadherin gene has not been described in human prostatic carcinoma but has been reported in endometrial and gastric cancers (Becker et al. 1993, Risinger et al. 1994). In some high grade prostatic carcinomas, E-cadherin levels may remain relatively normal while α-catenin may be absent (Isaacs et al. 1994). The absence of α-catenin also renders the E-cadherin system non-functional (Hulsken et al. 1994). For example, impaired E-cadherin function in the PC3 prostatic carcinoma cell line appears to be caused by homozygous deletion of α-catenin (Morton et al. 1993).

Previous studies have suggested that ‘host factors’ may down-regulate E-cadherin expression in carcinoma cells. This observation was made from experiments in which virus-transformed MDCK cells (expressing high levels of E-cadherin and exhibiting an adhesive
phenotype) were transplanted into athymic mice. These cells, which were originally shown to be non-invasive based upon *in vitro* assays, produced invasive metastatic tumors *in vivo* which contained both E-cadherin-positive and E-cadherin-negative cells. When these metastatic E-cadherin-negative cells were subsequently isolated and examined *in vitro*, adherent E-cadherin-positive cells were re-derived (Mareel *et al.* 1991). Although their precise nature was not specified, unknown ‘host factors’ were suggested to be involved in this down-regulation of E-cadherin. Further studies, in which micro-encapsulated virus-transformed MDCK cells were transplanted into athymic rodent hosts, demonstrated that the loss of E-cadherin expression *in vivo* required ‘immediate contacts between tumor cells and host cells or upon host factors that could not cross the capsule membrane’ (Vandenbossche *et al.* 1994).

In order to determine if stromal cells are capable of regulating E-cadherin expression, we have developed an *in vitro* model system in which epithelial cells are plated onto a confluent lawn of fibroblastic cells which have been derived from either a tumor or a non-tumor environment. In addition to human prostatic stromal cells, we have also used a rat Dunning-tumor derived stromal cell line (DT3RPF; a generous gift from Dr Wallace McKeehan, Texas A&M University, USA) and a benign mouse embryonic fibroblastic cell line in our *in vitro* system (Hayward *et al.* 1997a). Stromal cells were grown to confluence on positively charged microscope slides, after which 5000/cm² cells of the non-tumorigenic SV40T immortalized human prostatic epithelial cell line BPH-1 were plated onto the confluent stromal lawn. Co-cultures were then grown for 48 h. Control cultures composed of stromal cells alone, or BPH-1 cells alone were also grown on microscope slides for 48 h. Colony morphology (assessed by cytokeratin expression) and E-cadherin expression were examined using immunohisto-chemistry.

BPH-1 cells cultured for 48 h on a confluent lawn of benign fibroblasts (of either human or rodent origin) grew as discreet coherent colonies. Epithelial cells appeared to be contact inhibited. Epithelial colonies had smooth regular borders and epithelial cells appropriately expressed E-cadherin along cell membranes. Few single cells were observed. In contrast, BPH-1 cells cultured for 48 h on a confluent lawn of tumor-derived fibroblasts (either from human prostate tumors or from the DT3RPF cell line) grew as single cells or as small colonies with irregular borders. Many cells appeared to overlap, suggesting a loss of contact inhibition. E-cadherin expression was either markedly decreased or undetectable in epithelial cells grown in co-culture with the tumor-derived fibroblasts (Fig. 3).

In the adult prostate, the stromal smooth muscle apparently functions to maintain the surrounding epithelium in a homeostatic, growth-quiescent state. We have hypothesized that the undifferentiated stroma...
Associated with prostate cancer cells may promote epithelial proliferation and the loss of epithelial differentiation. Therefore, we have tested the hypothesis that ‘benign’ stroma may be able to ‘normalize’ malignant prostatic epithelium. The idea that interactions between benign stroma and malignant epithelium could possibly inhibit tumorigenic progression was examined by using the rat Dunning prostatic adenocarcinoma R3327 (DT). The stroma of this tumor is abnormal, being composed of fibroblastic cells with an absence of smooth muscle. In addition, the basement membrane between the epithelium and the stroma is often discontinuous or excessively reduplicated (Wong et al. 1992). These observations raised the possibility that interactions between the stroma and epithelium in this tumor system are abnormal. To test the hypothesis that the malignant epithelial cells might be modified by a more ‘normal’ stromal environment, small fragments or epithelial cell suspensions of the DT were grown for one month in male nude rodent hosts either alone or in combination with ‘normal’ stromal populations. These normal stromal populations included either urogenital sinus mesenchyme (UGM) or seminal vesicle mesenchyme (SVM) which are both potent inducers of epithelial differentiation (Cunha et al. 1983, 1991b) (Fig. 4). Grafts of DT alone demonstrated a characteristic histology, forming tumors that contained small ducts which were lined by one or more layers of undifferentiated squamous or cuboidal epithelial cells. In contrast, DT epithelial cells grown in association with UGM or SVM differentiated into tall columnar epithelial cells which were arranged in large cystic ducts (Hayashi et al. 1990, Hayashi & Cunha 1991, Wong et al. 1992, Hayashi et al. 1996, Tam et al. 1997). These changes in the histological appearance of the DT, which were induced by the normal mesenchyme, were also associated with marked changes in neoplastic growth (Hayashi & Cunha

Figure 4 Summary of the experimental protocol used to determine the effects of normal stroma (either seminal vesicle mesenchyme or urogenital sinus mesenchyme) on the proliferation and differentiation of the rat Dunning prostatic adenocarcinoma (adapted from Cunha et al. 1991a).
Tumor-associated stromal cells

There is a growing body of evidence suggesting that, as a carcinoma develops, changes occur in the surrounding connective tissue stroma that may serve to enhance the malignant potential of the nearby epithelium. The appearance of this so-called ‘tumor stroma’ has been demonstrated in a number of epithelial malignancies. For example, the stroma associated with invasive breast carcinoma is composed of activated or abnormal myofibroblastic cells which are found in close apposition to the tumor cell nests (Ronnov-Jessen et al. 1996). These myofibroblastic cells are unique to the ‘tumor stroma’ as they are not found in the normal breast tissue. However, the emergence of these myofibroblastic cells is certainly not specific to breast carcinoma. Myofibroblasts have also been identified in the stroma associated with cervical carcinoma, colon carcinoma, ovarian carcinoma and skin cancer (Ronnov-Jessen et al. 1996). Other phenotypic changes which have also been ascribed to these tumor-derived fibroblasts include changes in the migratory behavior of these cells in vitro (Schor et al. 1988a,b, 1991) and alterations in gene and protein expression. For example, stromal cells isolated from a number of carcinomas, including those arising in the breast, skin and lower gastrointestinal tract, have all been found to overexpress metalloproteinases such as MMP-2 and stromelysin-3 (Basset et al. 1990, Poulsom et al. 1992, 1993). In addition, proteins such as dipeptidyl peptidase IV (Atherton et al. 1992) and fibroblast activation protein alpha (Scanlan et al. 1994) also appear to be selectively expressed by tumor-associated fibroblasts. Because stromal cells contribute to the formation of the extracellular matrix, the appearance of a ‘tumor stroma’ may also lead to changes in the extracellular matrix surrounding a carcinoma (Bosman et al. 1993). Extracellular matrix proteins such as tenascin (Chiquet-Ehrismann et al. 1986) and hyaluronan (Ronnov-Jessen et al. 1996) are produced by these tumor-derived stromal cells and may enhance the invasive potential of malignant epithelial cells.

By altering the local environment of a carcinoma cell, the ‘tumor stroma’ may be capable of modulating malignant phenotype and behavior. This hypothesis has been examined in experiments using MCF-7 breast carcinoma cells in which these cells were placed in coculture with various types of fibroblastic cells (embryonic or adult fibroblasts, normal or tumoral fibroblasts) (Adam et al. 1994). The phenotype of the MCF-7 cells, including their expression of estrogen receptor, progesterone receptor, pS2 and cathepsin-D, was found to be dependent on the type of fibroblast with which they were co-cultured. This stromal modulation of epithelial cell phenotype appeared to take place through paracrine signaling mechanisms that were likely to be mediated by specific growth factors. Growth factors such as platelet derived growth factor (Ponten et al. 1994), insulin-like growth factors (IGF)-I and -II (Ellis et al. 1994, Manni et al. 1994, Singer et al. 1995), transforming growth factor-β1 (Ronnov-Jessen et al. 1996), interleukin-6 (Ronnov-Jessen et al. 1996), hepatocyte growth factor/epithelial scatter factor (Behrens 1993) and keratinocyte-growth factor (Yan et al. 1993) have all been identified as putative signaling molecules capable of modulating the function of carcinoma cells.

Stromal cells surrounding and within a tumor may have the capacity to facilitate tumor growth by supporting neovascularization. For a tumor to grow beyond a few millimeters in size, it must stimulate new blood vessel growth (Brem et al. 1993). This stimulation of new blood vessel formation has been termed angiogenesis. Quantitation of the angiogenic response (determination of ‘microvessel density’) can be performed using specific antibodies directed at antigens expressed in the newly formed blood vessels. Recent studies have demonstrated that microvessel density count may be of prognostic importance in several solid tumors, including human prostate cancer (Weidner et al. 1993, Hall et al. 1994, Silberman et al. 1997).

Studies in the prostate have shown that microvessel density count is significantly higher in adenocarcinoma specimens than in benign prostate (Bigler et al. 1993). Moreover, blood capillary architecture has been found to change in the progression from benign prostatic tissue to prostatic intra-epithelial neoplasia and finally to prostatic cancer (Ronnov-Jessen et al. 1996). For example, DT cells induced to differentiate by SVM demonstrated markedly decreased tumorigenic abilities with a significantly lower proliferation rate than the parental Dunning tumor cells. In a subsequent experiment, ducts from primary SVM+DT recombinants were grafted directly into new male hosts or were combined with fresh SVM to form secondary SVM+DT recombinants. Both types of recombinants exhibited minimal growth during a 3-month period and maintained a highly differentiated state (Fig. 4). Conversely, control grafts composed of DT alone formed large tumors which weighed 5-7 g during the same time period. The marked reduction in growth rate and the histological differentiation of the SVM-induced DT epithelial cells was found to be associated with a dramatic decrease in cellular proliferation as determined by [3H]thymidine labeling index (Hayashi & Cunha 1991). It should be noted that smooth muscle cells, which were apparently derived from the SVM, were found in close apposition to the highly differentiated, relatively growth-quiescent DT epithelium in these tissue recombinants (Wong et al. 1992).
carcinoma (Montironi et al. 1993). With respect to localization within a tumor, a higher degree of vascularization is found at the center of a prostatic tumor compared with the tumor edge (Siegal et al. 1995). Microvessel density has been associated with other well recognized prognostic indicators in human prostate cancer including tumor Gleason grade (Wakui et al. 1992) and pathological stage (Fregene et al. 1993, Brawer et al. 1994).

The angiogenic process in human prostate cancer appears to require the presence of stromal cells. This has been demonstrated in a co-culture system using the human prostatic tumor cell line, PC-3, where the induction of angiogenesis required the presence of stromal fibroblasts (Janvier et al. 1997).

The above data suggest that the degree of tumor neovascularization (which is dependent upon stromal cells) may be related to tumor cell invasion and metastasis in prostate cancer. Taken together, these suggest that alterations in the tumor-associated stromal cells may have an important role in prostate cancer progression. It is no surprise, therefore, that some investigators have suggested that without a ‘tumor stroma’ there would be no tumors (Bosman et al. 1993, Ronnov-Jessen et al. 1996).

Abnormal stroma as a mediator of tumorigenesis in the prostate

The idea that stromal cells may facilitate prostatic carcinogenesis has been investigated previously. Using an in vivo mouse prostatic reconstitution system, Thompson and colleagues infected either the urogenital sinus (prostatic anlagen) or its individual mesenchymal (UGM) or epithelial (UGE) components with a virus containing the myc and ras oncogenes. In prostatic reconstitutions containing uninfected UGM+infected UGE, epithelial hyperplasias were detected. Similarly, in prostatic reconstructions composed of infected UGM+uninfected UGE, stromal desmoplasias were observed. Carcinomas were found only in prostatic reconstructions in which both UGM and UGE were infected (Thompson 1990, Thompson et al. 1989, 1993a). These findings demonstrated that changes were required both in the epithelium and in the stromal microenvironment for prostatic carcinogenesis to occur.

Several studies examining the role of stromal cells in prostatic tumorigenesis have originated from the laboratory of Leland Chung. Camps et al. (1990) reported that co-inoculation of tumorigenic NbF-1 fibroblasts with human PC-3 prostatic carcinoma cells accelerated tumor growth and shortened tumor latency period. The interaction between fibroblasts and epithelial cells in this system was bi-directional – PC-3 cells reciprocally enhanced the tumorigenesis of sarcomatous NbF-1 fibroblasts. Similar studies have been extended to the human LnCaP prostatic carcinoma cell line (Chung 1991, Chung et al. 1991, Gleave et al. 1991, 1992, Wu et al. 1994). Co-inoculation of LnCaP cells with various non-tumorigenic fibroblasts demonstrated that fibroblasts differed in their ability to promote prostatic carcinogenesis (Gleave et al.}

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**Figure 5** Diagrammatic representation describing how both genetic and epigenetic determinants may influence prostatic cancer (CaP) progression. In this hypothesis, epigenetic factors may help to determine whether a particular tumor focus becomes a slowly growing, clinically insignificant cancer or a more rapidly growing, progressive cancer that will eventually threaten the host. PIN, prostatic intra-epithelial neoplasia.
Fibroblasts derived from rat urogenital sinus mesenchyme and human bone, but not NIH 3T3 cells, normal rat kidney fibroblasts or normal human lung fibroblasts, enhanced tumorigenesis of ‘non-tumorigenic’ (as described by Gleave and colleagues) human LnCaP cells in vivo. Conditioned media from bone fibroblasts, rat urogenital sinus mesenchyme cells or NbF-1 fibroblasts were also effective in stimulating growth of LnCaP cells in vitro, while conditioned media from 3T3 cells, normal rat kidney and normal human lung were ineffective in this regard. Once again, this effect was bi-directional, as LnCaP conditioned medium stimulated rat urogenital sinus mesenchyme growth by up to 275%. These data support the concept that fibroblasts can enhance tumorigenesis in the human prostate, and that the trophic effect of fibroblasts on prostatic carcinogenesis is not a property of fibroblasts in general but is restricted to selected fibroblastic cells.

As described above, we have hypothesized that initiation of prostatic carcinogenesis involves genetic alteration of the prostatic epithelium, after which there is a sequential disruption in the reciprocal homeostatic interactions between the prostatic smooth muscle and the associated epithelium (Cunha et al. 1996, Hayward et al. 1996b, 1997a). This altered signaling leads to the de-differentiation of both the emerging prostatic carcinoma cells and the surrounding smooth muscle. Even though the phenotype of the smooth muscle changes, the tumor-associated stromal cells are postulated to remain genetically normal (Fig. 5).

Using an in vivo model system, we have examined the effects of carcinoma-associated fibroblasts on adjacent epithelial cells. If our above hypothesis is correct, human carcinoma-associated fibroblasts (CAF) should have different effects on epithelial proliferation and differentiation than ‘normal’ fibroblasts derived from benign human prostatic tissue. We tested this hypothesis using a tissue recombination model in which benign or tumor-derived stromal cells isolated from human prostatic tissue were recombined with either BPH-1 cells (a non-tumorigenic, SV40T immortalized human prostatic epithelial cell line) (Hayward et al. 1995) or phenotypically normal human prostatic ductal organoids (fragments of acini and ducts derived from benign prostate which retain in vivo architecture). The resultant tissue recombinants were grown beneath the renal capsule of adult athymic nude rodent hosts. Control grafts were composed of either epithelial or stromal cells alone and were grown in conditions identical to the experimental tissue recombinants. The amount of tissue recombinant growth was determined by measuring wet weights. Histopathological features of tissue recombinants were determined in hematoxylin and eosin stained sections.

Control grafts containing either benign or tumor-derived stromal cells alone or epithelial cells alone demonstrated minimal growth during the experimental period. Tissue recombinants composed of normal prostatic fibroblasts+BPH-1 cells also exhibited minimal growth after sub-renal capsular grafting. In contrast, tissue recombinants composed of carcinoma-associated fibroblasts+BPH-1 cells exhibited striking growth after sub-renal capsular grafting, reaching wet weights as high as 5 g after 41 days (from an initial wet weight of approximately 10 mg). Morphometric analysis demonstrated that these tumors were predominantly epithelial (80%), with a histological appearance that was consistent with poorly-differentiated prostatic adenocarcinoma (Fig. 6).

Tumors were composed of poorly differentiated, irregular epithelial cords. In some areas, epithelium formed small glandular nests while in other areas epithelium appeared as single cells that were intermingled within a fibrous stroma. The epithelial nature of these cells was confirmed immunohistochemically using a wide spectrum anti-cytokeratin antibody. Furthermore, epithelial cells within these tumors exhibited nuclear staining with an antibody to SV40T antigen, confirming their BPH-1 origin. The majority of epithelial cells contained large, pleomorphic nuclei with large nucleoli. Stromal cells were intermingled between the epithelial cell nests throughout the tumor. In contrast to what was seen with CAF+BPH-1 tissue recombinants, none of the
Chung's studies utilized tumorigenic fibroblasts that differed from those of Chung and colleagues in several ways. First, unlike normal fibroblasts, those derived from a tumorigenic cell line (NbF-1) were capable of inducing an invasive phenotype in an immortalized but non-tumorigenic human prostatic epithelial cell line. The same fibroblasts were unable to induce this phenotype in genetically normal human prostatic epithelial cells, even though effects on epithelial differentiation were observed. These data suggest (1) that both genetic and epigenetic changes may be important in human prostatic carcinogenesis, (2) that carcinoma-associated fibroblasts were able to stimulate progression of an initiated epithelium while normal fibroblasts were incapable of stimulating progression and (3) that carcinoma-associated fibroblasts were incapable of causing initiation in a genetically normal epithelium. Recent data obtained using an in vitro co-culture system suggest that the carcinoma-associated fibroblasts may be capable of increasing proliferation and decreasing death rates of the BPH-1 human prostatic epithelial cells.

It should be noted that some of Chung's initial studies attempted to examine the ability of prostatic stromal cells to stimulate carcinogenic progression in vivo by co-inoculating prostatic stromal cells with prostatic epithelial cells in a rodent host. In these studies, a non-tumorigenic rat prostatic epithelial cell line (NbE-1 cells) was co-inoculated with a tumorigenic cell line of rat prostatic fibroblasts which formed sarcomas when grown alone (NbF-1 cells) (Chung et al. 1989). As early as nine days after transplantation, large ‘carcinosarcomas’ formed in which epithelium comprised only 2-5% of the tumor mass. The precise volume of epithelial growth was not calculated in these studies, and any histopathological changes that may have occurred in the epithelium as a result of co-inoculation with the tumorigenic NbF-1 cells were not specified. Furthermore, control experiments examining the in vivo characteristics of epithelial cells grown alone, or epithelial cells grown with a normal, non-tumorigenic stroma were not described. Moreover, in the tumor growth period was not varied for any of the experiments. Given the rapidity with which the tumors formed, as well as the primarily sarcomatous appearance of the tumors, it is likely that, instead of forming tumors themselves, the benign epithelial cells in these grafts remained benign epithelial cells 'trapped' within a NbF-1-derived sarcoma.

It is important to note that our experiments differ from those of Chung and colleagues in several ways. First, Chung's studies utilized tumorigenic fibroblasts that formed sarcomas when grown alone. Our stromal cell populations were genetically normal (by karyotypic analysis and comparative genomic hybridization) and were non-tumorigenic when grown alone. In fact, our carcinoma-associated fibroblasts exhibited virtually no growth during 6 weeks in vivo beneath the renal capsule. Secondly, the histological appearance of the tumors in our study was consistent with that of a poorly differentiated adenocarcinoma. Sarcomatous elements were not identified. This is in marked contrast to the primarily sarcomatous tumors (carcinosarcomas) described by Chung and colleagues. These important differences will enable us to utilize our model system to examine the role of stromal-epithelial interactions in the initiation and progression of human prostatic cancer.

**Mechanisms of stromal-epithelial signaling in the normal and neo-plastic prostate**

The data summarized above lead to two opposite but related ideas: (1) that carcinoma-associated stromal cells can promote prostatic carcinogenesis, and (2) that normal stromal cells may be able to inhibit prostatic carcinogenesis by inducing differentiation and decreasing proliferation of epithelium. These data indicate that a detailed understanding of the signaling mechanisms between stroma and epithelium could allow for the rational design of therapies aimed at inhibiting prostate tumor growth.

Many studies have attempted to identify the signaling molecules acting as paracrine mediators of stromal and epithelial interactions in the developing and adult prostate. In most studies to date, growth-quiescent adult prostatic tissues have been utilized, and therefore the signaling molecules that have been identified may be involved in homeostasis rather than in carcinogenesis (Cunha et al. 1992a, Cunha 1994). There is currently no detailed picture of the spatial and temporal expression patterns of many of the growth factors found in the prostate. Considerations such as the cell of origin of a particular growth factor (epithelium, smooth muscle or fibroblast), the precise localization of a growth factor or its receptor in relation to proximal, intermediate or distal ductal architecture, and the specific developmental stages when expression of a particular growth factor occurs have not been adequately examined.

In order to define better growth factor expression in the prostate, we have recently developed species-specific reverse transcription-PCR methods which examine the expression of growth factors in heterospecific tissue recombinants (i.e. rodent mesenchyme with human epithelium). Using this method, it has been possible to determine whether epithelial or stromal cells are expressing specific growth factors (Horseman et al. 1997,
Histological examination of the IGF-I knockout and IGF weights of 30-50 mg for wild-type fetal prostatic grafts. Of approximately 1-2 mg. This is compared with wet recovered tissue grafts were very small, with wet weights allowed to grow for one month. After this time period, the renal capsule of male nude mouse hosts. Grafts were type 1 receptor knockout mice were grafted beneath the prostatic rudiments harvested from both IGF-I and IGF recent experiments from our laboratory have shown that development and disease remains unclear. However, are the IGFs. The exact role of the IGFs in prostatic potentially influence prostatic growth and carcinogenesis (Thomson \textit{vivo}). In altered FGF ligand-specific responsiveness of the epithelium (Yan \textit{in vitro}) -IIIc splice variant in rat Dunning tumor cells may result of FGF-7 receptor expression from the FGF-R2-IIIb to the variant) is exclusively expressed by epithelium. This growth factor is expressed by mesodermally derived stromal cells, while its receptor (the FGF-R2 IIIB splice variant) is exclusively expressed by epithelium.

Previous studies have demonstrated that specific growth factors may play an important role in stromal-epithelial signaling in the normal developing prostate. Thus, it is possible that altered expression of these growth factors may contribute to the development of prostatic cancer. One such growth factor is FGF-7. During prostatic development, FGF-7 is capable of imitating some of the effects of testosterone. For example, the addition of exogenous FGF-7 in the absence of testosterone can induce ductal branching morphogenesis in organ cultures of neonatal rat ventral prostate and mouse seminal vesicle (Alarid \textit{et al.} 1994, Sugimura \textit{et al.} 1996). In addition, a neutralizing antibody directed against FGF-7 was able to inhibit androgen-induced ductal branching morphogenesis using this same rat ventral prostate model. Although stromal cell expression of FGF-7 may be regulated by androgens \textit{in vitro} (Yan \textit{et al.} 1992), RNAse protection assays of FGF-7 and its receptor (FGF-R2 IIIB) failed to show regulation of these genes by androgens \textit{in vivo} (Thomson \textit{et al.} 1997). In prostate cancer, the switch of FGF-7 receptor expression from the FGF-R2-IIIb to the -IIIc splice variant in rat Dunning tumor cells may result in altered FGF ligand-specific responsiveness of the epithelium (Yan \textit{et al.} 1993). A second family of growth factors which may potentially influence prostatic growth and carcinogenesis are the IGFs. The exact role of the IGFs in prostatic development and disease remains unclear. However, recent experiments from our laboratory have shown that IGF-I is required for normal prostatic growth. Fetal prostatic rudiments harvested from both IGF-I and IGF type 1 receptor knockout mice were grafted beneath the renal capsule of male nude mouse hosts. Grafts were allowed to grow for one month. After this time period, the recovered tissue grafts were very small, with wet weights of approximately 1-2 mg. This is compared with wet weights of 30-50 mg for wild-type fetal prostatic grafts. Histological examination of the IGF-I knockout and IGF type 1 receptor knockout grafts demonstrated that prostatic differentiation had occurred during this time period, with the appearance of a few small ducts which were lined by tall columnar secretory epithelium (A Donjacour, unpublished observations). These data suggested two important points: (1) that IGF-I was required for normal prostatic growth, and (2) that local production (or activation) of the ligand appears to be important (normal levels of circulating IGF-I in the nude mouse host did not induce normal prostatic growth in grafts of IGF-I knockout prostates). Previous studies have demonstrated inappropriately increased expression of IGF-II both in benign prostatic disease and in other carcinomas such as those arising in the breast (Ellis \textit{et al.} 1994, Manni \textit{et al.} 1994, Peehl \textit{et al.} 1995, Singer \textit{et al.} 1995, Tennant \textit{et al.} 1996, Dong \textit{et al.} 1997). In addition, IGF-I appears to be down-regulated in human prostate tumors (Peehl \textit{et al.} 1995, Tennant \textit{et al.} 1996, Dong \textit{et al.} 1997). When taken together, these data support further investigation into the regulation of IGF-I, IGF-II and the type 1 IGF receptor in benign and malignant prostatic disease.

Conclusions

The data summarized above suggest that both genetic and epigenetic factors are important in the progression of prostate cancer. Our recent \textit{in vivo} and \textit{in vitro} studies have demonstrated that: (1) carcinoma-associated fibroblasts do not form tumors when grown in the absence of epithelium, (2) carcinoma-associated fibroblasts stimulate progression of a genetically altered, non-tumorigenic human prostatic epithelium towards a carcinomatous phenotype, (3) the same fibroblasts are unable to stimulate initiation of a genetically normal human prostatic epithelium, and (4) stromal cells from non-malignant sources do not promote tumorigenesis in a genetically altered human prostatic epithelium under identical conditions. Our \textit{in vitro} studies have also demonstrated that the stromal microenvironment may be capable of regulating epithelial E-cadherin expression. These data do not discount the possibility that epithelial cells with severe genetic alterations can grow autonomously as malignant tumors. Instead, they imply that interactions with the stromal microenvironment are important determinants in the progression from a normal prostatic epithelium to an invasive carcinoma.

Prostate cancer can exist in two forms: either as a latent, clinically insignificant tumor or as an aggressive tumor which will progress without treatment. At the present time, we have been unable to identify the specific factors that determine the behavior of any given tumor focus. The evidence presented above strongly suggests that the stromal microenvironment may be capable of
modulating the biological potential of a particular tumor. This may be accomplished through regulation of tumor neovascularity or through specific phenotypic changes in the surrounding stromal cells that result in altered expression of certain enzymes, cellular adhesion molecules or growth factors.

The implications of our observations regarding tumor growth are potentially very important from both a diagnostic and a therapeutic perspective. For example, the ability to identify tumor-associated stromal populations which promote down-regulation of E-cadherin could be extremely important for identifying patients at risk for malignant progression of their prostatic adenocarcinoma. From a therapeutic viewpoint, the prospect of targeting therapy through the tumor-associated stroma to increase epithelial E-cadherin expression could be used to maintain prostatic tumors in a highly differentiated, non-invasive state. This is not a far-fetched possibility as studies in breast cancer patients have already demonstrated that agents such as 9-cis-retinoic acid, tamoxifen, IGF-I and tangeretin may up-regulate E-cadherin expression (Anzano et al. 1994, Bracke et al. 1994, Morton et al. 1995).

Traditional therapy for all epithelial malignancies, including prostate cancer, has been targeted at the malignant epithelial cell. Due to its genetic instability, this cell represents a ‘moving target’ for treatment. Although carcinoma-associated fibroblasts are abnormal, preliminary studies using karyotypic analysis and comparative genomic hybridization have demonstrated that these cells do not possess gross genetic alterations. Thus, they may provide a more stationary target at which to direct treatment.

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