A Novel Coculture Technique Demonstrates That Normal Human Prostatic Fibroblasts Contribute to Tumor Formation of LNCaP Cells by Retarding Cell Death

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Abstract

The microenvironment influences the progression of an epithelial malignancy. To examine the effect of fibroblasts on epithelial cells by direct cell-cell contact in vitro, a coculture system was designed to assess cell death and proliferation of two cell populations when grown together. We used a green fluorescent dye to stain fibroblasts and distinguish them from unstained epithelial cells by a flow cytometer. We show that tumor cell death is 5-fold less when cocultured with normal human prostatic fibroblasts than when cultured alone. In contrast, proliferation of tumor cells was similar when cocultured with normal human prostatic fibroblasts or when grown alone. The reduction in tumor cell death during coculture appears to play a significant role in promoting tumor formation. Combination of prostatic fibroblasts with LNCaP xenografts formed large tumors at a high frequency with a low apoptotic index in vivo, whereas, LNCaP xenografts alone formed small infrequent tumors with a high apoptotic index. Therefore, prostatic fibroblasts promote tumor formation by retarding the apoptotic pathways in tumor cells.

Introduction

Stromal cells affect the development of many organs (1). In addition to the critical role they play in normal development, it has been postulated that stromal cells may play an important role in regulating epithelial malignancies (2). For example, the stroma associated with invasive breast adenocarcinoma has been shown to be composed of activated or abnormal myofibroblastic cells, a stromal phenotype that is not present in tissues with normal breast epithelium (3). Because of these findings, many have investigated the effect of fibroblasts, a major cellular component of the stroma, on epithelial cell growth and differentiation (4–8).

To study the effect of fibroblasts on epithelial cells in vitro, experimental models have assessed both soluble and nonsoluble factors. Soluble factors have been examined by conditioned media experiments or cultures with a microporous membrane between the two cell types (4, 5, 9, 10). Nonsoluble factors have been examined by coculture experiments with a mixture of fibroblasts and epithelial cells. However, coculture of fibroblasts and epithelial cells in direct contact have been challenging to evaluate, because of the inability to differentially analyze each cell type separately (5, 6). To better examine the effect of fibroblasts on epithelial cells by direct cell-cell contact in vitro, we designed a coculture technique that used a vital green fluorescent dye, CMFDA, to differentially stain fibroblasts in cocultures with epithelial cells. Because of this differential staining, we were able to examine fibroblasts and epithelial cells separately and quantitatively for cell death and proliferation. Because this system used viable cells, fibroblasts and epithelial cells can be sorted after coculture for DNA, RNA, and/or protein analysis. In this study, we examined the effect of NHPFs on neoplastic prostate epithelial cells, LNCaP, by direct cell-cell contact both in vitro and in vivo. We show that NHPFs can contribute to tumor formation of these neoplastic epithelial cells by inhibiting cell death of LNCaP cells without affecting their proliferation.

Materials and Methods

Cell Lines. LNCaP cells are prostatic epithelial cells that were derived from a metastatic lymph node of a patient with advanced prostatic adenocarcinoma (11). These cells have lost their epithelial morphology and cellular polarity and have a spindle-like appearance that is characteristic of fibroblasts. Because fibroblasts have similar spindle-like morphological features, it is difficult to differentiate LNCaP cells from fibroblasts in culture by microscopic examination. LNCaP cells were obtained from American Type Culture Collection, and they were grown in complete DMEM (DMEM with 10% FCS both from Life Science Technologies, Inc., Grand Island, NY).

BPH-1 cells are SV40-T immortalized prostatic epithelial cells that intensely express epithelial-specific cytokeratin protein (12). Because of this phenotype, these epithelial cells were initially used for characterization of our coculture system.

Isolation of Primary Human Prostate Fibroblasts. Primary normal prostatic fibroblasts were obtained from a patient undergoing radical cystoprostatectomy for invasive transitional cell carcinoma of the bladder, without evidence of prostatic carcinoma. Prostate tissue was transported to the laboratory and immediately cut into small pieces (1 × 1 × 1 mm) using a scalpel and digested with collagenase type I (Sigma Chemical Co., 225 units/ml) and hyaluronidase (Sigma, 125 units/ml) as described previously (13). Briefly, after overnight digestion, the cellular mixture was vortexed to dissociate the tissue. The released epithelial structures and stromal cells were pelleted and washed to remove the proteolytic enzymes. Cells were grown to confluence in six-well dishes, after which the fibroblasts were separated from contaminating epithelium by differential trypsinization. Aliquots of each specimen were screened by immunocytochemistry with a wide spectrum anti-cytokeratin antibody (DAKO Corp., Carpinteria, CA) to confirm the absence of contaminating epithelial cells. Fibroblasts were used at second or third passage.

Staining of Prostatic Fibroblasts with a Fluorescent Dye and Preparation of Cocultures. Optimal staining conditions were determined by incubating 1 × 10⁵ NHPFs in various concentrations of a green fluorescent dye, CMFDA (Molecular Probes, Eugene, OR; 10 nm stock in DMSO). Ultimately, NHPFs were grown to confluence in six-well dishes and stained with CMFDA at different concentrations in serum-free DMEM for 45 min at 37°C. The
fibroblasts were washed and incubated with complete DMEM for 1 h and again washed to remove any remnant nonmetabolized CMFDA.

The fibroblasts that were fluorescently stained with 5 µM CMFDA were used for coculture. LNCaP cells that were growing in log phase were released from the plate, and 2.5 x 10^5 LNCaP cells were placed on top of the CMFDA-stained NHPFs. The cocultures with NHPF/LNCaP and control plates containing NHPF and LNCaP alone were incubated in medium with minimum serum (DMEM with 0.5% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin) for 1, 2, 3, and 4 days.

Flow Cytometric Analysis of Cocultures for Cell Death and Proliferation. The cocultures (NHPF/LNCaP) and the controls (NHPFs alone and LNCaP alone) were released from the plate by trypsinization, trypsin was deactivated, and cells were counted with a hemacytometer and centrifuged. The supernatant was removed, and the cells were resuspended in 1 ml of DMEM with minimum serum. Subsequently, samples were incubated with PI (Sigma, 25 µg/ml) and Hoechst 33342 (Molecular Probes, Eugene, OR; 5 µg/ml). After incubation with PI and Hoechst 3342, analyses for cell death and proliferation were performed at 6 and 45 min, respectively (14). Experiments were analyzed with a FACSStar Plus flow cytometer (Becton Dickinson, San Jose, CA) equipped with two argon lasers. The CMFDA and PI excitation took place at 488 nm and a laser tuned to 488 nm, and emission was collected through 530/30 nm and 630/22 nm, respectively. Hoechst 3342 was excited with multline UV at 354/363 nm from a second laser. Fluorescence was acquired logarithmically for the analysis of cell death at 480/10 nm and linearly at 451/15 nm for the analysis of cell proliferation. Emission separation was done with a dichroic filter (460 nm). On an Apple Computer (Cupertino, CA) with the Cell Quest software program (Becton Dickinson, San Jose, CA) we acquired and analyzed 10,000 events.

Immunocytochemistry. For the initial characterization of the coculture system, BPH-1 prostatic epithelial cells that express cytokeratin (12) were used to confirm the differential staining between the CMFDA-stained fibroblasts and the nonstained epithelial cells. The CMFDA-stained fibroblasts and the non-CMFDA-stained BPH-1 cells were sorted by a FACS based on high green fluorescence emission (CMFDA positivity) or low green fluorescence emission (CMFDA negativity). After sorting by FACS, the CMFDA-positive and CMFDA-negative cells were plated on glass-chambered slides (Fisher Scientific, Santa Clara, CA), grown in complete medium overnight, and fixed with 95% ethanol for 5 min. Both groups of cells were immunostained with a fibroblast specific marker (anti-vimentin; DAKO Corp.: 1:20 dilution) and an epithelial specific marker (pan anti-cytokeratin; DAKO Corp.: 1:100 dilution) (Fig. 1A). The nucleus was counterstained with hematoxylin. Positive controls included normal human bowel for cytokeratin and normal prostatic tissue for PSA.

Results

Determination of CMFDA Concentration for Staining Prostatic Fibroblasts. Morphology, growth characteristics, relative plating efficiency, and persistence of green fluorescence were used as parameters to determine the appropriate concentration of CMFDA for fibroblast staining in coculture. We determined that 5 µM CMFDA had the least toxic effect on prostatic fibroblasts, and these fibroblasts retained green fluorescence for an appropriate duration for the experiments in this study. CMFDA-stained and unstained fibroblasts have similar growth potential, for at least the first 3 days in culture (Fig. 1A), and exhibit a comparable relative plating efficiency (9 ± 1% versus 14 ± 1.3%). At this concentration, the CMFDA-stained fibroblasts retain an intensity of green fluorescence that is about 1000-fold higher than unstained fibroblasts for greater than 1 week (Fig. 1B) and can easily be distinguished when cocultured with unstained epithelial cells (Fig. 1C).

Flow Cytometry Separated CMFDA-stained Fibroblasts from Epithelial Cells. CMFDA-stained fibroblasts had a mean green fluorescence with a well-defined peak that was three log units higher than unstained cells and allowed them to be distinguished from unstained epithelial cells (Fig. 1C). The cocultured cells were separated by FACS based on high green fluorescence emission (CMFDA positivity) and low green fluorescence emission (CMFDA negativity) and immunostained with fibroblast- and epithelial-specific antibodies. As demonstrated in Figure 1D, the high green fluorescence emitting cells strongly reacted with the fibroblast-specific vimentin antibody, whereas there was no reactivity with the epithelial-specific cytokeratin antibody (the green fluorescence in Fig. 1D represents staining with CMFDA). Conversely, the low green fluorescence emitting cells were nonreactive to vimentin antibody, whereas they were strongly reactive with the cytokeratin antibody. Therefore, in this coculture system, the CMFDA-stained fibroblasts retain the green fluorescent dye without contaminating the unstained epithelial cells. This enabled us to accurately examine cell death and proliferation of fibroblasts and epithelial cells when cocultured together.

TUNEL Assay. Apoptotic cells within the xenografts were identified by using the Oncor ApopTag kit (Oncor, Gaithersburg, MD). Briefly, sections were deparaffinized, rehydrated, and incubated in Oncor Protein Enzyme (20 µg/ml) for 15 min at room temperature. Sections were then incubated in working terminal deoxynucleotidyl transferase enzyme buffer for 1 h at 37°C and washed, and apoptotic bodies were labeled using anti-digoxigenin fluoroscin. Sections were counterstained and mounted with Oncor Propidium Iodide/ Antifade (Oncor). Apoptotic index was determined by evaluating the percentage of apoptotic cells in 30 high-powered microscopic fields for each tissue section. None of the tumors showed any evidence of central necrosis at time of harvest. The periphery of the tumors was used for determining the apoptotic index, because apoptosis was pronounced in the periphery of the xenografts in both LNCaP and NHPF/LNCaP tumors.

Immunohistochemistry. Routinely processed, formalin-fixed, paraffin-embedded tissue blocks were cut into 5-µm sections and mounted on positively charged slides. Tissue sections were deparaffinized with xylene and rehydrated with graded alcohol solutions. After antigen retrieval in citrate buffer (pH 6.0), endogenous peroxidase was quenched in 3% hydrogen peroxide/PBS for 5 min. Slides were washed with PBS and water. Tissue sections for cytokeratin (combination of CAM 5.2 antibody at 1:100 dilution (Becton Dickinson) and AE1/AE3 antibody at 1:600 dilution (Boehringer Mannheim, Indianapolis, IN)) and PSA (polyclonal antibody at 1:3000 dilution; DAKO Corp.) were incubated for 30 min at room temperature with the primary antibody. After incubation with primary antibody, slides were washed with PBS and incubated with a biotinylated streptavidin-horseradish peroxidase secondary antibody (LSAB+: DAKO Corp.), after which reactivity was visualized using DAB+ (DAKO Corp.). Subsequently, slides were counterstained with hematoxylin. Positive controls included normal human bowel for cytokeratin and normal prostatic tissue for PSA.

J.G. and I.K. contributed equally to this work.
Fig. 1. CMFDA staining of prostatic fibroblasts.
A. Growth curve of CMFDA-stained (•) and unstained (□) cells incubated in complete DMEM. Bars, SE. B. CMFDA-stained fibroblasts (•) maintain green fluorescence at a high intensity for more than 1 week. Unstained fibroblasts (□) have low background fluorescence. C. Flow cytometry distinguished CMFDA-stained fibroblasts from unstained epithelial cells. Two distinct peaks were recognizable based on low green fluorescing cells (BPH-1 epithelial cells) and high green fluorescing cells (fibroblasts). Gated areas represent epithelial cells and fibroblasts that were used for analysis. D. After sorting by FACS, cells that display high fluorescence (CMFDA-stained fibroblasts) strongly immunoreacted with anti-vimentin fibroblast-specific antibody but did not immunoreact with epithelial-specific anti-cytokeratin antibody (green fluorescence is due to staining of cells with CMFDA). Conversely, low green fluorescing cells (BPH-1 cells) strongly immunoreacted with epithelial-specific anti-cytokeratin antibody but did not immunoreact with fibroblast-specific anti-vimentin antibody. Bar, 50 μm.
PROSTATIC FIBROBLASTS DECREASE LNCaP CELL DEATH

A.

Fig. 2. LNCaP cell death is decreased when cocultured with NHPFs, whereas proliferation remains unaltered. A. percentage of LNCaP cell death; or B. percentage of LNCaP cells in S phase when grown alone (■) or when cocultured with NHPFs (□).

NHPFs Inhibit Cell Death but Do Not Affect Proliferation of LNCaP Cells in Coculture. We examined the effect of NHPFs on the LNCaP prostate epithelial cells in vitro. In this coculture system, cell death and proliferation were examined by PI exclusion (14–16) and percentage of cells in S phase, respectively. Analysis for cell death (Fig. 2A) demonstrated that cell death in LNCaP grown alone on plastic was 5-fold higher than when LNCaP was cocultured with NHPFs (results are an average of six individual experiments, \( P = 0.0007 \), paired t test). The difference in LNCaP cell death was maximized on day 3 of the experiments. However, analysis of cellular proliferation (Fig. 2B) showed no difference when LNCaP was cultured on plastic alone or when cocultured with NHPFs. These data suggest that NHPFs promote growth of LNCaP in vitro by retarding cell death pathways of LNCaP, while not affecting their proliferative capacity. Despite retardation of LNCaP cell death by NHPFs in coculture, the LNCaP cell count was not significantly different when cocultured with NHPFs or when grown alone in vitro. The lack of LNCaP cell count difference could potentially be explained by the minimum serum (0.5% FCS) in the media in which the in vitro experiments were performed. The minimum serum media was designed to maintain the cells in culture without promoting growth. Therefore, to determine whether retardation of LNCaP cell death by NHPFs in vitro had any biological relevance, we carried out similar experiments in vivo.

NHPFs Stimulate Tumorigenesis of LNCaP Cells in Athymic Mice. It has been shown previously that LNCaP xenografts do not form tumors in athymic mice at s.c. sites (17). We examined whether the combination of NHPFs with LNCaP cells changed the tumorigenic potential of these epithelial cells. NHPFs did not form tumors when s.c. injected into athymic hosts (zero of eight in three different mice), and LNCaP xenografts formed small tumors in the athymic hosts at a low frequency [one of eight (13%) injection sites in three different mice, Fig. 3A]. In contrast, LNCaP cells, when combined with NHPFs, formed tumors at a high frequency [seven of eight (88%) in three different mice injected s.c., \( P = 0.01 \), Fisher’s exact test]. The growth of tumors was examined by measuring the greatest diameter of the xenografts over time. Both the LNCaP and recombinant xenografts (NHPF/LNCaP) had similar rates of growth up to week 7; however, with increased time, LNCaP xenografts shrunk to undetectable levels, whereas the recombinant xenografts maintained their growth and continued to expand (Fig. 3B). Because the in vitro results of NHPF/LNCaP cocultures indicated that NHPFs could reduce LNCaP cell death, we examined the xenografts for cell death at 9 weeks, a time when differential tumor expansion was noted.

To assess cell death in vivo, we performed the TUNEL assay at week 9 of the in vivo studies, a time when small tumors were occasionally detected on xenografts that contained LNCaP and NHPF/LNCaP (Fig. 3B). As shown in Fig. 4, the apoptotic index in the LNCaP xenograft was 5-fold higher than the NHPF/LNCaP xenograft (9.6 ± 1.5% versus 1.7 ± 0.3, \( P < 0.0001 \), t test). Therefore, our in vivo results recapitulated our in vitro findings. Collectively, these data demonstrate that NHPF can contribute to tumorigenesis by retarding apoptotic pathways of LNCaP cells.

To demonstrate that the s.c. tumors arose from LNCaP cells and not from NHPF or other contaminating host cells, the xenografts were examined immunohistochemically by a prostate-epithelial-specific antigen, PSA, and by an epithelial-specific antigen, cytokeratin. Virtually all cells within the tumor were immunoreactive with PSA and cytokeratin antibodies (data not shown). These data demonstrate that the s.c. tumors arose from LNCaP cells.

Discussion

In this study, we describe a novel in vitro coculture system that can be used to examine fibroblast-epithelial interaction in culture. Flow
Several investigators have examined the fibroblast/epithelial interaction in coculture previously (4–6, 9). The interaction between fibroblasts and epithelial cells in neoplastic progression was first examined by Chung and colleagues (7, 8). In their model system, they have shown that immortalized fibroblasts can affect tumor formation of neoplastic prostate cells. However, in vitro models to examine this type of interaction at a more cellular level have been lacking. For example, Pritchett et al. (5) examined the effect of bladder fibroblasts on the growth of two bladder epithelial cell lines, RT4 and EJ. They showed that cocultures of normal bladder fibroblasts with neoplastic bladder epithelial cell lines, RT4 and EJ, stimulated growth of the epithelial cells as assayed by total cell number in culture. However, cell cycle distribution and percentage of cellular death of the fibroblasts and epithelial cells in coculture could not be determined in their studies because of the inability to differentiate between the two populations in coculture. Another group assessed the effect of fibroblasts on growth of prostatic epithelial cells in coculture (6). In that study, the number of epithelial cells in coculture with accompanying fibroblasts was determined by subtraction of total cell number of fibroblasts plated on a control plate alone (6). This model assumes that the number of fibroblasts in the control plates and the coculture plates remain constant. However, this assumption may not be accurate because it has been demonstrated that paracrine signals from epithelial cells can dramatically change the growth and differentiation of the fibroblast population when grown together (8, 18, 19). Other investigators have examined the fibroblast/epithelial interaction in vitro by cocultures with a microporous membrane between the two cell types (4). The disadvantage of this system is that direct cell-cell contact, which may play an important role in fibroblast/epithelial interaction, cannot be examined.

In contrast to the previously described fibroblast/epithelial in vitro models, differential staining with CMFDA easily distinguishes fibroblasts from epithelial cells that are in direct contact. CMFDA-stained fibroblast morphology, proliferative capacity, and plating efficiency is comparable with unstained fibroblasts. In addition, the green fluorescent stain is retained in the stained fibroblasts for at least 8 days, allowing separate cellular measurements over an extended period of time. Because the stained cells used in our study are viable, each population can be examined for differential expression of RNA and proteins.

One limitation of this system is the amount of green fluorescence that is retained by the CMFDA-stained cells. Because the amount of green fluorescence in the CMFDA-stained cells is diminished over time (Fig. 1B), distinguishing fibroblasts from epithelial cells can be difficult, with cocultures for times exceeding 10 days. Under the present staining conditions, cellular effects in coculture must be monitored within this period of time.

In conclusion, we have developed a novel system to examine the interactions between fibroblasts and epithelial cells that are in direct contact in coculture. Using this system, we show that normal human prostatic fibroblasts contribute to tumorigenesis of LNCaP xenografts. Nourishment of the tumor mass could affect the frequency of cell death. For example, survival advantage could also result from endothelial cell recruitment would lead to regression of LNCaP xenografts. The regression of LNCaP xenografts could be attributed to apoptotic activities that were 5-fold higher than that observed in NHPF/LNCaP recombinant xenografts (Fig. 4). Strikingly, both in vitro and in vivo results demonstrate that NHPFs contribute to survival of NHPF/LNCaP recombinant xenografts. The in vitro results demonstrated that LNCaP cell death was less when cocultured with NHPFs than when LNCaP was cultured alone. In contrast, we demonstrated that LNCaP cells had a similar percentage of proliferating cells when cocultured with NHPFs or when grown on plastic alone. These results demonstrate that NHPFs may stimulate tumor formation of LNCaP cells by retarding cell death of this prostatic epithelial cell line.

As demonstrated by the xenograft studies, the retardation of LNCaP cell death in the NHPF/LNCaP cocultures that was observed in vitro correlated with tumor growth in vivo. Both LNCaP and NHPF/LNCaP xenografts had similar rates of tumor growth in athymic mice for the first 7 weeks of the experiment and diverged thereafter (Fig. 3B). The NHPF/LNCaP recombinant xenografts continued to expand at a rapid rate, whereas the LNCaP xenografts regressed (Fig. 3B). This suggests that NHPFs can contribute to survival of NHPF/LNCaP recombinant xenografts. The in vitro results demonstrate that LNCaP cell death in the NHPF/LNCaP cocultures that was observed in vitro was 5-fold higher than that observed in NHPF/LNCaP xenografts as demonstrated by the TUNEL assay. Bar, 50 μm.

Fig. 4. Apoptotic index in the LNCaP xenograft is 5-fold higher than in the NHPF/LNCaP recombinant xenograft as demonstrated by the TUNEL assay. Bar, 50 μm.
cells by inhibiting apoptotic pathways without affecting proliferative pathways in these neoplastic epithelial cells. Because tumor growth is a balance between apoptosis and proliferation, our study has important implications in better understanding the interactions between fibroblasts and epithelial cells in tumor development and progression.

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References

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