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

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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 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 prostatic epithelium.

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

After 5 days, the gels containing stromal cells had contracted, double.

days. For tPS cells, it took nearer to 8 days for the number of cells to
counts revealed that, in the coculture medium, neither bPS nor tPS
dense three-dimensional matrix in the gels within 5 days, although

But when bPECs were grown on bPS gels, they initially formed colonies
of tightly coherent morphology that developed within 5 days into a

plastic on both bPS and tPS gels (Fig. 1, and

In the absence of PECs, the extent of contraction seen in the
do not form complete monolayers, reflecting their malignant phenotype.

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.

when tPECs were grown on bPS, they developed an elongated
morphology with many pseudopodial extensions and a

Contraction of Collagen Gels. One striking feature of the exper-
timental 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 J).

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 1 Immunohistochemical characterization of collagen gel cocultures

<table>
<thead>
<tr>
<th>Marker and antigen</th>
<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
<th>PNT1A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PC3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>bPEC&lt;sup&gt;c&lt;/sup&gt;</th>
<th>tPEC&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>Cytokeratin 8</td>
<td>Dako</td>
<td>34bE12</td>
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<tr>
<td>PSA</td>
<td>Dako</td>
<td>1:50</td>
<td>SB, SF</td>
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<td>RB, SF</td>
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<tr>
<td>E-cadherin</td>
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<td>RB, SF</td>
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<sup>a</sup> 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.

<sup>b</sup> NS, diffuse cytoplasmic staining.

<sup>c</sup> J. M. Villette, St. Louis Hospital, Paris, France.

### Table 2 Immunohistochemical characterization of stromal cells

<table>
<thead>
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<th>Marker</th>
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<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
<th>bPS</th>
<th>tPS</th>
<th>% positive (n = 5)</th>
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<tbody>
<tr>
<td>Smooth muscle</td>
<td>α smooth muscle actin</td>
<td>IA4</td>
<td>Sigma</td>
<td>1:400</td>
<td>RB</td>
<td>SAB</td>
<td>DAB 50% 35%</td>
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</table>

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. Trans-
verse 10-μm frozen sections were cut using a Leica cryostat (Cryocut 1800)
and mounted on Superfrost Plus microslides (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 immunohisto-
chemical 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 incu-
bations 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 capt-
tured using Openlab image analysis software (Impruvision, Coventry, United
Kingdom). The intensity of staining was scored using the exposure times
needed to capture an image. The stromal cells were characterized for expres-
sion 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 micros-
copy, 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.

% positive (n = 5)

<table>
<thead>
<tr>
<th>Marker</th>
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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. 2A). On tPS gels, PNT1A cells formed a loosely associated layer at the surface of the gel (Fig. 2E). 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. 2C). 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. 2G), similar to that seen with PNT1A/bPS.

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. 2D). In contrast, Gleason score 6 tPECs cultured on tPS gels formed a tight monolayer at the surface of the gel (Fig. 2H). When sections were cut, this layer easily lifted intact from the surface of the gels. In contrast to 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. 2F).

Phenotype of Cell Lines Grown on Stromal Cell-populated Collagen Gels. In monolayers and on gels, both bPECs and tPECs stained more strongly for basal cytokeratin than for luminal 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/bPS 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
The 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 collagen 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

We thank Yorkshire Cancer Research for funding this project, Dr. Mike Sharrard for critical comments, and Kath Ramsey and Katie Hyde for technical help.

References

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