Advances in Brief

Primary Prostate Stromal Cells Modulate the Morphology and Migration of Primary Prostate Epithelial Cells in Type 1 Collagen Gels[1]

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Abstract

The effects of human primary prostatic stromal cells on the migration and morphogenesis of human prostatic epithelial cells, derived from tumor or benign prostatic hyperplasia tissue, were studied using a three-dimensional coculture system. Epithelial cells from tumor or benign tissue migrated efficiently into collagen gels populated with stromal cells from benign tissue. Only epithelial cells from benign prostate formed acinus-like structures that exhibited differentiated prostatic function and strong expression of membrane-associated E-cadherin. In gels populated by stromal cells from tumor tissue, migration of primary prostatic epithelial cells did not occur. In the absence of stromal cells, primary epithelial cells were unable to proliferate. This three-dimensional culture system allows closely controlled manipulation and analysis *in vitro* of interactions between prostatic epithelial and stromal cells.

Introduction

CaP[3] is the second most common cause of cancer-related deaths among men in the United States (1). The American Cancer Society estimates that ~198,100 new cases of CaP will be diagnosed during 2001. Although the lifetime chance of developing CaP is 1 in 6, only 1 in 30 will die of the disease. Identification of the tumors that will progress to metastasis requires an increase in our knowledge of the factors contributing to the development of the disease.

Because most prostate tumors arise in the glandular epithelial cells, these have been a major focus of research. However, it is clear that interactions between stromal, epithelial, and extracellular matrix components play an important role in defining the malignant phenotype, as shown in animal models and xenografts (2, 3). *In vivo* models provide a complex environment that makes the isolation of modulating factors difficult. *In vitro* studies have produced conflicting data, although the influence of stroma on epithelial cell invasion and motility has been established (4, 5). The progression to metastatic cancer is linked in pathological terms to a loss of acinar differentiation, but little is known about the cell-cell and cell-extracellular matrix interactions that are important in the regulation of acinar morphogenesis and progression to CaP. Olumi *et al.* (6), in an *in vivo* model, described how cancer-associated fibroblasts can direct the progression to tumorigenesis of initiated human prostate epithelium but do not have the same effect on normal primary prostate epithelium.

Recent studies have used collagen type 1, a major structural component of the stromal microenvironment, to generate a three-dimen-

sional collagen gel matrix culture system that allowed for the reconstruction of gland-like structures *in vitro* (2). In this study, we have used a modified collagen raft culture technique to examine the differential effects of primary stromal cells cultured from benign and tumor prostatic tissue on prostatic epithelial cells.

Materials and Methods

Cell Lines. PNT1A (7) and PC3, sourced from the European collection of Animal Cell Cultures (Porton Down, United Kingdom) were chosen to represent the behavior of normally derived and metastatic CaP epithelial cells when cocultured with stromal cells. PNT1A were routinely cultured in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine, PC3 were grown in Hams F-12 (ICN, Basingstoke, United Kingdom) supplemented with 7% FCS and 2 mM L-glutamine. All cell culture media were obtained from Life Technologies, Inc. (Paisley, United Kingdom), unless stated otherwise.

Primary Prostate Cell Culture. Tissue was obtained from consenting patients undergoing surgery for prostatic disease at York District Hospital. bPECs and stromal cells (bPS) were cultured from transuretheral resection biopsy chips, confirmed by pathology to be benign prostatic hyperplasia. tPECs and stromal cells (tPS) were cultured from radical prostatectomy samples. Histopathology confirmed that two tPEC samples were from Gleason score 6, and one was from Gleason score 8. All tPS cells were derived from Gleason score 6 tissue.

Primary cell cultures were prepared as described previously (8) using collagenase digestion and differential centrifugation. PECs were cultured in keratinocyte serum-free medium supplemented with 2% FCS, 2.0 mM L-glutamine, 50 μg/ml bovine pituitary extract, 5.9 ng/ml epidermal growth factor, and 1% antibiotic-antimycotic solution (coculture medium). Both bPS and tPS cells were cultured in RPMI 1640 supplemented with 10% FCS, 2.0 mM L-glutamine, and 1% antibiotic-antimycotic solution (stromal growth medium). PECs were used without passage or at passage 1. Stromal cells were used at passage 1.

Collagen Gel Coculture. Collagen type 1 (Collaborative Biomedical Products, Bedford, MA) was prepared at 2.0 mg/ml in reconstitution buffer (0.24 M NaHCO$_3$, 20 mM HEPES, 0.05 N NaOH, and 2.0 mM L-glutamine, pH 7.4) containing RPMI 1640 medium, 10% FCS, and 2×10^5 bPS or tPS cells/ml. Gels were also prepared omitting stromal cells. Collagen gel suspensions (0.5 ml) were plated in 24-well plates. After allowing the gels to set for 1 h at 37°C in 5% CO$_2$, 1 ml of stromal growth medium was added to each gel. The stromal cells were allowed to grow in the gel for 3 days. The medium was then removed. Epithelial cells were then seeded onto the surface of the collagen gels at 1×10^5 cells/ml in their respective growth medium and maintained at 37°C in 5% CO$_2$ for 15 days with a medium change every 2 days. In parallel, epithelial cells and stromal cells were plated onto 12-well plates and allowed to grow until semiconfluent. Inverted phase light microscopy was used to monitor the morphology of cells as monolayers, both on and within the gels. Gels were also monitored for contraction.

Proliferation of Stromal Cells in Collagen Gels. A parallel series of collagen gels was established to compare the growth of bPS and tPS in the coculture medium and in the stromal growth medium. This series of gels was also used to monitor the contraction of gels in the absence of epithelial cells. At days 5, 10, 15, and 20 days, the gels were digested using collagenase type 1 to release the stromal cells. After mixing with trypan blue, viable cells were counted using a hemocytometer.

Table 1 *Immunohistochemical characterization of collagen gel cocultures*

Marker and antigen	Clone	Source	Dilution	Secondary procedures[a]	PNT1A[b]			PC3[b]			bPEC[b]			tPEC[b]		
					ML	bPS	tPS	ML	bPS	tPS	ML	bPS	tPS	ML	bPS	tPS
Luminal epithelial																
Cytokeratin 8	M20	Sigma	1:100	RT	++	++	++	++	++	++	++	++	+	++	++	+
Basal epithelial																
Cytokeratins 1, 5, 10, 14	34bE12	Dako	1:50	RB, SF	+	+	+	+	+	+	+++	+++	+++	++	+++	+++
Prostate specificity and function																
PSMA		Gift[c]	1:100	RT	++	++	++	+	+	+	++	++	+	+	++	++
PSA	Rabbit	Dako	1:50	SB, SF	+	+	+	−ve	−ve	−ve	+	++	+	+	+	+
Cell adhesion																
E-cadherin	HECD-1	R&D	10μg/ml	RB, SF	−ve	−ve	NS	−ve	−ve	−ve	++	++	NS	NS	NS	++

[a] RT, 1:30 dilution of TritC-conjugated rabbit-antimouse; RB, 1:300 dilution of biotinylated rabbit-antimouse; SB, 1:300 dilution of biotinylated swine-antirabbit; SF, 1:300 dilution of FITC-conjugated streptavidin; ML, monolayer.

[b] NS, diffuse cytoplasmic staining.

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Analysis of Morphology and Migration of Epithelial Cells. Collagen gels were mounted in OCT (BDH, Poole, United Kingdom) and snap-frozen in liquid nitrogen. Care was taken to maintain the orientation of the gels. Transverse 10-μm frozen sections were cut using a Leica cryostat (Cryocut 1800) and mounted on Superfrost Plus microscope slides (BDH). Sections were air dried overnight and then fixed for 10 min in 1:1 methanol:acetone. Cells within the gels were then visualized by hematoxylin staining.

Immunohistochemistry. Serial sections were also cut for immunohistochemical characterization and used unfixed. Sections were blocked with either 1:20 rabbit serum or 1:20 swine serum for 20 min. Primary antibodies and secondary procedures, sources, and dilutions used are shown in Table 1. Antibodies and blocking solutions were prepared in 1% BSA/PBS. Primary antibody incubations were carried out for 1 h, and secondary antibody incubations were carried out for 30 min at room temperature. After each step, three washes with PBS were carried out. Counterstaining of cell nuclei was carried out using 1 μg/ml 4′,6-diamidino-2-phenylindole-2HCl (Sigma Chemical Co.). To establish the basal expression levels of each marker, the same procedure was carried out on monolayers of PECs. Samples were observed using a Nikon Eclipse TE300 fluorescent microscope, and images were captured using Openlab image analysis software (Improvision, Coventry, United Kingdom). The intensity of staining was scored using the exposure times needed to capture an image. The stromal cells were characterized for expression of smooth muscle actin as described by Lang *et al.* (5). Results are shown in Table 2.

Results

Growth of Stromal Cells in Collagen Gels. By phase microscopy, bPS and tPS seeded into the collagen matrix were seen to have established growth, developed elongated morphology, and formed a dense three-dimensional matrix in the gels within 5 days, although after the gels had been sectioned, the stromal cells were only visible when the plane of cut was directly through the nucleus. Stromal cell counts revealed that, in the coculture medium, neither bPS nor tPS proliferated, although they remained viable. This contrasted with the situation found when stromal cells were cultured in stromal growth medium. In the case of bPS cells, the population doubling time was 6 days. For tPS cells, it took nearer to 8 days for the number of cells to double.

Morphology of Cell Lines on Stromal Cell-populated Collagen Gels. After 5 days, the gels containing stromal cells had contracted, detached from the well to float free in the medium, making further observation with phase microscopy impractical. PNT1A and PC3 cells were used to optimize the coculture method, and 15 days was chosen

as the optimum time point. Culture beyond 15 days resulted in the disintegration of the gels. PNT1A cells formed a complete monolayer on the surface of bPS gels and retained the morphology seen when cultured on tissue culture plastic, which was polygonal and epithelial (Fig. 1A). In contrast, PNT1A cells on tPS did not form a contiguous sheet and became more elongated (Fig. 1I). In the absence of stromal cells, PNT1A grew as a monolayer on the surface of the gels.

PC3 cells showed similar morphology to that seen on tissue culture plastic on both bPS and tPS gels (Fig. 1, *B* and *J*), which was a fibroblast-like morphology with many pseudopodial extensions and little cell-to-cell contact. Neither in the presence of stromal cells nor in their absence did they form complete monolayers, reflecting their malignant phenotype.

Morphology of PECs on Stromal Cell-populated Collagen Gels. When bPECs were grown on bPS gels, they initially formed colonies of tightly coherent morphology that developed within 5 days into a typically epithelial cobblestone-like monolayer (Fig. 1C). By inverted phase microscopy, colonies of bPECs could be seen within bPS gels. In contrast, on tPS gels, bPECs were more elongated and did not form distinct colonies within the gel or on the surface (Fig. 1K). A loosely associated layer of cells was visible on the surface of the gel.

When tPECs were grown on bPS, they developed an elongated morphology with many pseudopodial extensions, and individual cells invaded into the gel (Fig. 1D). On tPS, the tPECs grew as an epithelial monolayer on the surface of the gel (Fig. 1L). In the absence of stromal cells, PECs did not proliferate, although they did initially adhere and spread out on the gel surface.

Contraction of Collagen Gels. One striking feature of the experimental system was the contrasting effects of different coculture combinations on the extent of collagen gel contraction. For example, more contraction was observed when PNT1A cells were cocultured with bPS than with tPS (Fig. 1, *E* and *M*), whereas PC3 cells with bPS or tPS contracted to the same extent (Fig. 1, *F* and *N*).

In the absence of PECs, the extent of contraction seen in the collagen gels was similar in bPS- and tPS-containing gels. However, the tissue source of stromal cells had a marked effect on the extent of gel contraction when combined with PECs. In cocultures of bPECs, more gel contraction was observed on bPS than tPS gels (Fig. 1, *G* and *O*). In contrast, when tPECs were added to the collagen gels, the situation was reversed; less contraction was seen on bPS than on tPS gels (Fig. 1, *H* and *P*).

Table 2 *Immunohistochemical characterization of stromal cells*

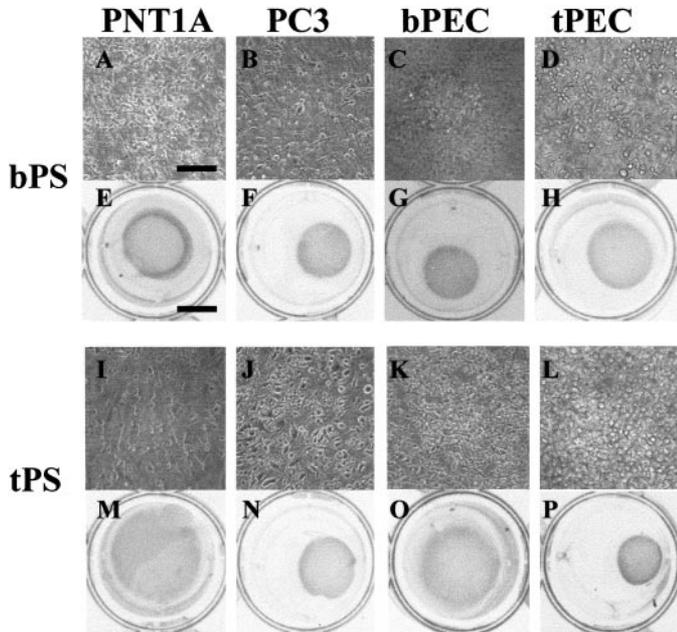| Marker	Antigen	Clone	Source	Dilution	Secondary procedures[a]	% positive (n = 5)	
						bPS	tPS
Smooth muscle	α smooth muscle actin	IA4	Sigma	1:400	RB, SAB, DAB	50%	35%

Fig. 1. Representative inverted phase light microscopy images demonstrating the morphology after 5 days of PNT1A, PC3, bPEC, and tPEC growing on gels containing bPS and tPS are presented (*A–D* and *I–L*). The extent of gel contraction is shown in photographs of individual wells from a 24-well plate (*E–H*) and (*M–P*). The images are representative of coculture experiments that were repeated three times. *Bars: A–D* and *I–L,* 50 μm; *E–H* and *M–P,* 500 mm.

Benign Tissue-derived Stromal Cells Allow the Migration of Benign or Tumor-derived Epithelial Cells into Collagen Type 1 Gels. Observations using phase light microscopy suggested that migration of cells into the gels had taken place. This was confirmed by sectioning and hematoxylin staining of frozen gels.

Five days after seeding the cells onto the gel surface, it was possible, by focusing through the gels, to see that PNT1A cells had migrated into bPS gels but not into tPS gels. Hematoxylin staining showed that PNT1A cells had migrated into bPS gels as single cells without forming apparent structures (Fig. 2*A*). On tPS gels, PNT1A cells formed a loosely associated layer at the surface of the gel (Fig. 2*E*). In the absence of stromal cells, PNT1A cells remained at the surface of the collagen gel.

The highly invasive PC3 cells migrated rapidly into all gels, irrespective of the presence of either bPS or tPS or the absence of stromal cells (Fig. 2, *B* and *F*). Individual cells were clearly visible within the gels by inverted phase light microscopy after 3 days when cocultured

with either bPS or tPS. A similar amount of migration was seen on both bPS and tPS gels (Fig. 2, *B* and *F*).

A significant and reproducible result was the formation of acinus-like structures within the collagen gel when bPECs were cocultured with bPS (Fig. 2*C*). No such acinus-like structures were observed in the absence of stromal cells. In contrast, in coculture experiments of bPEC/tPS, the cells formed a loosely associated layer at the surface of the gel (Fig. 2*G*), similar to that seen with PNT1A/tPS.

In coculture experiments using PECs derived from tumor tissue, the results reflected the Gleason score of the original tumor from which the PECs were derived. On bPS gels, both Gleason score 6 and 8 PECs migrated to a similar extent into the gels (Fig. 2*D*). In contrast, Gleason score 6 tPECs cultured on tPS gels formed a tight monolayer at the surface of the gel (Fig. 2*H*). When sections were cut, this layer easily lifted intact from the surface of the gels. In the case of the Gleason score 8 tPECs, migration into the gel did take place, as observed in PC3 cocultures (results not shown because they are identical to Fig. 2*F*).

Phenotype of Cell Lines Grown on Stromal Cell-populated Collagen Gels. PNT1A showed similar immunohistochemical characteristics in monolayers and on bPS and tPS gels. Staining for luminal cytokeratin was stronger than for basal cytokeratin and PSA and PSMA confirmed prostatic specificity and function. PNT1A were negative for E-cadherin when grown on bPS gels; however, they did show weak cytoplasmic staining on tPS gels. Fluorescent immunohistochemistry results obtained from PC3 cocultures showed no difference between monolayers and bPS and tPS gels (Tables 1 and 2).

Phenotype of PECs on Stromal Cell-populated Collagen Gels. In monolayers and on gels, both bPECs and tPECs stained more strongly for basal cytokeratin than luminal cytokeratin (Fig. 3, *A, B, F,* and *G*). This differential was even more pronounced when they were grown on tPS (Tables 1 and 2). PSA and PSMA expression was maintained (Fig. 3, *C, H, D,* and *I*).

A striking result in primary cell cocultures was obtained after staining with anti-E-cadherin. In both bPEC/bPS gels and Gleason score 6 tPEC/tPS gels, staining was strong and concentrated at cell-cell junctions (Fig. 3, *E* and *J*). Diffuse cytoplasmic staining was observed in cocultures of bPEC/tPS and Gleason score 6 tPEC/bPS. However E-cadherin staining was absent when Gleason score 8 tPECs were grown on either bPS or tPS gels.

Discussion

The primary goal of any valid *in vitro* model of tissue differentiation and disease must be to replicate the *in vivo* situation. Thus, the
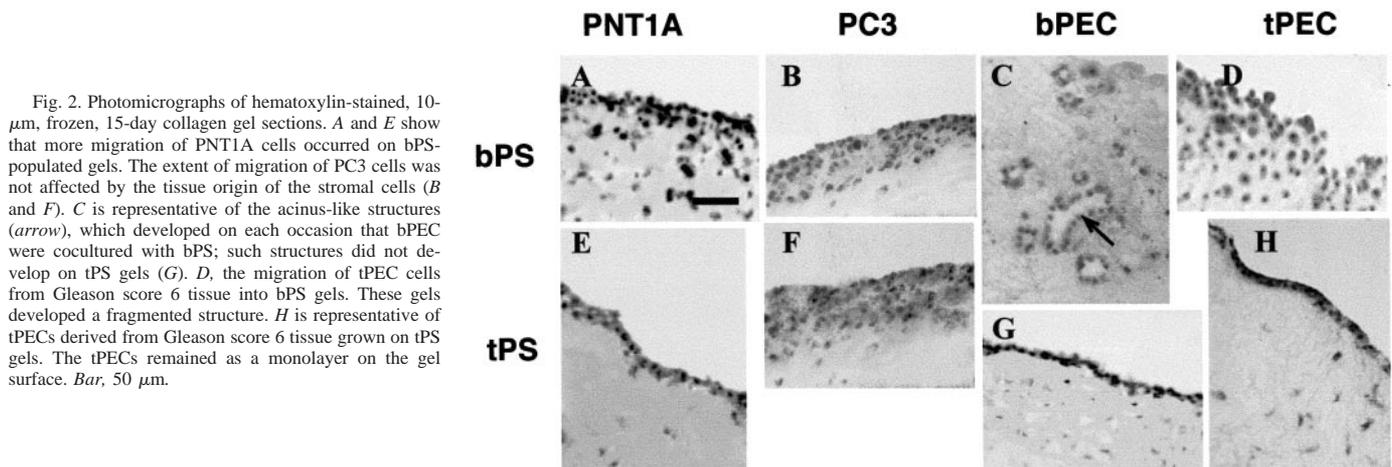
Fig. 2. Photomicrographs of hematoxylin-stained, 10-μm, frozen, 15-day collagen gel sections. *A* and *E* show that more migration of PNT1A cells occurred on bPS-populated gels. The extent of migration of PC3 cells was not affected by the tissue origin of the stromal cells (*B* and *F*). *C* is representative of the acinus-like structures (*arrow*), which developed on each occasion that bPEC were cocultured with bPS; such structures did not develop on tPS gels (*G*). *D*, the migration of tPEC cells from Gleason score 6 tissue into bPS gels. These gels developed a fragmented structure. *H* is representative of tPECs derived from Gleason score 6 tissue grown on tPS gels. The tPECs remained as a monolayer on the gel surface. *Bar,* 50 μm.
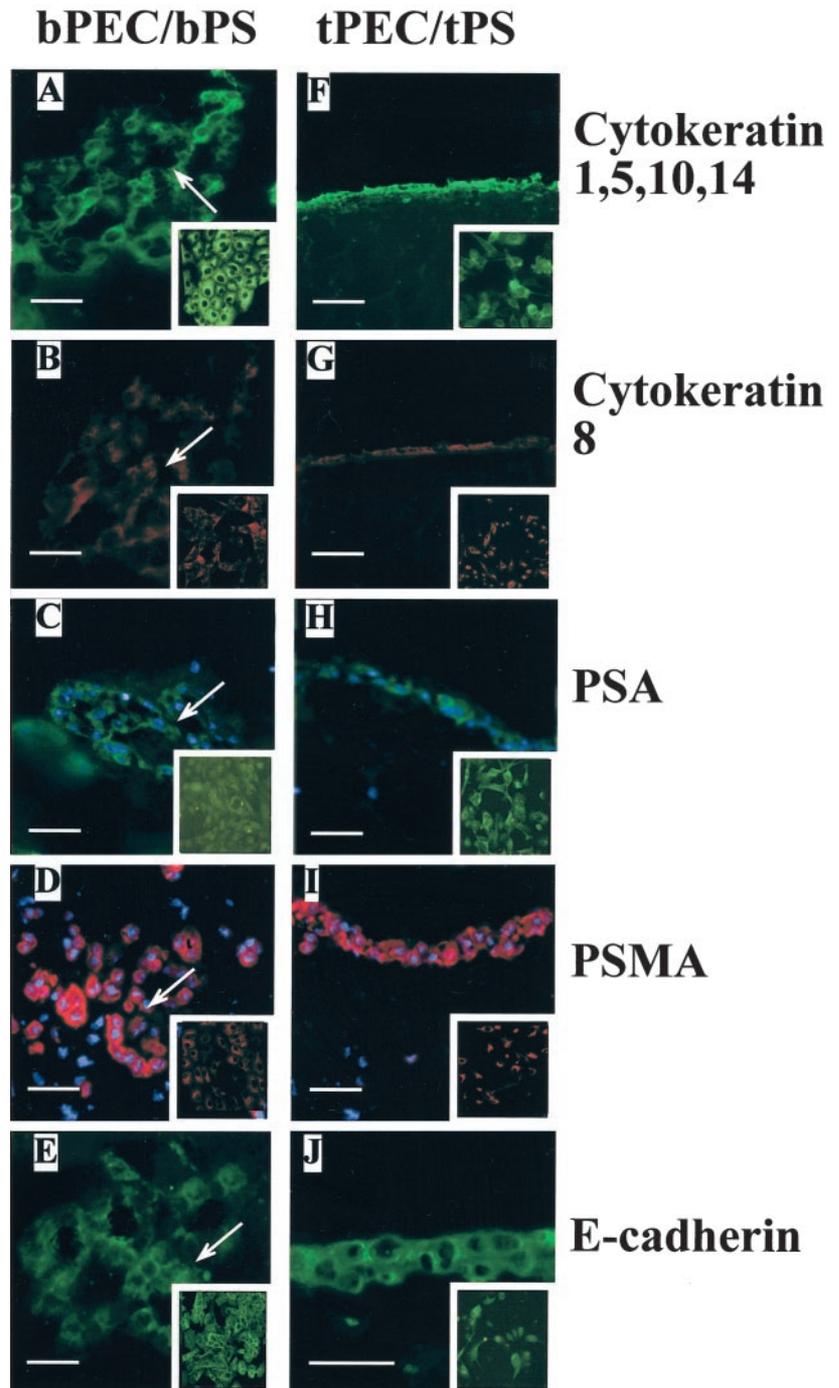
bPEC/bPS   tPEC/tPS

Fig. 3. Typical fluorescent immunostaining of bPEC/bPS and Gleason score 6 tPEC/tPS 15-day, 10-μm, unfixed frozen sections. *Insets,* immunostaining of monolayer PEC cultures. *Arrows,* acinus-like structures. In both cocultures, staining for cytokeratins 1, 5, 10, an 14 (luminal cytokeratins; *A* and *F*) was more intense than staining for cytokeratin 8 (basal cytokeratin; *B* and *G*). Representative staining for prostate-specific markers PSA (*C* and *H*) and PSMA (*D* and *I*) are shown. *E,* strong expression of E-cadherin concentrated at cell junctions of the acinus-like structures. Strong E-cadherin staining at sites of cell-to-cell contact was also found in tPEC/tPS gels (*J*). *Bars: A–J,* 50 μm.

formation of differentiated acinus-like structures only when nonmalignant epithelial and stromal cells were cocultured was an important observation in our three-dimensional collagen culture system. PSA and PSMA expression were clearly observed in the presence of strong intercellular adhesions. Further work will determine the full degree of similarity of these acinus-like structures to those present in normal human prostate tissue. Acinus-like structures were not observed when either the epithelial or stromal cells were derived from tumor tissue or in the absence of stromal cells, emphasizing the importance of normal stromal/epithelial interactions in the maintenance and formation of differentiated acini. Tumor progression is accompanied by the loss of normal glandular morphology and is undoubtedly influenced by the change in stromal/epithelial interactions.

The lack of differentiated acinus-like structures when SV40 immortalized normally derived (PNT1A), PC3, and primary tumor epithelial cells were introduced into the cocultures could be explained by a multitude of factors. In the former case, immortalization by SV40 probably ensures that the ability to differentiate morphologically *in vitro* is destroyed. The lack of differentiated acinus-like structures when tumor cells were introduced into the cocultures could be explained by changes in cell-to-matrix adhesion, cell-to-cell adhesion, motility, and invasion. It is most likely that a combination of all these factors occurs, because they are interdependent. Our coculture system will allow individual assessments of such factors at a cellular level.

It has been shown recently that integrin-to-matrix interactions are critical for acinar morphogenesis (9). Changes in integrin profiles

associated with the progression of CaP (10, 11) may contribute to the altered morphologies observed here. In cocultures of nonmalignant epithelial and stromal cells, strong intercellular E-cadherin expression was noted, matching that seen in intact prostate tissue. Tissue expression of E-cadherin is, however, progressively lost with increasing Gleason score (10). This was reflected by tumor-derived epithelial cells grown in the presence of tumor stroma. Epithelia derived from Gleason 6 tumors expressed cell-to-cell E-cadherin, whereas those derived from Gleason 8 tumors had lost expression. Clearly, the Gleason score 8 epithelial cells behaved more like PC-3, by migrating into both malignant and nonmalignant stroma, which might be expected because both are derived from poorly differentiated tumors. Therefore, our system will also provide a useful tool for studying the behavior and interactions of cells derived from different grades of cancer and their potential effects on cellular architecture and cancer progression.

The significance of malignant stroma has not been studied widely, although there is some preliminary evidence of genetic changes relative to normal stroma in human prostatic tissue (12). The tissue origin of the stromal cells in our cocultures had a distinct effect on the extent to which prostate epithelial cells migrated into the collagen gels. When stromal cells were derived from nonmalignant tissue, the epithelial cells migrated into the gels. On stromal cells derived from Gleason score 6 CaP, only epithelial cells derived from poorly differentiated cancers migrated into the gels. Further study using stromal cells derived from poorly differentiated tumors would extend our understanding of the role that the stroma plays in the progression to a malignant phenotype.

A number of phenotypic changes have also been assigned to malignant stroma such as increased motility *in vitro* (13) and increased expression of matrix metalloproteinase 2 in breast tumor stroma (14). In CaP, malignant stroma produces less HGF (or scatter factor) than nonmalignant (15). HGF is an important paracrine growth factor that influences cell differentiation, adhesion, invasion, and motility (16). This finding provides one explanation why epithelial cells preferentially migrated into collagen gels populated with nonmalignant stromal cells. Future work should establish the contribution of HGF.

More recently, Olumi *et al.* (6) found that *in vivo* culture of tumor-associated fibroblasts and initiated PECs led to the loss of normal acinus morphology. *In vitro*, the normal morphology of skin epidermis, recreated using collagen raft cultures similar to our own, was lost in the presence of tumor stroma (17). These results are consistent with those presented here, suggesting that the ability of epithelial cells to differentiate *in vitro* requires nonmalignant cells.

In prostate and other cancers (5, 18), malignant epithelial cells are known to be more invasive and motile than normal epithelial cells, and such *in vitro* differences undoubtedly contribute to the results found here. The factors that are responsible for these findings have not been established, although expression of matrix metalloproteinases is of great importance for breast epithelial invasive potential and morphology (19).

In summary, prostate cell reconstitution in three-dimensional col-lagen gel cocultures has revealed distinct differences between tumor and normal tissue-derived stromal cells, emphasizing the critical role of prostatic stroma in cancer progression.

Acknowledgments

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