Mesenchymal-Epithelial Interactions between Normal and Transformed Human Bladder Cells

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ABSTRACT

Epithelial cells obtained from normal human urothelium, a cell line derived from a papillary bladder carcinoma, and cells derived from an invasive carcinoma were grown in a serum-free fully defined medium. The interaction between these cell types and normal bladder stromal cells obtained by explant culture in serum were investigated in mixed cultures. These studies showed that normal urothelium was not responsive to the growth factors produced by cultured bladder fibroblasts and the cells did not grow at increased rates in association with living fibroblast layers. Cells derived from a papillary human bladder carcinoma cell line also did not associate well with fibroblast layers or show marked stimulation of growth by preformed layers of fibroblast cells. On the other hand, cells of the EJ carcinoma line, originally derived from a patient with highly invasive disease, easily infiltrated fibroblast layers and were strongly stimulated to grow by the presence of the stromal cells. This model system might therefore be used to determine key elements associated with malignant progression in human bladder carcinoma.

INTRODUCTION

The maintenance of tissue architecture depends upon the controlled interaction between epithelial and mesenchymal cells. It is widely accepted that alterations in these homeostatic controls occur during the process of oncogenic transformation. A critical change in cellular environment occurs during the evolution of an invasive carcinoma when tumor cells breach the basement membrane and begin to divide in the underlying stroma, which contains mesenchymal cells. The ability to proliferate in the stromal environment is therefore critical to tumor progression.

We have taken advantage of our recently developed techniques for the selective growth of bladder urothelium or stromal cells (1) to investigate these interactions in greater detail. Normal urothelial cells and cell lines derived from papillary [RT4 (2)] and invasive bladder cancers [EJ6 (3)] were cultured in direct contact with living bladder fibroblasts to investigate the effects of the mesenchymal cells on cell growth and morphology. The epithelial cell types were also grown on the extracellular matrix produced by fibroblasts or the medium conditioned by them. In this way, we were able to determine the effects of various stromal components on the behavior of urothelial cells with different tumorigenic and invasive potentials.

MATERIALS AND METHODS

Cell Cultures. Cultures of human bladder fibroblasts were obtained from fetal ureter explants grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) that contained 10% heat-inactivated fetal calf serum (Tissue Culture Biologicals, Tulare, CA) and 2% penicillin/streptomycin, as described previously (1). Cell cultures were grown in 60-mm dishes (Falcon, Oxnard, CA) previously rinsed with 0.1% gelatin (Sigma Chemical Co., St. Louis, MO) and formed a confluent layer containing 2.5 x 10^6 cells after 7 days. Positive immunostaining with an anti-vimentin monoclonal antibody (Biogenex Laboratories, Dublin, CA) confirmed that the cells were of mesenchymal origin. Changing the medium to LD (1) serum-free medium (1) when the cells reached confluence arrested fibroblast growth but maintained cell viability. One cell strain (LD412) was used between passages 10 and 18 for all the experiments reported here to standardize the results; however, similar data were generated with independently isolated strains of fibroblasts.

Fibroblast-conditioned medium was prepared from confluent cultures of cells which were washed 2 times with PBS and 1 time with LD medium (see below) and then incubated for 48 h with LD medium. The conditioned medium was centrifuged at 1000 x g for 10 min to remove cellular debris and then diluted 1:1 with fresh LD medium before addition to test cells. The fibroblast layers were recycled up to 3 times for the production of conditioned medium. In some experiments, epithelial cell types were added directly to living cultures of fibroblast layers containing 1-6 x 10^6 cells/60-mm dish. These layers were washed with PBS and LD medium as described above before addition of test cells. The extracellular matrices produced by fibroblasts were prepared from confluent cultures incubated for 6-10 days with daily additions of a final concentration of 25 μg/ml ascorbic acid. Matrices were prepared by lysis of the producer cells in 0.025 M NaOH and were washed and stored in water as described previously (4).

LD medium is a chemically defined medium containing calcium chloride, ethanolamine, phosphoethanolamine, adenine, cholestrin, insulin, and hydrocortisone added to a 50:50 mixture of Dulbecco's modified Eagle's medium and MCDB 153 (Irvine Scientific, Irvine, CA) that includes trace elements and vitamins (1). It does not contain epidermal growth factor, bovine pituitary extract, serum, or any other undefined proteins.

Normal urothelial cell strains derived from fetal ureter tissue were obtained at second passage from 20-week-old abortuses and grown in LD medium; they have a doubling time of approximately 40 h in log phase (1). RT4 is a tumor cell line derived from a recurring papillary low grade stage T2 transitional bladder tumor (2) obtained from the American Type Tissue Collection (ATCC HTB2). EJ cells were isolated from a high grade (G3) invasive bladder carcinoma (3) and were obtained from Dr. Eric J. Stanbridge (University of California, Irvine, CA). The tumor cell lines had doubling times of 33 and 33 h, respectively, in log phase (1). All urothelial cell lines were grown and maintained in serum-free LD medium.

Urothelial cells were seeded at densities that would allow log phase growth to near confluence under optimum condition in 4 to 8 days for growth experiments. Medium was changed every 48 h and observations of growth patterns and representative photomicrographs were recorded prior to harvesting. Cells were harvested with a 0.05% trypsin solution in isotonic phosphate buffer (Difco Industries, Detroit, MI). Cells were counted with a Coulter Counter (Coulter Electronics, Hialeah, FL) and viabilities were determined by the trypsin blue dye exclusion method. Plateating efficiencies for each concentration and condition were calculated by harvesting representative dishes 24 h after seeding and counting the number of viable cells in the dish.

The number of urothelial cells in dishes containing mixtures of cells was determined by immunostaining or DNA staining and measuring the positive-staining fraction of the mixed populations in a flow cytometric.
All experiments were repeated at least twice with each experimental point determined in triplicate.

Cell Staining with Monoclonal Antibody. The percentage of fetal uroepithelial cells or RT4 cells in mixtures containing both epithelial and mesenchymal cells was determined by immunostaining with the murine monoclonal antibody URO 5 (Cambridge Research Laboratories, Cambridge, MA), which is specific for stratified epithelial cells (5) and does not stain cultured mesenchyme (1). Trypsinized cell suspensions of the mixtures containing 0.5 × 10^6 cells were centrifuged and washed with 0.05% Tween 20 in PBS to prevent clumping. Cells were then incubated with 40 μl of URO 5 (0.02 mg/ml in PBS) at 25°C for 1 h, washed with 1 ml of PBS, and incubated in 40 μl of goat anti-mouse IgG conjugated with fluorescein isothiocyanate (0.182 mg/ml diluted in PBS) (Tago Inc., Burlingame, CA), at 25°C for 1 h. Cells were washed in 1 ml of PBS, centrifuged, resuspended in 1 ml of PBS, and stored at 4°C for 1 to 3 h prior to flow cytometric analysis. Corresponding negative controls (i.e., cells incubated in the absence of a primary antibody) were run for each sample.

DNA Staining. The number of EJ cells in the mixed culture dishes was calculated based upon the percentage of aneuploid cells in the sample, since EJ is an approximately tetraploid cell line. Samples containing 0.5 × 10^6 cells were fixed in decreasing dilutions of methanol (50%, 30%) (VWR Scientific) for 30 min on ice, treated with 0.2 mg/ml RNase (Sigma) at 37°C for 10 min, and stained with 100 μl of propidium iodide (0.05 mg/ml in 1.12% sodium citrate solution; Sigma) for at least 15 min prior to running of the sample. Samples were stored at 4°C.

Flow Cytometric Analysis. Samples were run on a Cytofluorograf 501H flow cytometer (Ortho Diagnostic Systems, Inc., Westwood, MA), equipped with a 2150 computer and using a 488 nm argon laser. Cells were gated on the basis of forward versus right angle scatter to eliminate dead cells and cell clusters from analysis. Green fluorescence was measured on a 515–530-nm filter. Red fluorescence was measured on a 590–630-nm filter. Each histogram for RT4 and urothelial cell mixtures was obtained from the analysis of 10^6 cells and the percentage of cells defined as staining positive was the percentage of sample the green fluorescence of which was greater than that of the fibroblast control sample (see Fig. 1). Calibration curves were constructed by staining samples of known compositions with URO 5 (see Fig. 2). The percentage of uroepithelial cells or RT4 cells in each experimental group culture was then determined through extrapolation from this curve. The total number of urothelial cells could then be calculated by multiplying this percentage by the total number of cells in the mixture.

The percentage of EJ cells in mixtures was defined as the percentage of aneuploid cells in the culture. Each histogram was obtained from the analysis of 10^6 cells and the percentage of near-tetraploid cells was defined as the percentage of sample the red fluorescence of which was greater than that of fibroblasts analyzed alone. The number of EJ cells was calculated by multiplying the percentage of aneuploid cells by the total cell count.

Total numbers of urothelial cells were calculated and compared with the original number of cells seeded in each condition. Control experiments (not shown) showed that the fibroblast layers containing 1–6 × 10^6 cells/60-mm dish maintained their starting densities and showed no appreciable loss of viability over a 10-day period.

**RESULTS**

Growth Measurements. Previous experiments had shown that human bladder fibroblasts would grow in medium containing serum but were unable to grow in the LD serum-free medium designed for the growth of uroepithelial cells (1). Control experiments (not shown) showed that fibroblast layers containing 1–6 × 10^6 cells/60-mm dish maintained their starting densities and showed no appreciable loss of viability over a 10-day period in serum-free medium. The stable layers of mesenchymal cells were therefore usable as substrates for the various epithelial cell types to be used in these experiments.

The feasibility of determining the numbers of normal urothelial and RT4 cells cocultured with mesenchymal cells was tested using mixtures of known composition, which were stained with a monoclonal antibody and analyzed in the flow cytometer. A standard calibration curve was first constructed for the various cell mixtures by staining trypsinized layers of epithelial or mesenchymal cells that were grown separately and then mixed in known proportions for analysis in the flow cytometer. The monoclonal antibody URO-5 stains uroepithelial cells, but not mesenchymal cells, in this system (1) and Fig. 1 shows a typical result of URO-5 staining of a mixture of 75% fibroblasts and 25% normal urothelial cells. A calibration curve is shown in Fig. 2, which allowed for the determination of the percentages of mesenchymal and epithelial cells to be made in unknown mixtures.

Cells of the EJ line do not stain with URO-5 and we therefore used DNA staining by propidium iodide to distinguish the tumor cells from the normal fibroblasts, based on their increased DNA content (Fig. 3). Fig. 3 shows the curve obtained from the analysis of a known mixture of 50% fibroblasts and 50% EJ cells and data such as these were used to construct a calibration curve (Fig. 4). Mixtures of two known compositions were run in each experiment in order to determine the relative numbers of EJ cells and fibroblasts at the end of the coculture experiments.

Effect of Fibroblasts on the Growth of Normal and Transformed Bladder Cells. We next tested the effects of the extracellular matrix produced by fibroblasts, medium which had previously been conditioned by fibroblasts, and living fibroblasts on the growth and division of the various urothelial cell types (Table 1). The epithelial cells were seeded at two initial seeding densities, which varied with the cell type being utilized and

**Fig. 1.** Analysis of a mixture of 75% fibroblasts and 25% urothelial cells by flow cytometry. The cell mixture was stained with the URO-5 antibody and analyzed for green fluorescence. **Shaded area,** fluorescence in mixtures stained with URO-5; **unshaded area,** cells incubated without primary antibody.

**Fig. 2.** Calibration curve to determine percentage of mesenchymal and epithelial cells. Mixtures of known compositions were analyzed after URO-5 staining as in Fig. 1 and the results are plotted as the percentage of cells showing positive fluorescence after subtraction of the results obtained with fibroblasts alone. Points, triplicate determinations ± SD (bars).
which were selected to give either little or no increase in cell number after a 6–8-day incubation period in serum-free medium.

Normal urothelial cells showed no increase in cell number when they were seeded at 0.25 × 10^6 cells/dish, but an approximately 2-fold increase in cell number was seen when dishes contained extracellular matrix or when the cells were incubated in medium previously conditioned by bladder fibroblasts. On the other hand, the presence of a living fibroblast layer did not result in any increase in epithelial cell number. When the experiment was repeated with 0.5 × 10^6 cells seeded, the fold increase in the number of cells on plastic was increased, presumably due to the production of an autologous growth factor at sufficiently high concentrations to allow some cell division. The presence of extracellular matrix, fibroblast-conditioned medium, or living fibroblasts did not substantially increase the growth of the cells under these conditions. The results shown in Table 1 are for a single experiment done with one isolate of normal human urothelial cells. The absolute numbers of cells at day 6 differed with two separate isolates of epithelial cells, but the same trend was observed and the presence of living fibroblasts did not substantially increase the growth rate of the urothelial cells. Thus bladder mesenchymal cells, their extracellular matrix, or conditioned medium were not stimulatory for normal urothelial cells. Table 1 also shows that the normal epithelial cells did not significantly alter the number of fibroblasts present in mixed cultures.

The growth rate of the RT4 cell line, which was originally obtained from a low grade papillary tumor, was not altered much by the presence of extracellular matrix or fibroblast-conditioned medium (Table 1). However, the cells were more affected by the presence of living fibroblast layers, since the number of cells was increased by a factor of 10 if the cells were grown on the mesenchymal cells rather than on plastic, on extracellular matrix, or in the presence of fibroblast-conditioned medium. Thus the RT4 cells were responsive to the presence of a living fibroblast layer. As found with normal urothelial cells, the RT4 line had no measurable effect on the growth of an underlying fibroblast layer.

In contrast to these results, the cells of the EJ carcinoma cell line were markedly stimulated by the presence of fibroblast-conditioned medium or the presence of a living layer of human bladder fibroblasts (Table 1). If the cells were seeded at 0.01 × 10^6 cells/60-mm dish, the number of cells after 8 days was increased 150-fold by the presence of fibroblast-conditioned medium and even more by living layers of fibroblasts. A similar trend was seen in the dishes seeded with 0.05 × 10^6 cells, although the magnitude of the effect was diminished. Thus cells of the invasive carcinoma cell line were markedly stimulated by factors produced and secreted by mesenchymal cells. The EJ cells also had a negative effect on the number of fibroblasts present in mixed cultures, presumably due to their degradative activity.

Morphology of Cells Growing on Living Fibroblasts. The morphologies of the different urothelial cells growing on fibroblast layers were also markedly different. Normal urothelial cells remained associated together when seeded on sparse or condensed extracellular matrix. The morphologies of the different urothelial cells growing on fibroblast layers were also markedly different. Normal urothelial cells remained associated together when seeded on sparse or condensed extracellular matrix.

Table 1 Effect of culture conditions on the growth of urothelial cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of cells seeded × 10^6</th>
<th>Plastic</th>
<th>Matrix</th>
<th>Fibroblast-conditioned medium</th>
<th>Living fibroblasts</th>
</tr>
</thead>
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<tr>
<td>Normal urothelium</td>
<td>0.25</td>
<td>0.8 ± 0.06</td>
<td>2.1 ± 0.29*</td>
<td>1.6 ± 0.27*</td>
<td>0.7 ± 0.04</td>
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<tr>
<td></td>
<td>0.50</td>
<td>2.5 ± 0.08</td>
<td>4.4 ± 0.39*</td>
<td>2.3 ± 0.34*</td>
<td>1.1 ± 0.17*</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.5 ± 0.03</td>
<td>0.8 ± 0.13</td>
<td>1.5 ± 0.49*</td>
<td>0.9 ± 0.21*</td>
</tr>
<tr>
<td>RT4</td>
<td>0.25</td>
<td>0.5 ± 0.09</td>
<td>1.2 ± 0.34</td>
<td>1.4 ± 0.21*</td>
<td>1.4 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>2.0 ± 0.24</td>
<td>1.1 ± 0.52</td>
<td>2.8 ± 0.43*</td>
<td>2.9 ± 0.15</td>
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<tr>
<td></td>
<td>0.05</td>
<td>0.8 ± 0.09</td>
<td>1.7 ± 0.42</td>
<td>1.4 ± 0.22*</td>
<td>2.0 ± 0.24</td>
</tr>
<tr>
<td>EJ</td>
<td>0.25</td>
<td>0.6 ± 0.24</td>
<td>1.7 ± 0.47</td>
<td>1.4 ± 0.23*</td>
<td>2.0 ± 0.24</td>
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<tr>
<td></td>
<td>0.50</td>
<td>2.0 ± 0.24</td>
<td>1.7 ± 0.47</td>
<td>1.4 ± 0.23*</td>
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<td>0.05</td>
<td>0.8 ± 0.09</td>
<td>1.7 ± 0.42</td>
<td>1.4 ± 0.22*</td>
<td>2.0 ± 0.24</td>
</tr>
</tbody>
</table>

* P < 0.01.
* P < 0.05.
* P < 0.02.
MESENCHYMAL-EPITHELIAL INTERACTIONS IN UROTHELIUM

thick fibroblast layers and did not spread out on top of the mesenchymal cells (Fig. 5 A and B). A similar result was also observed with the RT4 cells, which formed tight cell-cell connections between each other and did not associate well with the fibroblasts in the mixed culture (Fig. 5C). On the other hand, cells of the EJ cell line, which were markedly stimulated to divide by the fibroblast layers (Table 1), easily infiltrated the interstices between the fibroblasts and tended to spread more easily on the stromal cells (Fig. 5, D and E). These results were similar to our in vivo observations following injection of the two tumor lines directly into the nude mouse urinary bladder (6), since the EJ, but not the RT4 cells, were able to infiltrate and grow in the mesenchyme of the bladder and, ultimately, to metastasize from this site.

DISCUSSION

Many studies have focused on the interactions between normal mesenchymal and epithelial cells (e.g., Refs. 7 and 8) but there has been less work on the effects of oncogenic transformation on these interactions. In these experiments we have measured the response of normal urothelial cells and cells derived from noninvasive and invasive carcinoma cells for their interactions with bladder mesenchymal cells in culture. We

Fig. 5. Photomicrographs of urothelial cell types seeded onto living cultures of bladder fibroblasts of the LD 412 strain. A, normal urothelium growing on sparse fibroblast layers; B, normal urothelium growing on heavy fibroblast layers; C, RT4 cells on thick fibroblast layers; D, EJ cells 4 days after seeding on thin fibroblast layers; E, EJ cells 6 days after seeding on heavy fibroblast layers x 100.
selected these tumor lines because they have xenograft morphologies in the nude mouse similar to those of the parent tumors even after prolonged culture (9) and because we previously had examined the invasive and metastatic activities of the various cell lines in a nude mouse model in which the cells were implanted directly into the mouse urinary bladder (6). These earlier experiments demonstrated clear differences in the invasive activities of the cells; the RT4 cells were not invasive or metastatic in contrast to the EJ carcinoma line. It is remarkable that the tissue culture models now described seem to recapitulate this behavior in vitro.

The serum-free medium developed for the growth of urothelial cells allowed us easily to grow normal and transformed urothelial cells and then to investigate the interaction between them and mesenchymal cells. The normal fibroblasts did not appear to stimulate the growth of bladder epithelium significantly and indeed did not serve as a good substrate for the cells in culture. In this regard the results are different from those of Green et al. (7), who found that the growth and differentiation of epidermal keratinocytes was dependent on the presence of fibroblasts. Normal bladder epithelial cells tended to associate closely together and not to spread on preformed layers of bladder fibroblasts. This relationship between the two cell types as regards the failure to both produce the growth-stimulatory substances and allow infiltration of the epithelial cells into the stroma may reflect the situation as it occurs in vivo.

The cells derived from the RT4 tumor also did not grow in a spread-out configuration on the mesenchyme and were only weakly responsive to growth-promoting effects of the mesenchymal cells. Intimate cell-cell contact was necessary for this effect to be apparent, since the medium previously conditioned by the bladder fibroblasts was not capable of stimulating strong papillary cell growth.

On the other hand, cells of the EJ line were stimulated to grow very considerably by medium conditioned by fibroblasts or by layers of these cells. The cells also tended to infiltrate the layers and to grow in close proximity to the mesenchyme in a manner similar to that observed in the nude mouse bladder (6). These experiments therefore suggest that invasive carcinoma cells may differ from normal and papillary epithelial cells in their ability both to adhere to and to associate intimately with stroma and to respond to growth-promoting factors. It was somewhat surprising that none of the epithelial cell types tested appeared to stimulate the growth of the supporting fibroblast layers, particularly since tumors are often infiltrated with mesenchymal cells. This may be due to the fact that the serum-free medium developed for the growth of epithelial cells (1) is not capable of supporting fibroblast growth even in the presence of putative growth factors elaborated by epithelial cells. On the other hand, the decrease in fibroblast cell number elicited by EJ cells was similar to the human fibrosarcoma-induced destruction of smooth muscle cell cultures we have previously observed (10). Thus, invasive cancer cells seem to recapitulate their behavior in vivo by the in vitro dissolution of mesenchymal cell layers.

The model system which we have developed appears to be particularly suitable for the investigation of cell-cell interactions between urothelial cells in a systematic way. Knowledge of these interactions is critical to our understanding of the nature of tumor progression, since a key step in malignant progression is the ability of transformed cells to grow within the mesenchyme and to respond to the particular microenvironment present within the tissue of infiltration. In this regard, it is obviously highly significant that many recent studies have shown that the growth and biological behavior of implanted tumor cells are dependent markedly on the site of implantation (11). Tumor cells will often metastasize only if implanted orthotopically, suggesting that there are strong interactions between the tissues within the environment of the tumor cell (11). We are therefore beginning an analysis of the factors produced by the bladder fibroblast cells and are attempting to define the adhesive molecules produced by the mesenchyme.

REFERENCES

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