Carcinoma-associated Fibroblasts Direct Tumor Progression of Initiated Human Prostatic Epithelium

Aria F. Olumi, Gary D. Grossfeld, Simon W. Hayward, Peter R. Carroll, Thea D. Tlsty, and Gerald R. Cunha

ABSTRACT

The present study demonstrates that fibroblasts associated with carcinomas stimulate tumor progression of initiated nontumorigenic epithelial cells both in an in vivo tissue recombination system and in an in vitro coculture system. Human prostatic carcinoma-associated fibroblasts grown with initiated human prostatic epithelial cells dramatically stimulated growth and altered histology of the epithelial population. This effect was not detected when normal prostatic fibroblasts were grown with the initiated epithelial cells under the same experimental conditions. In contrast, carcinoma-associated fibroblasts did not affect growth of normal human prostatic epithelial cells under identical conditions. From these data, we conclude that in this human prostate cancer model, carcinoma-associated fibroblasts stimulate progression of tumorigenesis. Thus, carcinoma-associated fibroblasts can directly tumor progression of an initiated prostate epithelial cell.

INTRODUCTION

The majority of human cancers are carcinomas that, by definition, arise from epithelial cells that line glands, ducts, and surfaces of organs (1). Consequently, the focus of research to date has been on epithelial cells, or more specifically genetic changes that occur in epithelial cells as they progress from normal to malignant. Multiple genetic alterations are necessary for this transformation to occur (2). However, several lines of investigation suggest that concomitant changes also occur in cells surrounding the epithelial malignancy (3). These cells form the stroma or supportive base for the epithelial layer and are composed of fibroblastic, smooth muscle, inflammatory, endothelial, and nerve cells. Changes in these stromal cells have been postulated to enhance several tumorigenic phenotypes of the epithelial cell (4–7).

The conceptual framework pointing to a role for stromal cells in tumorigenesis comes from the study of embryological development where instructive and permissive interactions (in addition to genetic determinants) are necessary for programming and maintaining epithelial structure and function. The embryonic epithelial and instructive stromal (mesenchymal) cells exchange a reciprocal molecular dialogue that ensures proper organ development and function (8–10). The permissive adult counterparts of these epithelial and stromal interactions are believed to provide the regulatory signals that maintain homeostasis. Malignant transformation of adult epithelial cells disrupts such homeostatic regulation including control of tissue architecture, adhesion, cell death, and proliferation. If the reciprocal molecular exchange between the epithelial and stromal cells is modified during transformation, this could result in stromal cells that receive and transmit altered molecular signals.

Studies of fibroblasts in the vicinity of the malignant lesion also support a role for stromal cells in tumorigenesis (11–13). The fibroblast is a major cell type of the stromal compartment, and as such is intimately involved in orchestrating the stromal half of the dialogue in tissue homeostasis. Modification of fibroblasts in the stroma immediately adjacent to transformed epithelial cells has been documented in several tumor systems (14–18). The phenotypic and genotypic expression patterns of these CAFs are under intense investigation. For example, Ronnov-Jessen et al. (3, 19) demonstrated that the histology and growth characteristics of mammary CAFs were different from those of fibroblasts associated with normal breast epithelial cells. They described the presence of “activated” or abnormal myofibroblasts associated with invasive breast carcinoma cells. Other phenotypic changes ascribed to CAFs include abnormal migratory behavior in vitro (20) and altered expression of growth factors such as platelet-derived growth factor, insulin-like growth factors I and II, transforming growth factor-β1, hepatocyte growth factor/epithelial scatter factor, and keratinocyte growth factor (18, 21–25). Although these phenotypic changes have been documented in CAFs, their consequences or contributions to tumor growth and development have not yet been investigated.

We hypothesized that CAFs may affect tumor progression in non-tumorigenic epithelial cells. To test this hypothesis, we designed a set of experiments that are conceptually equivalent to the study of genetic determinants of tumorigenesis. A general test of oncogenicity is the introduction of a mutated gene into an already altered (initiated/immortal) but nontumorigenic cell line. If a tumor forms, the mutation has contributed to tumor progression. A more stringent test of oncogenicity examines the effect of a mutated gene on a normal cell. If a tumor forms in this case, the mutation has accomplished both initiation and progression of a tumor. To determine whether CAFs can influence the progression and/or initiation of prostate epithelial cells, we grew CAFs with either initiated (TAg-HPE) or NHPE cells, both in vitro and in vivo, and measured changes in tumorigenesis and tumorigenic indicators (morphology, cell death, and proliferation), respectively.

MATERIALS AND METHODS

Culture Conditions

NHPEs and NHPE cells immortalized with SV40-large T antigen (TAg-HPE; Ref. 26) were grown in complete DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Life Technologies) at 37°C in a 5% CO₂ incubator. The medium was devoid of antibiotics except where indicated. NHPE cells were grown in a customized medium that contained DME H16 50%/Ham's F12 50% mix (Life Technologies), 2.5% heat-inactivated dextran-coated/charcoal-stripped FCS, epidermal growth factor (10 ng/ml; Life Technologies), bovine pituitary extract (10 mg/ml; Clonetics, Walkersville, MD), insulin (1 mg/ml; Clonetics), transferrin (5 mg/ml; Sigma Chemical Co., St. Louis, MO), phosphoethanolamine (0.1 mM; Sigma), cholora toxin (0.2 mg/ml; Sigma), penicillin (100 units/ml; Life Technologies), and streptomycin (100
mg/ml; Life Technologies). Cultures were routinely checked for Mycoplasma contamination and found to be devoid of contamination.

Animals
Homozygous athymic CD1 male nude mice (Simonsen, Gilroy, CA; Tacronic Farms, Germantown, NY; Charles River, Hollister, CA; Harlan, Indianapolis, IN) and homozygous athymic male RNU nude rats (Harlan) were obtained and used for tumorigenicity assays.

Cell Preparation
Isolation of Normal Prostatic Cells. Human prostatic tissue was obtained from patients undergoing surgery at the University of California, San Francisco. NHPE cells were derived from adult patients with benign prostatic hyperplasia in whom there was no evidence of prostate cancer. Tissue was obtained from these patients at the time of transurethral resection of the prostate. Such epithelium may not be identical to that from younger patients prior to the development of benign prostatic hyperplasia. However, there is no evidence to suggest that benign prostatic hyperplasia is a premalignant state. Therefore, due to the scarcity of prostatic tissue from young males, we have included epithelial cells derived from benign hyperplasia in our normal population. NHPFs were obtained from an adult patient undergoing cystoscopy for invasive transitional cell carcinoma of the bladder in which there was no histopathological evidence of carcinoma in the prostate.

Tissue specimens were cut into small pieces (1 × 1 × 1 mm), and prostatic epithelial organoids were separated from surrounding stromal cells by methods described previously (27). Briefly, the tissue was enzymatically digested with collagenase type 1 (225 units/ml; Sigma) and hyaluronidase (125 units/ml; Sigma) in RPMI 1640 (with 25 mM HEPES and 10% FCS; Life Technologies) for 2 to 3 h. After incubation in situ, the organoids were collected by gravity sedimentation and washing as described previously (28). The epithelial organoids were frozen in DMSO until further use (29). Fibroblast and smooth muscle-specific markers (vimentin, collagen type IV, fibronectin, and smooth muscle α-actin) were not detected around the intact organoids, indicating that the digestion was complete and that stromal contamination of the ductal organoids was minimal (30).

The supernatant containing the stromal cells was centrifuged at 250 × g for 5 min. The pellet was resuspended and plated in six-well dishes in RPMI 1640 with 1 nM testosterone, a medium that encourages preferential fibroblastic outgrowth. After allowing the cells to grow to confluence, they were briefly trypsinized to release the fibroblasts, which were then removed and expanded in RPMI 1640 with 5% FCS. Microscopy and immunocytochemistry were used to determine the purity of the fibroblastic fraction. Specimens were screened by immunofluorescence with a wide spectrum anticytokeratin antibody (Sigma) to confirm their fibroblastic nature. If needed, fibroblast enrichment by differential trypsinization was repeated. Fibroblasts were used at the second or third passage.

TAG-HPE Prostatic Epithelial Cell Line. TAG-HPE has been used to designate the human prostatic epithelial cell line BPH-1 (26). This clonally derived line was generated by SV40-T immortalization of cells derived from a surgery specimen of prostatic tissue with benign hyperplasia. Malignancy was not detected by histopathological examination. These parental epithelial cells were isolated using techniques described above. This cell line is nontumorigenic, as demonstrated, in that tumors do not develop after growth periods of up to 1 year in an athymic rodent, although viable cells can be detected in the graft site. The TAG-HPE designation is used here to emphasize that these are genetically initiated (by SV40-T) human prostatic epithelial cells.

Isolation of Prostatic CAFs. Primary prostatic CAFs were obtained from prostate tumors from three patients. At the time of radical prostatectomy, tumors were identified in the prostate using histopathological analysis of stained frozen sections. A fresh tissue fragment immediately adjacent to frank carcinoma was used to obtain primary CAFs for growth in culture. Specimens were cut into small fragments, and fibroblasts were separated from contaminating epithelial cells by enzymatic digestion as described above. These CAFs were indistinguishable from normal prostatic fibroblasts morphologically, immuno- cytochemically, and by growth characteristics, as indicated by population doubling times (Fig. 1 and Table 1).

Protocol
In this study, experiments were organized into two parallel series. In the first series, fibroblast/epithelial interactions were examined in vivo using tissue recombinants. In the second series, the same interactions were examined for morphology, viability, and proliferation using an in vitro coculture system. To measure tumor progression, CAFs were combined with initiated TAG-HPE cells. To measure tumor initiation, CAFs were combined with NHPE cells.

Preparation of in Vivo Tissue Recombinants
In this model system, fibroblasts (either NHPFs or CAFs) were mixed with epithelial (either NHPE or TAG-HPE) cells and cast in a collagen gel that was then grafted into a host animal. The tissue recombinant was retrieved after a designated period of time and analyzed.

Tissue recombinants were made by mixing five human prostatic epithelial organoids or 1 × 10^5 TAG-HPE cells with 2.5 × 10^5 human prostatic fibroblasts in 50 μl of type I rat tail collagen (31). The resultant gels were incubated overnight in a 5% CO2, humidified incubator at 37°C in complete RPMI 1640 with 1 nM testosterone and subsequently placed beneath the renal capsule of male athymic rodents. Control tissue specimens consisted of 3.5 × 10^5 cells of each type of fibroblast (CAFs or NHPFs), 3.5 × 10^5 TAG-HPE cells, or 50 NHPE organoids in collagen gel alone. Tissue specimens were grown for 20–85 days. Wet weight of each tissue recombinant was recorded at harvest. Tissues were formalin-fixed, paraffin-embedded, and analyzed histologically.

Preparation of in Vitro Cocultures
In this model system, fibroblasts (either NHPFs or CAFs) were grown to confluence, and different epithelial cells (NHPE or TAG-HPE) were then layered on top. A green fluorescent dye, CMFDA (Molecular Probes, Eugene, OR), was used to stain the fibroblast population prior to addition of the unenriched epithelial population. Fluorescent microscopy as well as flow cytometry easily distinguished the CMFDA-stained cells from the unenriched cells (Fig. 2) and allowed separate measurements of each population. The differential staining was maintained for a minimum of 4 days with no detrimental effects on proliferation and plating efficiency (32).

To prepare a coculture, 1 × 10^5 primary prostatic fibroblasts were grown to confluence in six-well dishes in complete DMEM over a 3-day period. Staining was accomplished by incubation in serum-free DMEM containing 5 μM CMFDA (10 mM stock in DMSO; Sigma), 100 units/ml penicillin, and 100 mg/ml streptomycin for 45 min at 37°C. Subsequently, the medium was aspirated, and the fibroblasts were washed twice with HBSS and further incubated with complete DMEM for 1 h. Epithelial cells growing in log-phase were released from the tissue culture plate by trypsinization, and 2.5 × 10^5 cells were plated on the confluent CMFDA-stained prostatic fibroblasts. The cocultures were incubated with minimum serum medium (DMEM + 0.5% FCS + penicillin 100 units/ml and streptomycin 100 ng/ml) for 1–4 days per period. Morphology, viability, and proliferation of each cell type independently or in coculture with its partner were assessed as described below.

Measurement of Tumorigenic Indicators
Morphological Analysis. For in vivo tissue recombinants, morphology of the tissue recombinants was assessed by H&E staining of paraffin-embedded tissues and by immunohistochemistry. H&E processing was done in a standard fashion. Tissue recombinants were examined for expression of cytokeratin and SV40-T antigen using epoxide retrieval immunohistochemistry (33).

After deparaffinization and dehydration of tissue sections, epoxide retrieval was performed for all specimens using microwave heating for 10 min in 0.01 M Tris-base buffer at pH 9–10. After overnight incubation in the appropriate blocking serum, tissue sections were incubated with either a wide spectrum anticytokeratin antibody at a 1:100 dilution (DAKO Corp.) or the Pab101 antibody to SV40-T antigen at a 1:40 dilution (a generous gift from Dr. John Lehman, Albany Medical College, Albany, NY). After washing, tissue sections

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were incubated with a biotinylated secondary antibody (Amersham Life Science, Inc., Arlington, IL). The avidin-biotin-peroxidase system (Vector Laboratories; Burlingame, CA) was used to detect reactivity using 3,3′-diaminobenzidine (Sigma) as the chromagen and hematoxylin as the counterstain. Prostate-specific antigen expression was determined in NHPF/NHPE tissue recombinants using a rabbit polyclonal antibody (DAKO Corp.) according to the manufacturer’s specifications. Antigen retrieval was not required for this antibody. For in vitro cocultures, light microscopy and CMDFA staining were used to examine the relationship of the epithelial cells to the fibroblasts.

**Viability.** For in vivo tissue recombinants, apoptotic carcinoma cells were identified 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 strength TD enzyme buffer for 1 h at 37°C and washed, and apoptotic bodies were labeled using anti-digoxigenin-fluorescein. Sections were counterstained and mounted with Oncor Propidium Iodide/Fluorescein (Oncor). Apoptotic index was determined using fluorescence microscopy as the percentage of apoptotic cells in six microscopic fields at ×400. For in vitro cocultures, epithelial cell death was determined by measuring relative PI uptake using flow cytometry. This method is useful because viable cells are able to exclude PI, whereas apoptotic and necrotic cells are not (34–36). Coculture experiments were carried out serially for 1-, 2-, 3-, and 4-day incubations. Maximal effect of prostatic CAFs on proliferation and cell death of TAg-HPE cells was seen on day 3 (data not shown). The cocultured cells and the appropriate controls were released from the plates by trypsinization and centrifuged. The cells were resuspended in 1 ml of minimum serum medium, and samples were incubated with PI (25 μg/ml; Sigma) and Hoechst 33342 stain (5 μg/ml; Molecular Probes) for 6 min. Experiments were carried out in triplicate and analyzed on a dual-laser FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) equipped with two argon lasers. The CMFDA and PI excitation took place with the first laser tuned to 488 nm. Emissions of CMFDA and PI were collected at 530/30 nm and 630/22 nm, respectively. Hoechst 33342 was excited with multiline UV at 354–363 nm from a second laser, and its fluorescence was acquired at 451/15 nm logarithmically after incubation. Ten thousand events were acquired and analyzed on a Macintosh computer with the Cell Quest software program (Becton Dickinson).

**Proliferation.** For in vivo tissue recombinants, proliferation measurements in paraffin-embedded samples of the tumor explants were performed using

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Fig. 1. Characterization of human epithelial and fibroblastic populations. Phase contrast and fluorescent photomicrographs of NHPE, NHPF, TAg-HPE, and CAFs are presented. NHPE represents an epithelial organoid in culture prior to cellular outgrowth. The epithelial cells (NHPE and TAg-HPE) were strongly reactive with anticytokeratin antibody (B and H), whereas they were weakly reactive or nonreactive with antivimentin antibody (C and L). Cytokeratins are specific to epithelial cells, whereas vimentin is normally expressed in fibroblasts and not epithelium. In contrast, fibroblasts were strongly reactive to antivimentin antibody (F and L) and nonreactive to the anticytokeratin antibody (E and K). Bar, 50 μm.
Ki-67, a proliferation-specific marker (37), as described previously (38). For in vitro cocultures, determinations were performed as above with the exception of a 45-min incubation in Hoechst 33342. Fluorescence intensity was displayed in a linear scale. For tissue culture, population doubling times and relative plating efficiency for cells grown in monolayer were determined as described previously (39).

Tumorigenesis

To assess the growth of tissue recombinants in vivo, collagen gels containing the indicated cells were grafted beneath the renal capsule of athymic mouse or rat hosts 50–120 days of age. Grafts were removed after 20–85 days as indicated. All animal protocols were approved by the University of California San Francisco Committee for Animal Research.

Morphometric Analysis

Representative sections from those tissue recombinants composed of prostatic CAF/TAg-HPE cells that had been examined previously for cytokeratin expression by immunohistochemistry were chosen for morphometric analysis. Digitized images were acquired from several different microscopic fields. Random lines were drawn across multiple fields, and cell nuclei intersected by these lines were designated as epithelial or stromal based upon the presence or absence of cytokeratin staining. At least 1000 cells/slide and three slides/specimen were counted in this fashion to determine the percentage of epithelial cells and the percentage of stromal cells.

Karyotypic Analysis

Cell cultures at 50–80% confluence were incubated in medium containing Colcemid (0.0625 μg/ml) for 4 h. Mitotic cells were removed from plates, swollen in 0.075 M KCl hypotonic solution, and fixed onto slides. Chromosomes were G-banded with 0.05% trypsin-0.02% EDTA and Giemsa stain (Fisher Scientific).

Comparative Genomic Hybridization

In brief, probes were prepared by nick translation as described previously (40). Two hundred ng of fluorescein-labeled test DNA, 200 ng of Texas Red-labeled normal reference DNA, and 20 μg of unlabeled Cot-1-digested DNA were mixed, precipitated, and resuspended in 10 μl of hybridization mixture. This probe mix was denatured and immediately added to a slide of normal lymphocyte metaphase spreads that had been denatured, dehydrated, and air dried. The probe mix was allowed to hybridize to the metaphase spreads for 72 h. Slides were then washed and counterstained with 4',6-diamidino-2-phenylindole.

Statistical Analysis

Cell death and proliferation indices were compared using the t test. Wet weights of the various tissue recombinants were compared using the Kruskal-Wallis test (nonparametric ANOVA).

RESULTS

Normal and Initiated Prostatic Epithelial Cells, along with Normal and Carcinoma-associated Prostatic Fibroblasts, Compose the Tissue Components of the Oncogenic Model Systems. We examined stromal/epithelial interactions in prostatic tumorigenesis by

<table>
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<tr>
<th>Cell type</th>
<th>Ploidy</th>
<th>Proliferation potential (passages)</th>
<th>Population doubling time (h)</th>
<th>% colony plating efficiency</th>
<th>% cell death</th>
<th>% cells in S-phase</th>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>8.5</td>
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<tr>
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<td>42.0</td>
<td>32.0</td>
<td>9.8</td>
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* ND, not determined.

Table 1 Characterization of fibroblasts and epithelial cells

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<th>85 days</th>
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* N/A, not applicable.

CAFs used in CAF/NHPE tissue recombinants were CAF#2 (n = 3), CAF#3 (n = 2), and one CAF (n = 1) from a recent patient not analyzed in this study.

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combining fibroblastic and epithelial cells both in vivo and in vitro. To determine whether CAFs were capable of stimulating tumor progression, they were grown with initiated epithelial cells. To further test whether CAFs were capable of stimulating both tumor initiation and progression, they were grown with normal epithelial cells.

NHPE cells were isolated as organoid fragments of prostatic ducts and acini from histologically benign prostate tissue. Organoids contained basal cells that expressed cytokeratins 5 and 14 and luminal cells that expressed cytokeratins 8, 18, prostate-specific antigen, and prostatic acid phosphatase. This indicated that the epithelial cells were well differentiated. The typical organoid morphology of the NHPE cells in culture is seen in Fig. 1. These cells have limited proliferative capacity and represent a noninitiated epithelial population.

Stromal cells from normal human prostate were characterized using antibodies to vimentin, actin, myosin, and cytokeratin. Nearly all of the cells expressed vimentin, whereas 10–20% also expressed actin or myosin, which is characteristic of smooth muscle cells typically found in the stroma of the prostate. Thus, these cell populations represent a combination of fibroblasts and myofibroblastic cells that are derived from the stroma of the prostate. For simplicity, these cells are termed NHPFs. NHPFs did not express cytokeratins that are specific for epithelial cells (Fig. 1). The population doubling times, plating efficiency, and karyotype were comparable with other primary human fibroblasts (Table 1). These cells represent normal fibroblastic populations.

The initiated epithelial cell population (TAg-HPE) is represented by the human prostatic cell line BPH-1. As described previously, these cells expressed epithelial-specific cytokeratins in a pattern consistent with luminal epithelial cells in vivo and demonstrated nuclear reactivity with the antibody Pab 101 that detects SV40-T antigen. TAg-HPE cells retained many of the enzyme systems expressed in the prostate. The cobblestone morphology and growth characteristics of these cells are shown in Fig. 1 and Table 1. Karyotypically, these cells are abnormal with a mean chromosome count of 76. Although these cells have acquired some of the markers of transformation, the TAg-HPE cells are nontumorigenic when grown alone either s.c. or beneath the renal capsule of an athymic host for up to 1 year (Table 2; Ref. 26). The TAg-HPE designation underscores that the cells are initiated (by the effects of the SV40-T antigen) and are derived from human prostatic epithelium.

CAFs were indistinguishable immunocytochemically from the NHPFs described above. In Fig. 1, the exponentially growing NHPFs and CAFs had an identical morphological appearance typical of fibroblasts (long and spindle-shaped). However, upon prolonged culture at confluence, the CAFs formed foci in which the cells grew on top of each other, a phenotype that is reminiscent of transformed cells and is not seen with NHPFs. CAF#1, CAF#2, and CAF#3 demonstrated population doubling times that ranged from 22 to 48 h (Table 1). The chromosomal complement of all CAFs, as analyzed by karyotypic analysis and comparative genomic hybridization, was normal.

The baseline morphologies, viability, proliferative indices, and tumorigenicity for each cell population individually were measured and are described in Fig. 1 and Tables 1 and 2, respectively.

CAFs Cause Dramatic Tumor Progression When Grafted as Tissue Recombinants with Initiated Epithelial Cells in Vivo. Three major hallmarks of tumor progression are increased growth, neoplastic tissue morphology, and invasive ability. All three were assessed in our model system.

Control grafts of NHPE cells, TAg-HPE cells, NHPFs, and CAFs, grown individually in collagen gels for 20 to 85 days exhibited minimal growth with a median wet weight of 10 mg or less (Table 2; Ref. 26). Upon histological examination, TAg-HPE cells formed keratinized pearls. These data are consistent with previous reports that TAg-HPE cells are nontumorigenic (26) when grown alone in athymic hosts (even up to 1 year). Control grafts of each type of fibroblast alone (NHPFs and CAFs) demonstrated benign-appearing fibroblastic stroma.

Tissue recombinants composed of CAF/TAg-HPE cells exhibited striking growth with a maximum wet weight approaching 500 times that seen with any of the other tissue recombinants. The median wet weight of these tissue recombinants was 97 mg after 20 days of growth, 198 mg after 42 days of growth, and 180 mg after 85 days of growth. Assessment of two other tumorigenic indicators, cell death and proliferation, demonstrated a low cell death fraction (1.5 ± 0.9%) and a high proliferative fraction (43.9 ± 5.3%), which is to be expected in a tumor. In contrast, control tissue recombinants composed of normal fibroblasts/initiated epithelial cells (NHPF/TAg-
HPE) demonstrated minimal growth after 20 days with a median wet weight identical to that of TAg-HPE cells alone (Table 2). Even after 90 days, the median wet weight of these tissue recombinants did not significantly change. The small epithelial contribution to these tissue recombinants precluded a reliable assessment of epithelial cell death or proliferation in vivo.

The histological appearance of the CAF/TAg-HPE tissue recombinants resembled poorly differentiated adenocarcinomas with areas of squamous differentiation (Fig. 4, A–C). This histology was consistent for all of these tissue recombinants regardless of the wide range of wet weights of CAF/TAg-HPE grafts. Tumors contained poorly differentiated, irregular epithelial cords of varying sizes. Epithelium formed small nests resembling glands in some areas of the tumor and appeared as single cells that were intermingled within a fibrous stroma in other areas. The epithelial nature of these cells was confirmed by immunostaining with a wide spectrum anticytokeratin antibody (Fig. 4B). Furthermore, nuclear immunoreactivity with an antibody to SV40-T antigen confirmed the derivation of the tumors from the grafted TAg-HPE cells (Fig. 4C). Intermixed with these poorly differentiated epithelial cells were areas of squamous epithelium with occasional keratinization (Fig. 4A). Many epithelial cells contained large, polymorphic nuclei with large nucleoli. Stromal cells were intermingled between the epithelial cell islands throughout the tumor.

Morphometric analysis of CAF/TAg-HPE tissue recombinants was performed to assess the percentage of epithelial and stromal cells. Eight representative tissue recombinants composed of CAF/TAg-HPE were chosen. Wet weights of these tissue recombinants ranged from 97 to 5130 mg. Sections were stained with a wide-spectrum anticytokeratin antibody to identify the epithelial components in the tissue recombinants. These tissue recombinants were composed of 80 ± 3% (± SD) epithelium and 20 ± 3% stroma. The contribution of epithelial cells to the tumor mass was similar for all time points analyzed.

Invasive potential is often assessed by the establishment of tumor growth at distant sites with time (i.e., metastases). To determine whether these tissue recombinants had metastatic potential, we attempted to grow the grafts for a prolonged time period (Table 2). CAF#4 and CAF#5 had plating efficiencies that were similar to the other CAF populations described above and exhibited population doubling times of 30.9 and 26.5 h, respectively. The analysis was terminated because of wasting of the host. At the time of sacrifice (85 days), the CAF/TAg-HPE tissue recombinants had completely surrounded both host kidneys, leaving little identifiable normal renal tissue (see Fig. 3A) and an animal that was severely compromised. Metastases were not detected. Consequently, a time period of 42 days was chosen for the remaining analyses. Metastases were not detected under these experimental conditions.

In contrast to the malignant histological appearance of the CAF/TAg-HPE recombinants, the NHPF/TAg-HPE recombinants appeared benign. Histologically, the epithelial cells in NHPF/TAg-HPE tissue recombinants formed small solid cords with few single cells as demonstrated in Fig. 4D. Epithelial cells were surrounded by a benign appearing fibroblastic stroma, which made up the majority of the tissue recombinant.

Tissue recombinants composed of CAF/TAg-HPE were significantly larger than grafts of TAg-HPE alone and tissue recombinants of NHPF/TAg-HPE after 20 days of growth (P = 0.0015), 42 days of growth (P = 0.0013), and 85 days of growth (P = 0.0002).

CAFs Fail to Cause Tumorigenesis When Grafted as Recombinants with Normal Epithelial Cells in Vivo. Although CAFs demonstrated a dramatic ability to affect tumor progression in initiated epithelial cells, they did not elicit extensive tumorigenesis in normal epithelial cells under these conditions. Tissue recombinants composed of CAF/NHPE cells demonstrated minimal growth after 42 days with a median wet weight of 10.5 mg (n = 6).
However, the histological appearance of the epithelial cells was different from that seen when combined with normal fibroblasts. Epithelial cells combined with CAFs formed ductal structures with a stratified squamous lining (Fig. 4E). Such differentiation is abnormal for prostatic epithelium. Again, the epithelial structures were surrounded by a benign-appearing fibroblastic stroma, which made up the majority of the small tissue recombinants. Grafts of NHPF/NHPE tissue recombinants also exhibited minimal growth (4 mg; Table 2). In contrast to the CAF/NHPE tissue recombinants, epithelial cells in the NHPF/NHPE tissue recombinants formed ductal structures lined by tall columnar epithelial cells that expressed prostate-specific antigen. This appearance is characteristic of normal prostatic epithelium (Fig. 4F).

CAFs Cause Altered Epithelial Morphology, Decreased Cell Death, and Increased Cell Proliferation When Cocultured with Initiated TAg-HPE Cells in Vitro. Coculture of CAF/TAg-HPE cells resulted in altered epithelial morphology, decreased epithelial cell death, and increased epithelial proliferation compared with coculture of NHPF/TAg-HPE (Fig. 5). TAg-HPE cells in coculture with CAFs had robust growth, which produced an abundance of cells (Fig. 5). Fluorescent-stained CAFs cocultured with unstained TAg-HPE cells demonstrated a "honeycomb" appearance. The TAg-HPE cells had grown through the fibroblastic monolayer to reach the bottom of the culture dish. There was complete displacement of CAFs, as demonstrated by islands of unstained cells within the green fluorescence-stained fibroblastic monolayer (Fig. 5A). In control cocultures composed of NHPF/TAg-HPE cells, the TAg-HPE cells grew as a monolayer on top of the normal fibroblasts. Displacement of NHPFs was not observed (Fig. 5A). A second tumorigenic indicator, TAg-HPE cell death, was lower when these initiated epithelial cells were cocultured with CAFs as compared with coculture with NHPFs (Fig. 5B). Likewise, a third indicator, cellular proliferation, was also altered in this system. TAg-HPE cell proliferation was increased when in coculture with CAFs as compared with coculture with NHPFs (Fig. 5C). These data demonstrate that CAFs elicited tumorigenic phenotypes in TAg-HPE cells when grown in coculture. Increased epithelial growth into the fibroblastic layer (reminiscent of invading cells), decreased epithelial cell death, and increased epithelial proliferation are all hallmarks of a tumorigenic population.

CAFs Fail to Alter Tumorigenic Indicators When Cocultured with Normal Epithelial Cells in Vitro. Because CAFs enhanced phenotypes associated with tumor progression when grown with immortalized TAg-HPE cells, we asked whether CAFs could enhance a tumor initiation phenotype when grown with genetically normal epithelial cells. When NHPE cells were layered on confluent lawns of CAFs, the epithelial population of cells did not expand. Effects on morphology, cell death, and proliferation were difficult to measure because of a paucity of epithelial cells. To examine these parameters on a cellular basis, microscopic examination of NHPE colonies growing on CAFs revealed an extremely low incidence of mitosis and a lack of pyknotic nuclei (as determined by Hoechst 33258 staining, data not shown). This confirms that proliferation and cell death were low in NHPE cells cocultured with CAFs. In control cocultures of NHPE cells growing on a confluent layer of NHPF, the epithelial cell population demonstrated minimal expansion, growing as single cells and ultimately forming small colonies.

These data demonstrate that although CAFs can have a significant effect on tumorigenic indicators of initiated epithelial cells (TAG-HPE), there was a negligible effect on growth of genetically normal epithelial cells. Furthermore, we also observed that there was no effect of the NHPFs on either the normal or initiated prostatic epithelial cells, as assessed by measuring viability and proliferative indices.

Although the absolute differences in proliferation and cell death observed in the CAF/TAg-HPE cocultures were statistically significant, they were not dramatic. However, prolonged exposure to these subtle differences in decreased cell death and increased proliferation may result in a dramatic cumulative effect in tumor size over time, as indicated in the in vivo model described above.

DISCUSSION

The results of the present study demonstrate that CAFs stimulate tumor progression of initiated epithelial cells both in vivo and in vitro. Human prostatic CAFs grown with initiated prostatic epithelial cells dramatically stimulated the growth and altered the histology of the TAg-HPE population. This effect was not detected when normal prostatic fibroblasts were grown with TAg-HPE cells under the same experimental conditions. In contrast, CAFs did not stimulate the

![Fig. 5. Morphology, cell death, and proliferation of TAg-HPE cells in coculture with CAFs or NHPFs. A, phase and fluorescent images of TAg-HPE cocultures with different fibroblastic populations. The unstained TAg-HPE cells are distinguished from the CMFDA/green fluorescence-stained fibroblasts in coculture. In CAF/TAg-HPE cocultures, the TAg-HPE cells displaced the CAF (green fluorescent cells), reaching the bottom of the plate. Cocultures appeared as “honeycombs.” In NHPF/TAg-HPE cocultures, the TAg-HPE cells grew on top of the fibroblastic monolayer (green lawn of cells) without displacing the fibroblasts. Bar, 50 μm. B, cell death of TAg-HPE cells in coculture. A significantly lower percentage of cell death was observed in TAg-HPE cells when cocultured with CAFs as compared with NHPFs. Percentage of TAg-HPE cell death when cocultured with different fibroblasts were: CAF#1, 8.2%, n = 5, P = 0.0062; CAF#2, 8.4%, n = 3, P = 0.05; and CAF#3, 8.7%, n = 3, P = 0.04 versus NHPF, 12.6%; unpaired t test comparison. C, proliferation of TAg-HPE cells in coculture. A significantly higher percentage of TAg-HPE cells were in S phase when cocultured with CAFs as compared with NHPFs. The percentage of TAg-HPE cells in S phase when cocultured with different fibroblasts were: CAF#1, 17.2%, n = 5, P < 0.0001; CAF#2, 17.1%, n = 3, P < 0.0001; and CAF#3, 21.8%, n = 3, P < 0.0001 versus NHPF, 11.0%, n = 12. Bars, SE.](image-url)
growth of normal epithelial cells under identical conditions. From these data we conclude that in this human prostate model system, CAFs can stimulate progression of tumorigenesis.

**Progression and Initiation.** Demonstration of tumor progression necessitates increased epithelial growth (a balance between proliferation and apoptosis), neoplastic tissue morphology, and invasive ability. Indeed, stimulation of TAg-HPE growth by CAFs was the most striking phenotype, readily detected upon observation both in vitro and in vivo. Epithelial proliferation generated the majority of the tumor bulk in vivo. Neoplastic tissue morphology was evident in these carcinomas generated in CAF/TAg-HPE grafts. Cytological appearance was consistent with malignancy, demonstrating pleiotropy, increased nuclear:cytoplasmic ratio, prominent nucleoli, and lack of cellular polarization. These features are all hallmarks of tumor cells. Tissue architecture was also altered in that there was a lack of normal glandular structures containing luminal and basal cells. Malignant cell growth was typical of adenocarcinoma in that cells formed nests that resembled poorly differentiated glands with disrupted tissue architecture in some areas, whereas in many areas, cells grew individually and infiltrated the stroma. The carcinomatous appearance was not evident when initiated TAg-HPE cells were grown with normal fibroblasts under these same in vivo conditions. Finally, the appearance of CAF/TAg-HPE cocultures in vitro and the tissue recombinants in vivo were both consistent with a tumorigenic phenotype in that the epithelium breached the fibroblastic layer in each case. Demonstration of tumor initiation requires an additional event beyond the increased growth, altered morphology, and invasion necessary for tumor progression. Because CAFs can stimulate these latter properties in genetically altered TAg-HPE cells, they would have unmasked an initiating event in NHPE if such an event had occurred. Although growth of NHPE cells was not stimulated by CAFs, we believe this is not a limiting event. These same NHPE cells can be stimulated to grow and differentiate when combined with instructive urogenital sinus mesenchyme (30). Although CAFs did not promote growth of normal epithelial cells, CAFs did alter epithelial histology. This suggests that abnormal signaling from CAFs is not sufficient to stimulate tumor formation. Epithelial cells in the CAF/NHPE tissue recombinants were benign in appearance, exhibiting a normal nuclear:cytoplasmic ratio, ductal morphology, and lacking local invasion. However, replacement of normal tall columnar cells with an abnormal multilayered stratified squamous appearance suggests that abnormal stromal signals from CAFs are being detected by the NHPE cells on some level. Taken together, these data suggest that CAF signals can affect both normal and genetically altered epithelial cells but that the outcome of such signaling is dictated by the genetic composition of the epithelial cells.

**Fibroblasts in Development, Wound Healing, and Tumorigenesis.** Although the term “fibroblast” is often used in a generic sense, molecular, biochemical and morphological distinctions between different types of fibroblastic cells are becoming evident. In this regard, fibroblasts appear to exhibit a “plastic” phenotype that allows these cells to adapt to normal and pathological situations (41). This has been elegantly demonstrated during embryonic development, where fibroblasts appear to instruct normal epithelial development in a variety of organ systems including the prostate, uterus, and gut (9, 42, 43). This instructive phenotype is characteristic of embryonic fibroblasts because mature adult fibroblasts are rarely able to recapitulate this specialized function.

Tissue reaction to injury (wound healing), as well as certain benign soft tissue proliferative disorders, also represents circumstances in which “reactive” fibroblasts with unique phenotypic characteristics have been described (44–46). These lesions have been characterized by the appearance of so-called “myofibroblasts,” which share structural and biochemical properties with both “normal” fibroblasts and smooth muscle cells. Myofibroblasts begin to accumulate during the early stages of wound healing, are most abundant during wound contraction, and gradually disappear (possibly through apoptotic mechanisms) during the later stages of scar formation (41, 46). In contrast, myofibroblasts appear to be a consistent feature of various fibromatoses. Studies of myofibroblasts in these pathological conditions have revealed a further diversity in fibroblast phenotype because these cells can be categorized based upon their expression of proteins such as vimentin, actin, myosin, and desmin. There is little conclusive evidence as to the cell of origin of these myofibroblasts or to the chemical signals that trigger their accumulation (45). However, it is likely that these cells arise according to specific physiological needs as a result of modified signals from the microenvironment (41).

Finally, neoplasia represents a third circumstance in which the phenotype of “carcinoma-associated” fibroblasts differs from that of fibroblasts found in normal tissue. Previous work has documented such differences including the abnormal expression of smooth muscle actin (similar to that seen in myofibroblasts associated with wound healing), inappropriate secretion of proteolytic enzymes including metalloproteinases (metallomethionine-2 and stromelysin-3; Ref. 14, 47), and the production of extracellular matrix proteins such as tenascin and hyaluronan (15, 48). These differences may enhance the invasive potential of malignant epithelial cells. CAFs may also be capable of modulating the phenotype of nearby epithelial cells. Specifically, epithelial steroid receptor expression (11), as well as the expression of cellular-adhesion molecules (49), may be regulated by CAFs through paracrine signaling mechanisms.

Although changes in CAFs have been described previously, this study gives evidence that the presence of such cells in proximity to an initiated epithelium has important biological consequences. We propose that these CAFs direct progression of the epithelial tumor. In the model system described herein, these CAFs stimulate progression of epithelial tumorigenesis. CAFs consistently do not form tumors when grafted or inoculated alone. They are genetically normal, as determined by comparative genomic hybridization and karyotypic analysis, and they demonstrate a finite life span in culture (Table 1). Moreover, by the criteria examined here, these fibroblasts were morphologically and immunocytochemically indistinguishable from normal fibroblasts.

**Previous Studies.** It should be noted that others have examined previously fibroblast/epithelial interactions in tumor growth and development using immortalized or tumorigenic fibroblastic cell lines rather than the mortal fibroblasts used in this study (Refs. 25 and 50–57; see Fig. 6). These studies have demonstrated that fibroblastic cell lines, as well as initiated fibroblasts that have been altered by viral or chemical carcinogens, can enhance tumor take and growth when co-inoculated with carcinoma cells. This occurs even under conditions in which the carcinoma cells are inoculated below their tumorigenic dose. These initiated and/or tumorigenic fibroblastic cell lines are distinct from the CAFs described herein because the cell lines described previously formed tumors (sarcomas) when injected alone, contained genetic alterations, were immortal, and/or differed from normal fibroblasts in multiple tumorigenic phenotypes.

Fibroblast/epithelial interactions in tumor growth and development have also been studied by co-inoculating normal or CAFs with tumorigenic epithelial cells. Such fibroblasts have been shown to regulate carcinoma cell growth, differentiation, and tumorigenesis, either in a positive or negative fashion (54, 58–63). It must be recognized that the epithelial cells used in these previous studies also differed from those used in the present study. The TAg-HPE cells, although immortalized, have been shown to be nontumorigenic by rigorous criteria. Thus, although previous studies have demonstrated that fi-
broblasts can regulate carcinoma cell growth, the present study is the first to demonstrate that primary, phenotypically normal fibroblasts associated with a human epithelial malignancy can stimulate progression of a nontumorigenic epithelial cell (Fig. 6).

Variability of CAF Phenotype. The CAF/Tag-HPE tissue recombinants presented above demonstrated reproducible variability. Although the clinical stage and Gleason grade of all tumor specimens used to isolate the different CAF populations were similar, the ability of individual CAF isolates to promote tumorigenesis varied. This was not unexpected given the heterogeneous clinical behavior of prostate tumors, even within similar clinical stage and grade strata. The variability in tumorigenesis that was encountered was not a function of the growth period of the tissue recombinants but was dictated by the CAF sample used in the particular tissue recombinant (Table 1). This underscores tumor to tumor heterogeneity. It should also be noted that different CAF subpopulations within the same specimen gave rise to tumors of varying wet weights. This observation likely reflects the variable contribution of normal fibroblasts, CAFs, and smooth muscle cells to the stroma surrounding a prostatic malignancy. Intratumoral variability was not a function of experimental conditions because grafting tissue recombinants containing the same CAF subpopulation on different days gave similar results.

Complementarity of Model Systems. The in vivo and in vitro systems described in this study generated complementary results. All changes in the measured tumorigenic indicators from both systems contributed to tumor growth. CAFs stimulated histological alteration, retarded cell death, and enhanced proliferation of the initiated epithelial cells both in vivo and in vitro. Tissue recombinants composed of CAF/Tag-HPE demonstrated wet weights as much as 500-fold greater than tissue recombinants composed of NHPF/Tag-HPE. The percentage of Tag-HPE cells in S-phase of the cell cycle doubled in coculture with CAF. This would predictably lead to a substantial difference in tumor growth over time. Likewise, the retardation of cell death that was seen when Tag-HPE cells were cocultured with CAF could also have a significant effect on tumor size over time. This 2-fold increase in cell viability (reduced cell death) in conjunction with a 2-fold increase in proliferation would be expected to have a dramatic effect in vivo. Indeed, this is what we observed (Fig. 3). Thus, results from each system validated and recapitulated results from the other. Although the present experiments analyzed the contribution of CAFs to the stimulation of tumorigenesis, other stromal components were not precluded from participation. Endothelial cells, inflammatory cells, and others may have also contributed to the milieu that enhanced tumorigenesis in the in vivo system.

Novel Aspects of the Model Systems. There are several important advantages to the model systems described in the present study: (a) human cells were used to investigate the tumorigenic process. This is important because these cells may be more representative of human cancer than animal models; (b) we used separate but complementary in vitro and in vivo models. Because our in vitro system was validated by our in vivo approach, we will be able to use the in vitro system in the future to examine specific variables that are likely to affect the tumorigenic process; (c) our model systems allowed us to manipulate the genetic and epigenetic components individually. Genetic change is critical in tumorigenesis. However, mutational accumulation in malignant cells occurs on the background of interaction with the tumor microenvironment. Although the microenvironment is often viewed as supportive and responsive, our data demonstrate that it has a more active role in the tumorigenic process. In this regard, the multistep progression that epitomizes carcinogenesis involves genetic alterations in the epithelium as well as epigenetic contributions from the surrounding supportive stromal tissue. Our data highlight the necessity of both components to this process and demonstrate that neither component alone is sufficient to induce tumor progression. The model systems described herein allow for the study of genetic and epigenetic influences independently. The observations set forth in the present study have important implications with respect to the diagnosis and treatment of malignant disease.

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