Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

Differential Influence of Normal and Cancer-Associated Fibroblasts on the Growth of Human Epithelial Cells in an *In vitro* Cocultivation Model of Prostate Cancer

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Abstract

The prostate is composed of a number of different cell populations. The interaction between them is crucial for the development and proper function of the prostate. However, the effect of the molecular cross talk between these cells in the course of carcinogenesis is still unclear. Employing an approach wherein immortalized epithelial cells and immortalized human fibroblasts were cocultured, we show that normal associated fibroblasts (NAF) and cancer-associated fibroblasts (CAF) differentially influenced the growth and proliferation of immortalized human prostate epithelial cells. Whereas NAFs inhibited the growth of immortalized epithelial cells but promoted the growth of metastatic PC-3 cells, CAFs promoted the growth of immortalized epithelial cells but not of PC-3. Cytokine arrays revealed that NAFs secreted higher levels of tumor necrosis factor-α compared with CAFs whereas CAFs secreted higher levels of interleukin-6 (IL-6) compared with NAFs. The growth-inhibiting effects of NAFs were counteracted by the addition of IL-6, and the growth-promoting effects exerted by the CAFs were counteracted by tumor necrosis factor-α. Furthermore, CAFs induced the migration of endothelial cells in an IL-6–dependent manner. Here, we show that normal fibroblast cells have a protective function at very early stages of carcinogenesis by preventing immortalized epithelial cells from proliferating and forming new blood vessels whereas CAFs aid immortalized epithelial cells to further develop. (Mol Cancer Res 2009;7(8):1212–23)

Introduction

Prostate cancer is the second leading cause of cancer-related death in men in western countries (1). Little is known about the initiation of prostate carcinomas. They progress from an early androgen-dependent, organ-confined state to a highly invasive, androgen-independent metastatic disease state (2). The invasive adenocarcinoma arises from a state called prostatic intraepithelial neoplasia (reviewed in ref. 3). Its prominent feature comprises the androgen-dependent proliferation of the luminal epithelium (4). Several factors, such as age, diet, race, environmental factors, steroid hormones, and genetic predisposition, contribute to the development of prostate cancer (2). From all of which age seems to be the most prominent risk factor, probably due to the altered production of steroid hormones (5, 6), which might contribute to the favorable microenvironmental conditions for the development and progression of prostate cancer (7, 8). A correlation between decreased testosterone levels and an increase in proinflammatory cytokines including interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), and IL-1β were observed in aging men (9, 10).

Inflammation is a crucial part of the immune response of the organism against pathogens. Chemokines and cytokines are the mediators of this defense. They are secreted by infected cells to infiltrate monocytes, which are then activated to macrophages, which in turn secrete further cytokines and chemokines to attract other cells of the immune system to destroy the invaders. Dysregulation of this fine-tuned secretion and degradation of immune regulators leads to constant inflammation, which can develop into autoimmune diseases or cancer. Diverse proinflammatory cytokines were observed to be increased in the serum of prostate cancer patients among others, IL-6 (11-13) and TNFα (14). It is widely accepted that the initiation and the progression of prostate cancer are associated with chronic or recurrent inflammation (15). However, thus far, the contribution of the prostate stroma to the regulation of proinflammatory mediators in prostate cancer and the possible mechanisms by which they operate have not been fully elucidated.

The prostate is made of the epithelium and the stroma, which consists of smooth muscle cells (SMC), fibroblasts, nerves, and lymphatics (16). The stroma plays an important role during embryonal development of diverse structures in the prostate. In adult tissues, stromal cells are responsible for the maintenance of a homeostatic equilibrium and in controlling cell size and cell functions of the epithelium they surround. This is achieved through modifications of the extracellular matrix (17, 18). In the case of disturbed homeostasis regulation, stromal cells can contribute to the initiation and progression of cancer (19). During carcinogenesis, the stroma undergoes several changes. Altered fibroblasts, termed cancer-associated fibroblasts (CAF), appear in the close proximity of the tumor, the amount of SMCs decreases, the bioavailability of growth factors increases, inflammatory cells are infiltrated, angiogenesis increases, and stromal protease inhibitors are lost (20-23).

Received 2/26/09; revised 6/2/09; accepted 6/3/09; published OnlineFirst 8/11/09.
Grant support: Center of Excellence grant from Flight Attendant Medical Research Institute, European Community FP6 grant LSHC-CT-2004-503576, and Yad Abraham Center for Cancer Diagnosis and Therapy.
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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).
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doi:10.1158/1541-7786.MCR-09-0073

Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

IL-6 is a pleiotropic cytokine involved in the regulation of growth and differentiation of cancer cells. It interacts with its receptor IL-6R, and the subsequent stimulated Janus-activated kinase/signal transducers and activators of transcription pathway promotes the growth of hormone-refractory prostate cancer cell lines (24, 25). Upon IL-6 treatment, the hormone-dependent LNCaP cells are either inhibited or promoted in their growth depending on experimental conditions such as passage number or serum composition (26). IL-6 stimulates the growth of prostate cancer cells and of prostate cancer in mice through the activation of the androgen receptor (27). The corresponding up-regulation of IL-6R correlates with the proliferation of prostate cancer cells *in vivo* (28). IL-6 has also been shown to be an autocrine and paracrine growth modulator for other cancer types, such as human hepatocellular carcinoma (29), mammary carcinomas (30), cervical carcinomas (31), or lung cancer (32).

TNFα is a cytokine that serves two contradictory functions, either apoptosis or survival. It exerts its pleiotropic actions via the two receptors of the TNF receptor superfamily, TNFR1 and TNFR2 (33, 34). Both receptors are involved in the regulation of the immune system and in inflammation but via different mechanisms. Whereas TNFR1 contributes to the divergent cellular events, such as survival or apoptosis, depending on the complex formed at the intracellular domain (35), TNFR2 controls the access of TNFα to TNFR1, especially at low TNFα concentrations (36). There is evidence showing that tumors and stromal cells surrounding the tumor secrete TNFα. This secretion is usually correlated with a poor prognosis, loss of hormone responsiveness, and cachexia (reviewed in ref. 37).

Tumors that reach a size of ~1 to 2 mm^3 have an increased requirement for nutrients and oxygen. An "angiogenic switch" in the tumor cells caused by hypoxia is the prerequisite for the up-regulation of the expression of proangiogenic proteins. This induces sprouting of preexisting capillaries resulting in the formation of new blood vessels (38). The formation of blood vessels begins with the assembly and tube formation of endothelial cells, which mostly stem from the bone marrow (39). Tumor stromal cells aid in the process of angiogenesis by up-regulation of connective tissue growth factor (40), and CAFs were shown to recruit blood monocytes into tumor tissue via overexpression of Chemokine (C-C motif) ligand 2 (CCL-2) by endogenous IL-6 (41).

Previously, we have created an *in vitro* transformation model by immortalizing primary prostate cells through overexpression of the human telomerase catalytic subunit (hTERT). In this way immortalized cells retained almost all normal features, which makes this system a very valuable tool to investigate very early events of tumorigenesis (42). Furthermore, this system allowed us to investigate the individual effects exerted by fibroblasts on epithelial cells, thereby elucidating their particular contribution to carcinogenesis. Recently, we showed that normal associated fibroblasts (NAF) and CAFs show a different biological behavior in terms of colony formation and proliferation. Furthermore, the secreted protease inhibitor WFDC1, which can inhibit the proliferation of tumor and senescent cells, was found to be highly up-regulated in NAFs compared with CAFs (43). Similar observations were made for breast cancer fibroblast and normal breast fibroblasts (44).

Here, we used a cocultivation system of immortalized human prostate fibroblasts and immortalized human prostate epithelial cells to monitor the proliferation of the epithelial cells. NAFs negatively regulated the growth of epithelial cells, whereas CAFs promoted it. Indeed, we found that prostate NAFs and prostate CAFs secreted different cytokines that were responsible for the opposing effects on the survival of immortalized epithelial cells and on the recruitment of endothelial cells. Our data suggest an important function of normal stromal fibroblast in preventing the development of tumors initiated by very early epithelial cell changes.

Results

NAFs and CAFs Differentially Influenced the Growth of Immortalized Human Epithelial Prostate Cells EP156T and Androgen-Refractory Prostate Cells PC-3

Human immortalized epithelial cells ectopically expressing a firefly-luciferase vector ($EP156T^{-luc}$) were cocultivated with an increasing number of human immortalized fibroblasts expressing a green fluorescent protein (GFP) vector (NAF^{-GFP} or CAF^{-GFP}). Four or eight days later, the cells were lysed and the intensity of the GFP fluorescence and the activation of luciferase were measured. NAFs slightly inhibited the growth of the epithelial cells $EP156T^{-luc}$ after 4 days of cocultivation. After 8 days of cocultivation, a significant decrease in the growth of the epithelial cells could be measured, correlating to the number of cocultivated NAF^{-GFP} (Fig. 1A, *left graph*). CAFs, on the opposite, slightly increased the growth rate of epithelial cells after 4 days and strongly promoted growth after 8 days of cocultivation (Fig. 1B, *left graph*). To ensure that the effects were not due to an overgrowth of NAFs or a diminished growth of CAFs in the coculture, we also measured the growth of the fibroblasts by measuring GFP. Both fibroblasts cocultivated with $EP156T^{-luc}$ grew linearly, which means that the observed effects were not due to an impaired growth of either fibroblast cell type (Fig. 1A and B, *right graphs*).

Next, we wanted to know if NAFs or CAFs exerted similar effects on prostate cancer cells as on immortalized epithelial cells. Therefore, we cocultivated PC-3 cells with NAFs or CAFs for 4 or 8 days. PC-3 cells did not impair the growth of NAFs or CAFs. This was concluded from the linear growth of increasing numbers of fibroblasts cocultured with PC-3, as was monitored by measuring GFP fluorescence (Fig. 1C and D, *right graphs*). Interestingly, NAFs had a significant growth-promoting effect on PC-3 already after 4 days of cocultivation. This effect was dramatically enhanced after 8 days of cocultivation linearly with increasing numbers of NAFs (Fig. 1C, *left graph*). Remarkably, CAFs had no effect on the growth of PC-3 cells neither after 4 days nor after 8 days of cocultivation (Fig. 1D, *left graph*). To ensure that the reverse effect that NAFs and CAFs exerted on PC-3 cells was not due to the impaired growth of the fibroblasts, we checked the growth rates of the fibroblasts between days 4 and 8 in monoculture. Both NAFs and CAFs grew linearly in monoculture, concluding that the reverse effects were not due to an overgrowth of PC-3 cells (Supplementary Fig. S1). Based on these results, we suggest that epithelial cells at different stages of carcinogenesis respond differently to the same environmental stimuli.

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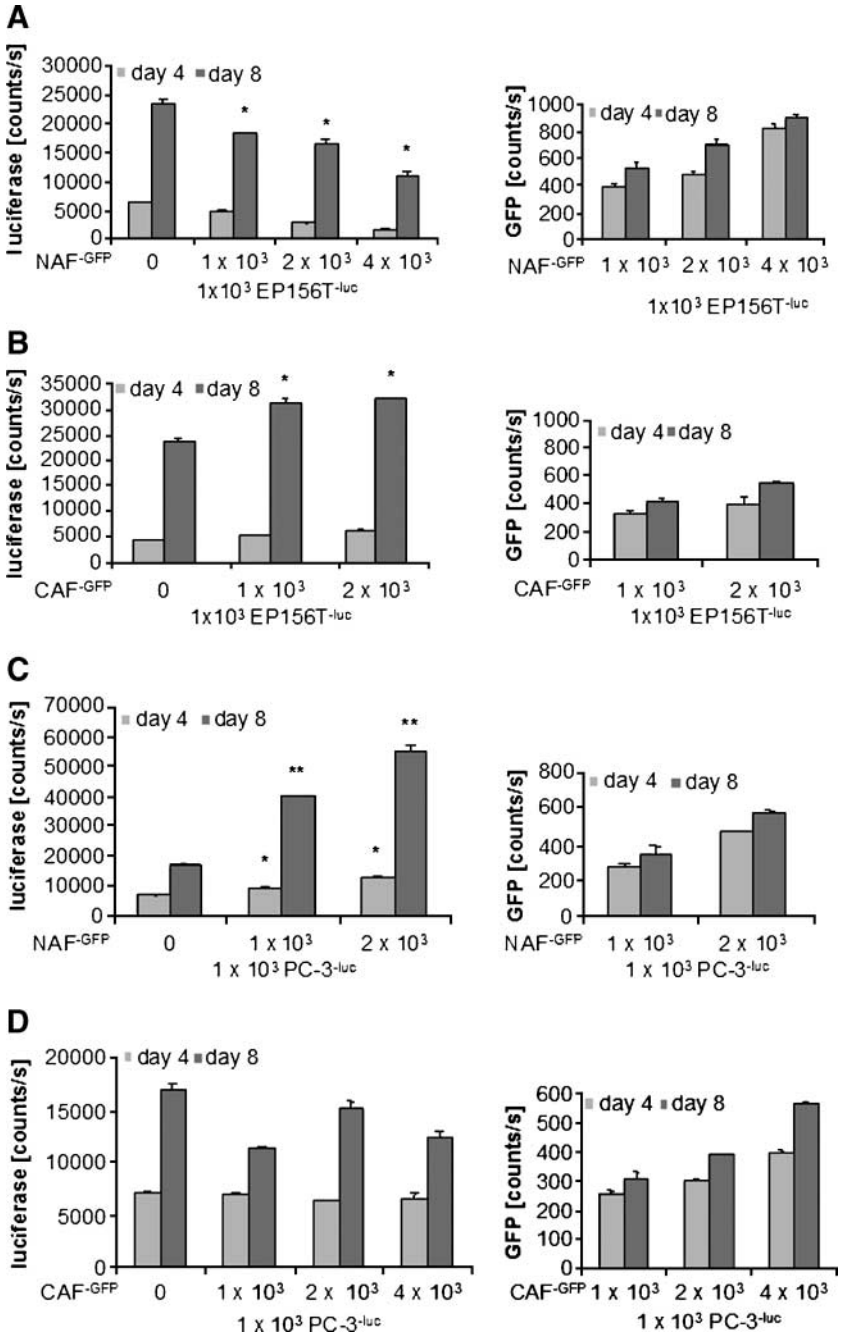

FIGURE 1. Cocultivation of fibroblasts with epithelial cells (EP156T^{-luc}). EP156T^{-luc} cells (1×10^3) were either cocultivated with increasing numbers of NAF (**A**) or CAFs (**B**) for 4 d (*gray columns*) or 8 d (*black columns*). NAFs inhibited the growth of epithelial cells, statistically significant after 8 d of cocultivation; *, $P < 0.05$ (**A,** *left graph*). CAFs promoted the growth of epithelial cells statistically significant after 8 d of cocultivation (**B,** *left graph*). The growth of NAFs or CAFs in cocultivation was monitored by the measurement of GFP fluorescence. Increasing amounts of NAFs or CAFs showed a linear increase in GFP fluorescence (**A, B,** *right graph*). Error bars, SD of the means of three independent experiments. 1×10^3 PC-3 cells were cocultivated with increasing numbers of NAFs (**C**) or CAFs (**D**) for 4 d (*gray columns*) or 8 d (*black columns*). NAFs promoted the growth of PC-3 cells statistically significant after 4 d (*, $P < 0.05$) and after 8 d (**, $P < 0.005$; **C,** *right graph*), whereas CAFs had no influence on the growth of PC-3 cells (**D,** *left graph*). The growth of NAFs or CAFs in coculture was monitored by measuring of GFP fluorescence. Increasing amounts of NAFs showed a linear increase in GFP fluorescence (**C, D,** *right graph*). One representative experiment of three independent experiments. Error bars, SD of the means of duplicates.

Conditioned Medium from NAFs Inhibited and Conditioned Medium from CAFs Promoted Epithelial Cell Growth

To confirm the results obtained from the cocultivation assays and to determine whether factors secreted from fibroblasts or a cell-cell contact were responsible for the effects described above, the proliferation of the epithelial cells treated with conditioned media was determined by using the Wst-1 assay. Because possible secreted factors could be very unstable in the conditioned medium, the experiment was shortened to 12 or 24 h. The proliferation of EP156T^{-luc} after the application of the conditioned medium agreed with the growth regulation observed in the cocultivation experiment. Twelve hours after the application of conditioned medium, the proliferation of epithelial cells was inhibited by conditioned medium from NAFs and enhanced by conditioned medium from CAFs. The effect was less pronounced after 24 hours (Fig. 2). From these experiments, we concluded that secreted factors were responsible for the differential effects exerted by NAFs or CAFs on epithelial cells.

NAFs Secreted High Amounts of TNFα, whereas CAFs Secreted High Amounts of IL-6

To identify the factors secreted from fibroblasts, we subjected conditioned media from NAFs or CAFs to a cytokine

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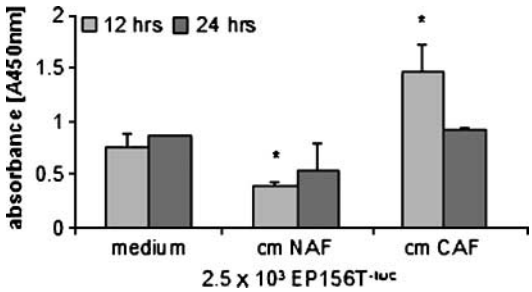

FIGURE 2. Proliferation of EP156T^{-luc} in fibroblast-conditioned media. EP156T^{-luc} cells (2.5×10^3) were treated with conditioned medium from NAFs (*gray columns*) or with conditioned medium from CAFs (*black columns*) for 12 or 24 h. Proliferation was measured with the Wst-1 assay, and absorbance was determined at 450 nm wavelength. The 12-h treatment with conditioned medium from NAFs inhibited the proliferation of epithelial cells, whereas treatment with conditioned medium from CAFs promoted the proliferation of epithelial cells that are statistically significant (*, $P < 0.05$).

array that comprised 42 different cytokines. As seen in Fig. 3A, the most prominent cytokines that were secreted by both types of fibroblasts were GRO, IL-8, and CCL-2. CAFs secreted higher levels of IL-6 compared with NAFs, and NAFs secreted higher levels of TNFα compared with CAFs (Fig. 3A). We confirmed these results by performing ELISAs for the secretion of TNFα and IL-6. Supernatants from NAFs or CAFs were collected at early passages (p14-p16), middle passages (p18-p20), or late passages (p22-p24) 1 day after addition of fresh medium to confluent cells. TNFα was secreted by NAFs at amounts of around 1,000 pg/mL at early passages, down to around 700 pg/mL at middle passages, and further down to around 350 pg/mL at late passages. CAFs secreted only small amounts of TNFα at early passages of around 100 pg/mL, which increased to around 400 pg/mL at middle passages and late passages (Fig. 3B, *left graph*). The secretion of IL-6 from CAFs increased with the number of passages from around 800 pg/mL at early passages up to 1.5 to 1.6 ng/mL at middle and late passages. NAFs also secreted IL-6 at significantly lower amounts from around 600 pg/mL at early passages, down to 300 pg/mL at middle passages, and interestingly up again to around 1 ng/mL at late passages (Fig. 3C, *left graph*). Therefore, we did the following experiments with NAFs and CAFs at middle passages. The secretion pattern corresponded with the expression of the respective mRNA (Fig. 3B and C, *right graphs*). From these results, we suggested that TNFα

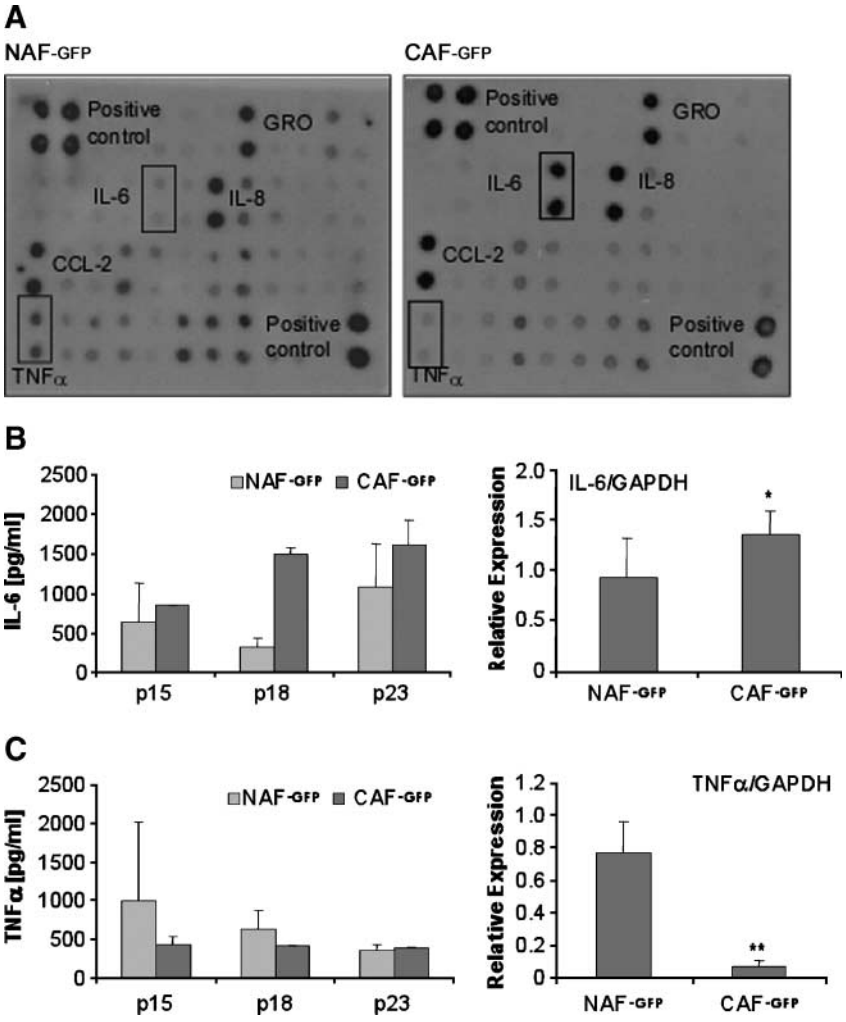

FIGURE 3. NAFs and CAFs secrete different cytokines. Cytokine arrays of secreted factors from NAFs at a middle passage (p19; *top array*) and of secreted factors from CAFs at a late passage (p24; *bottom array*). Cytokines that are profoundly secreted by the fibroblasts GRO, IL-8, and CCL-2 are indicated. Differences are marked with a box. NAFs additionally secreted TNFα, and CAFs additionally secreted IL-6 (**A**). ELISAs for TNFα (**B**) and IL-6 (**C**) were done with supernatants from NAFs (*gray columns*) and CAFs (*black columns*) at early passages (p14-p16), middle passages (p18-p20), and late passages (p22-p24). The secretion of TNFα from NAFs decreased with advanced passages, and CAFs secreted significantly lower amounts of TNFα (**B,** *right graph*). The secretion of IL-6 from CAFs increased with advanced passages. NAFs also secreted IL-6 in significantly lower amounts at early and middle passages, which approximated the amount of IL-6 secreted by CAFs at late passages (**C,** *left graph*). Error bars, SD of the means of three biological independent experiments. The mRNA expression level was measured by quantitative real-time PCR. The expression of TNFα mRNA was statistically significantly (**, $P < 0.005$) increased in NAFs compared with CAFs (**B,** *right graph*), and the expression of IL-6 mRNA was statistically significantly (*, $P < 0.05$) increased in CAFs compared with NAFs (**C,** *right graph*). Error bars, SD of the means of three independent experiments.

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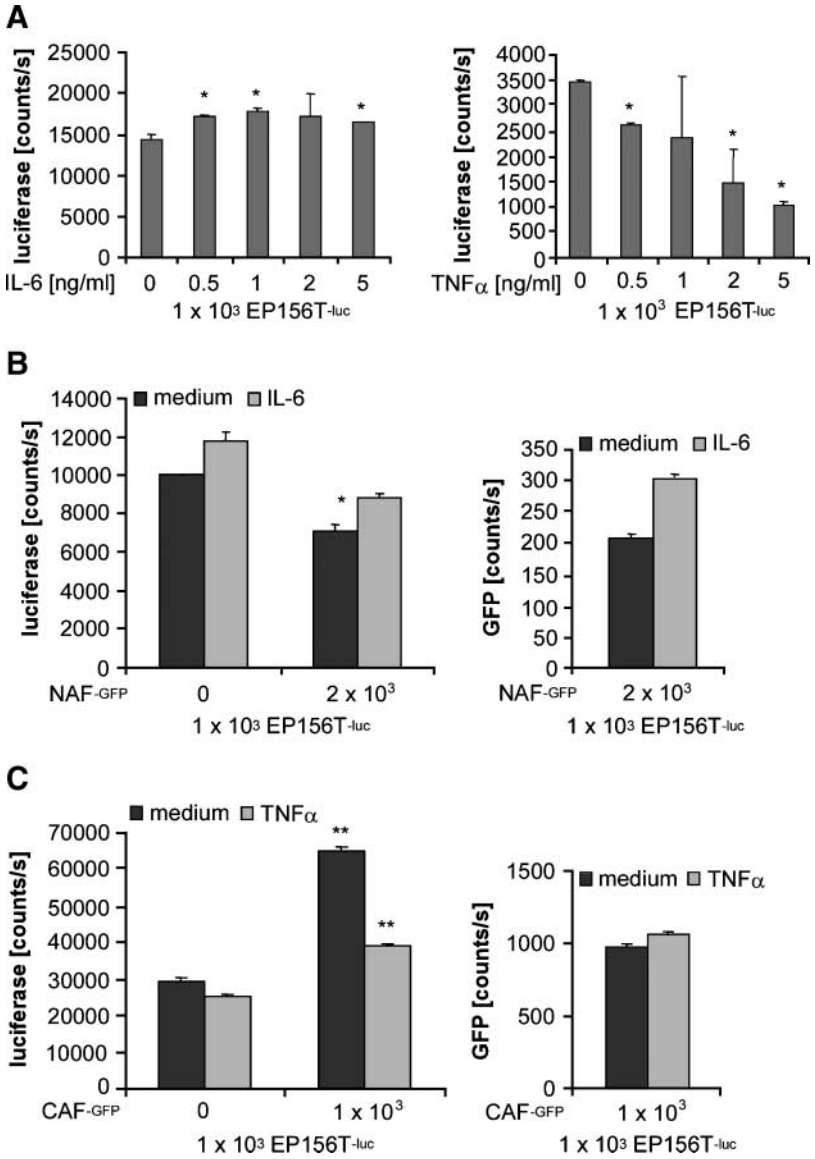

FIGURE 4. IL-6 promotes and TNFα inhibits the growth of EP156T^{-luc}. Growth of 1 × 10^3 EP156T^{-luc} was monitored by measuring luciferase activity 8 d after addition of either 0.5, 1, 2, or 5 ng/mL IL-6 (**A,** *left graph*) or 0.5, 1, 2, or 5 ng/mL TNFα (**A,** *right graph*) to the medium. TNFα inhibited the concentration-dependent growth of the cells, whereas IL-6 increased the growth of epithelial cells independent of the concentration used. Error bars, SD of the means of three biologically independent experiments. Coculture of 1 × 10^3 EP156T^{-luc} with 2 × 10^3 NAFs was supplemented with IL-6 for 4 d. The growth of epithelial cells was monitored by measuring luciferase activity. NAFs decreased the growth of epithelial cells in nontreated cell medium (**B,** *left graph, black column*). Addition of IL-6 led to an increase in growth of epithelial cells in medium by 18% and in coculture with NAFs by 25% (**B,** *left graph, gray column*). Growth of NAFs was monitored by measuring GFP. IL-6 increased the growth of NAFs by 45% (**B,** *right graph, gray column*) compared with the growth in normal medium (**B,** *right graph, black column*). Coculture of 1 × 10^3 CAF^{-GFP} with 1 × 10^3 EP156T^{-luc} was supplemented with TNFα for 8 d. CAFs increased the growth of epithelial cells by 120% (**C,** *left graph, black column*). Addition of TNFα led to a decrease in growth of epithelial cells by 14% in medium and 39% in coculture (**C,** *right graph, gray column*). TNFα had no effect on the growth of CAFs in coculture with epithelial cells (*right graph, gray column*) compared with the growth in normal medium (**C,** *right graph, black column*).

might be involved in the growth inhibitory effect exerted by NAFs and IL-6 might be the factor responsible for promoting epithelial cell growth.

Exogenously Added TNFα Inhibited the Growth of EP156T Cells whereas Exogenously Added IL-6 Promoted It

Next, we wanted to investigate the effect that TNFα or IL-6 had on the growth of epithelial cells. Therefore, we added recombinant TNFα or recombinant IL-6 to EP156T^{-luc} for 4 or 8 days. The concentrations used in this assay were 0.5, 1, 2, or 5 ng/mL TNFα or IL-6. As can be seen in (Fig. 4A, *left graph*), IL-6 promoted the growth of EP156T^{-luc} independent of the concentration used. TNFα exerted a concentration-dependent growth-inhibiting effect on EP156T^{-luc}, with the most profound inhibitory effect when 5 ng/mL TNFα were added (Fig. 4A, *right graph*). Based on these data, we concluded that TNFα and IL-6 were the likely candidates for the above-described effects on the growth of epithelial cells.

Consequently, we supplemented NAFs with IL-6 or CAFs with TNFα when cocultured with EP156T^{-luc}. IL-6 up-regulated the growth of NAFs by 45% when cocultured with epithelial cells. The growth of epithelial cells in monoculture was up-regulated by 18%. NAFs decreased the growth of epithelial cells, which was reversed by 25% by IL-6 (Fig. 4B). TNFα had no influence on the growth of CAFs. The growth of epithelial cells in monoculture was inhibited by 14% by TNFα. CAFs caused a significant increase in the growth of epithelial cells. When TNFα was added, this growth was diminished by 39% (Fig. 4C). Based on this, we concluded that the addition of the particular cytokines had the potential to reverse the effects exerted by NAFs or CAFs on epithelial cells.

To reinforce our findings, these experiments were confirmed with conditioned media. Thus, conditioned medium from NAFs

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was supplemented with 5 ng/mL IL-6 and conditioned medium from CAFs with 50 ng/mL TNFα or left untreated. Conditioned media and control medium were then added to serum-starved EP156T^{luc} for 24 hours, and the proliferation of epithelial cells was measured by the Wst-1 assay. As can be seen in Fig. 5A, conditioned medium from NAFs inhibited the proliferation of epithelial cells; when IL-6 was added, this effect was reversed by 84%. TNFα inhibited the growth of epithelial cells by 19% in cell culture medium; when added to conditioned media from CAFs, the growth was inhibited by 69%. These results further confirmed the results obtained from the cocultivation experiments and strengthened the hypothesis that TNFα secreted from NAFs acts as a growth-inhibiting factor and IL-6 secreted from CAFs as a growth-promoting factor in prostate epithelial cells.

To further strengthen the observation that NAFs inhibited proliferation of prostate epithelial cells, we measured the number of dead epithelial cells in the presence of conditioned medium from NAFs or TNFα and also in the presence of a TNFα-inhibitor. Conditioned medium from NAFs induced cell death of epithelial cells, which was reduced by BAY117082 (Fig. 5B, *left graph*). Also, when TNFα was added to EP156T, cells inhibited by BAY117082 died (Fig. 5B, *right graph*).

To rule out the possibility that the observed effects were specific for the cells from the patient from which they were isolated, epithelial cells from a different patient, designated EP153T, were subjected to conditioned media from NAFs or CAFs for 12 or 24 hours and a Wst-1 proliferation assay was done. Similar results to those observed for EP156T cells were observed. NAFs inhibited the proliferation of EP153T, which was reversed by the addition of IL-6, and CAFs increased the proliferation of EP153T which was counteracted by TNFα (Fig. 5C). This indicated that the effects that fibroblasts exerted on epithelial cells were not patient specific.

Next, we wanted to know if other stromal cells also had a growth-regulating influence on the epithelium. Therefore, we treated EP156T and EP153T cells with conditioned medium from SMCs for 12 and 24 hours. We could observe that SMCs also inhibited the growth of EP153T and EP156T cells 12 hours after addition of the conditioned medium. This effect was also reversed by exogenous IL-6. From these results, we could conclude that the nonmalignant stroma had a tumor-protecting function at very early stages of carcinogenesis (Fig. 5D). To confirm that the growth-inhibiting effect by SMCs could also be exerted by TNFα, we measured the release of TNFα and also IL-6 from SMCs by an ELISA assay. Both cytokines were released by SMCs, whereas TNFα was released in substantially higher amounts than IL-6, similar to the amounts which were released by NAFs (Supplementary Fig. S2).

Furthermore, we wanted to know if the growth-inhibiting effect of NAFs and the growth-increasing effect of CAFs were general mechanisms. Thus, we tested the effects of the fibroblasts on LNCaP cells, another metastatic prostate cancer cell line, and on the non–small cell lung cancer cell line H1299. Both cell lines did not respond to the conditioned media from NAFs or CAFs in terms of proliferation (data not shown), strengthening our hypothesis that NAFs have a protective function at rather very early stages of carcinogenesis.

The Migration of Endothelial Cells toward CAFs Was Dependent on IL-6 and Counteracted by TNFα

Tumors that reach a size of ~1 to 2 mm^3 undergo an "angiogenic switch" triggering the formation of new blood vessels because of an increased requirement for nutrients and oxyen (38). The formation of blood vessels begins with assembly and tube formation of endothelial cells, which mostly stem from the bone marrow (39).

We wanted to examine whether NAFs and CAFs exerted differential effects on the recruitment of endothelial cells. Therefore, we did migration assays of bone marrow–derived endothelial cells toward increasing numbers of NAFs or CAFs or toward conditioned media or control media through a transwell. Approximately, the same number of endothelial cells migrated toward NAFs as toward the control medium. Migration of endothelial cells toward conditioned medium from NAFs was slightly lower but not significantly. Notably, increasing numbers of CAFs recruited increasing numbers of endothelial cells. These effects were dramatically enhanced when endothelial cells were allowed to migrate toward conditioned medium from CAFs (Fig. 6A). From this experiment, we concluded that endothelial cells are recruited to the tumor environment by a soluble factor secreted by the CAFs.

Next, we examined whether the ability of CAFs to recruit endothelial cells is mediated through IL-6 secretion. Therefore, NAFs were treated with either 5 ng/mL IL-6 or left untreated, and the endothelial cells were allowed to migrate toward them for 24 hours. Nontreated NAFs caused a very slight decrease in migration of endothelial cells compared with the migration to the cell culture medium by 13%. Remarkably, the addition of IL-6 caused an increase in endothelial migration of 38% (Fig. 6B).

Complementary, CAFs were treated with TNFα or left untreated. CAFs increased the migration of endothelial cells by 116%. Addition of TNFα reduced the effect by 33% (Fig. 6C). These results suggest that TNFα and IL-6 are also involved in the regulation of the migration of endothelial cells.

Discussion

Prostate cancer mostly arises from a condition called prostatic intraepithelial neoplasia, which is an abnormal change in prostate epithelial cells that eventually become malignant (reviewed in ref. 45). It is well accepted that the microenvironment of the tumor largely contributes to the initiation and progression of cancer. Stromal-epithelial interactions play a critical role in the development of benign prostatic hyperplasia in the progression of tumors and in metastasis (reviewed in ref. 46). Introduction of immortalized epithelial cells or cells from benign prostatic hyperplasia into mice led to the formation of tumors when cocultivated with CAFs, but not with normal fibroblasts (21, 47). These effects were associated with an increased cancer cell proliferation and enhanced angiogenesis, but not increased fibroblast proliferation, as was shown by s.c. injection of MCF-7 breast cancer cells into mice in cocultivation with CAFs or normal fibroblasts (48). *In vitro* studies revealed some of the factors that might be involved in the differential effects that fibroblasts exert on

Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

epithelial cells or benign prostatic hyperplasia cells, such as insulin-like growth factor-I (49), CXCL12 (50), or transforming growth factor-β (51). Here, we also show that stromal fibroblast cells differentially contribute to the proliferation of human immortalized prostate epithelial cells. NAFs inhibited the proliferation of epithelial cells but not of metastatic prostate cancer cells (PC-3), whereas CAFs promoted the growth of human immortalized epithelial cells but not of PC-3 cells.

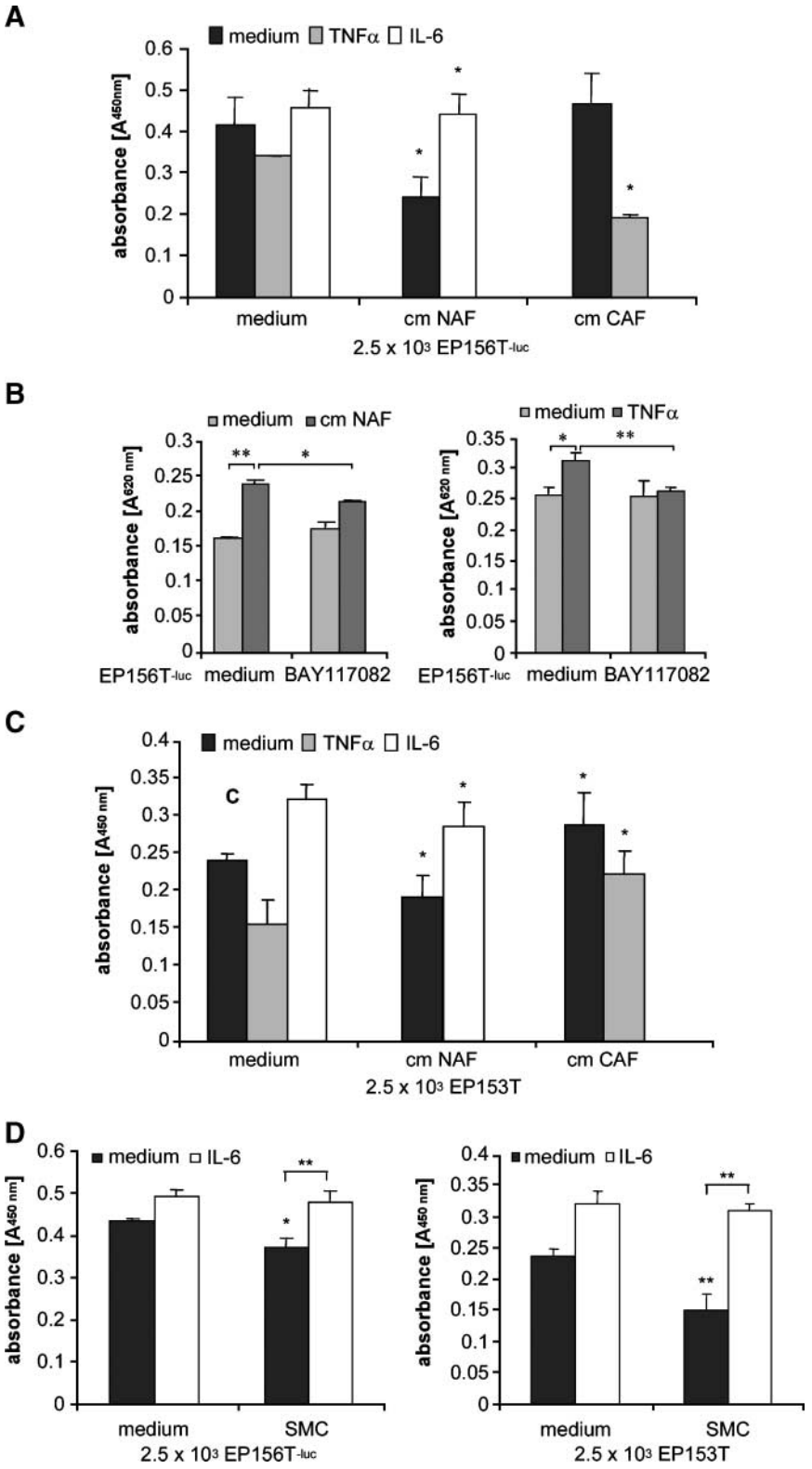

FIGURE 5. IL-6 and TNFα reverse the effects of conditioned media from NAFs or CAFs on the proliferation of immortalized epithelial cells. EP156T^{-luc} epithelial cells (2.5×10^3) were treated with conditioned media from NAFs (**A,** *middle columns*) or CAFs (*right columns*) for 12 h or were cultured in normal growth medium (*left columns*). Growth medium and conditioned media (*black columns*) were supplemented with TNFα (*gray columns*) or IL-6 (*white columns*). NAF conditioned medium inhibited the growth of epithelial cells, whereas CAF conditioned media promoted the growth of epithelial cells. These effects could be reverted by addition of IL-6 to NAF conditioned media or by addition of TNFα to CAF conditioned medium. One representative example of three independent experiments. Error bars, SDs of the means of triplicates (*, $P < 0.05$; **, $P < 0.005$; **A**). EP156T^{-luc} epithelial cells (2.5×10^3) were treated with conditioned media from NAFs (**B,** *left graph, dark gray columns*) or with TNFα (**B,** *right graph, dark gray columns*) for 12 h. Additionally, an inhibitor against TNFα BAY117082 was added accordingly. Cell death was determined by trypan blue staining and measured in an ELISA reader at a wavelength of 620 nm. The intensity of staining correlates with the amount of dead cells. Conditioned medium from NAFs increased the number of dead cells that are statistically significant (**, $P < 0.005$), which was inhibited by the inhibitor BAY117082 (*, $P < 0.005$). Similarly, adding TNFα to the cells resulted in cell death (**, $P < 0.005$), which was inhibited by the inhibitor BAY117082 (**, $P < 0.005$). One representative experiment. Error bars, SD of the means of triplicates. EP153T epithelial cells (2.5×10^3) were treated with conditioned media from NAFs (**A,** *middle columns*) or CAFs (*right columns*) for 12 h or were cultured in normal growth medium (*left columns*). Growth medium and conditioned media (*black columns*) were supplemented with TNFα (*gray columns*) or IL-6 (*white columns*). NAF conditioned medium inhibited the growth of epithelial cells, whereas CAF conditioned media promoted the growth of epithelial cells. These effects could be reverted by addition of IL-6 to NAF conditioned media or by addition of TNFα to CAF conditioned medium. One representative example of three independent experiments. Error bars, SDs of the means of triplicates (*, $P < 0.05$; **C**). Conditioned medium from SMCs was added to immortalized EP156T^{-luc} epithelial cells (**D,** *left graph, black columns*) or to immortalized EP153T epithelial cells (**D,** *right graph, black columns*). Growth medium and conditioned medium were supplemented with exogenous IL-6 (*white columns*). Proliferation was measured by Wst-1 assay. Conditioned medium from SMCs inhibited the proliferation of both epithelial cells; this was reversed by IL-6. One representative experiment. Error bars, SD of the means of triplicates (*, $P < 0.05$; **, $P < 0.005$; **D**).

Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

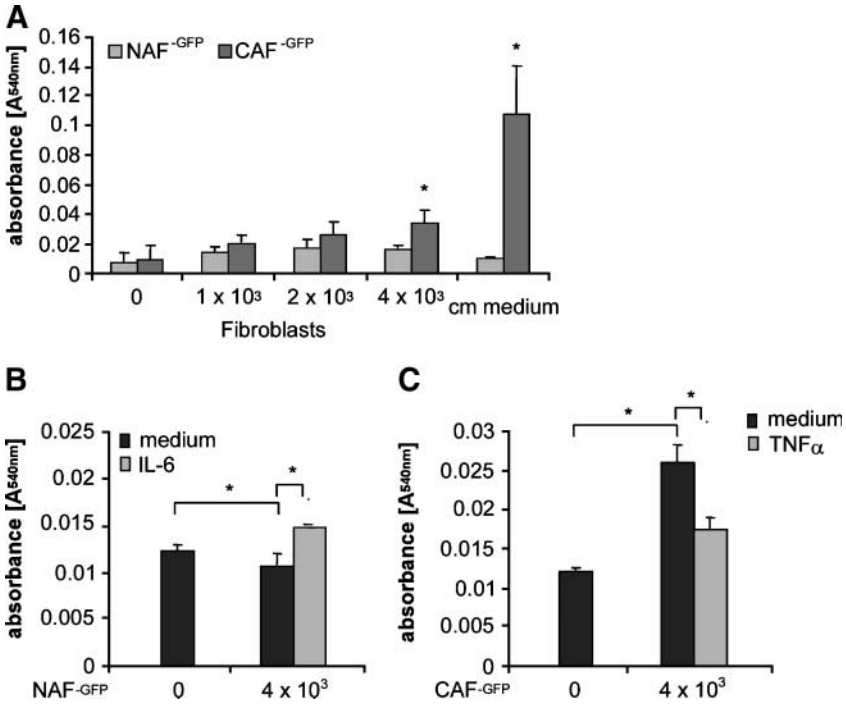

FIGURE 6. NAFs differentially regulate the migration of endothelial cells. Endothelial cells were allowed to migrate toward an increasing number of NAFs or NAF conditioned medium (*gray column*) or an increasing number of CAFs or CAF conditioned medium (*black column*). Endothelial cells migrated toward a high number of CAFs or CAF conditioned medium but not toward NAFs or NAF conditioned medium. Error bars, SD of the means of three biologically independent experiments (*, $P < 0.05$; **A**). NAFs (4×10^3) were seeded, and the medium was supplemented with IL-6. NAFs in normal growth medium (*black columns*) inhibited the migration of endothelial cells. Addition of IL-6 promoted the migration of endothelial cells by 38%. One representative of three biologically independent experiments. Error bars, SD of the means of duplicates (**B**). CAFs (4×10^3) were seeded, and the medium was supplemented with TNFα. CAFs in normal growth medium (*black columns*) promoted the migration of endothelial cells, which was inhibited by TNFα by 33% (*gray columns*). One representative of three biologically independent experiments. Error bars, SD of the means of duplicates (**C**).

Conditioned media from NAFs or CAFs had the same growth-inhibiting or growth-promoting effects on the proliferation of epithelial cells as were observed in the cocultivation experiments. This is indicative of the action of soluble factors secreted into the medium but does not rule out the possibility that additional cell-cell contacts play a role in the observed effects. This includes the possibility of an altered distribution of certain receptors on the cell surface, which might be influenced by the microenvironment, as well. For example, IL-6R was found to be functional only in LNCaP cells but not in DU145 or in PC-3 cells, but dexamethasone could induce the formation of soluble IL-6sR in DU145 and PC-3 cells postulating that the IL-6sR might modulate the behavior of not only the prostate tumor cells but also the surrounding cells (52). Another group showed that PC-3 cells were not responsive to exogenous IL-6, whereas growth could be inhibited by an anti–IL-6 monoclonal antibody claiming that IL-6 acted as an autocrine growth factor for PC-3 (53). This could explain why PC-3 cells were not stimulated to grow by CAFs as were immortalized epithelial cells. Similarly, when the human non–small cell lung cancer cell line H1299 was treated with conditioned media from lung-derived NAFs or CAFs, no change in proliferation could be observed (data not shown). This is in agreement with results from Bihl et al., who showed an up-regulation of IL-6 but no proliferative response to exogenous IL-6 of this cell line and other lung cancer cell lines (54).

Cytokine arrays revealed that CAFs secreted IL-6 in high amounts and NAFs secreted TNFα. Both cytokines were shown to be up-regulated in the sera of prostate cancer patients at different stages of malignancy but not in the sera of healthy patients (11-13). All in all, IL-6 was shown to be a cancer-promoting cytokine, and results from Palmer et al. suggested a unique role for IL-6 in the progression of prostate cancer (55). However, contradictory observations were made for the actions of TNFα, either tumor promoting or tumor inhibiting, depending on the cell type and the stage of malignancy (reviewed in ref. 37).

Strikingly, the secretion of IL-6 from NAFs approximated the amounts of IL-6 secreted by CAFs at the late passage after it had decreased at the middle passage. Recently, it was shown that senescence leads to a senescence-associated secretory phenotype, including the enhanced secretion of IL-6 and IL-8 (56). We could not rule out the possibility that NAFs had entered a senescence state at this stage, but this could explain the observed secretion pattern from NAFs.

Therefore, we focused on IL-6 as the putative cytokine exerting a growth-promoting effect on immortalized cells, and we considered TNFα as the possible growth-inhibiting cytokine at early and middle passages. Adding recombinant IL-6, up to a concentration of 5 ng/mL, which closely resembles the concentration secreted by the CAFs, promoted the growth of immortalized epithelium. Higher concentrations did not induce epithelial cells to grow, which implies that the concentration of cytokine might be crucial for the regulation of growth.

Supplementing the medium of cocultivated epithelial cells and CAFs with TNFα dramatically diminished the growth of the immortalized epithelial cells. This suggests that TNFα has the ability to counteract the growth-promoting role IL-6 has, at least at the very early stages of carcinogenesis. This makes TNFα a potential powerful molecule for the treatment of very early changes in prostate cancer formation. Indeed, TNFα was already tested in clinical trials and proved to be successful, also in advanced disease, when given locally in combination with γ-irradiation but was toxic when given systemically (57).

Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

The addition of IL-6 to NAFs in coculture with immortalized epithelium promoted the growth of epithelial cells and NAFs. This enhancement of growth of NAFs by the added IL-6 in coculture should have led to a dramatic decrease in the growth of epithelial cells in the coculture. The fact that IL-6 promoted the growth of epithelial cells implies that the action of IL-6 itself can neutralize the effect that NAFs exerted. Additional IL-6 might overwrite the effect of TNFα, suggesting a concentration-dependent function of TNFα. Furthermore, it was recently shown that the antiproliferative effect of IL-1β and TNFα on the human prostate cancer cells LNCaP was enhanced in coculture with normal human fibroblasts WI-38 by IL-6 (26). From this, it cannot be ruled out that other factors might play a synergistic role in the effects that NAFs and CAFs exert on epithelial cells, especially because some cytokines that are proproliferative or proangiogenic, like CCL-2, are secreted from both NAFs and CAFs. Moreover, there might be cytokines differentially secreted from the fibroblasts that also have an effect on the observed effects that were not covered in our used cytokine array.

Interestingly, NAFs promoted the growth of metastatic prostate cells PC-3. This is not in contradiction to the inhibition of growth of immortalized epithelial cells but rather in agreement with earlier publications stating that prostate cell lines derived from different stages of malignancy responded differently to the actions of TNFα. For example, whereas LNCaP cells underwent apoptosis after treatment with TNFα, normal prostatic epithelial cells and PC-3 cells were not susceptible to apoptosis (58). This is in contradiction to the results we obtained that, when LNCaP cells were treated with conditioned media from CAFs or NAFs, no response in proliferation could be observed (data not shown), concluding that not only the stage of malignancy of the cells has a major effect on its response to the microenvironment but also the experimental conditions.

The involvement of fibroblasts in angiogenesis is still poorly understood. However, angiogenesis is a very critical phase of the development of prostate cancer. To this end, we wanted to investigate the involvement of the microenvironment in the recruitment of endothelial cells to normal or CAF as a first step in angiogenesis. We found that increasing numbers of CAFs or CAF conditioned medium recruited endothelial cells, whereas NAFs or NAF conditioned medium had no effect on the migration of endothelial cells. Furthermore, IL-6 triggered the migration of endothelial cells when added to NAFs, whereas TNFα inhibited the migration when added to CAFs, suggesting that these factors secreted by stromal fibroblasts were responsible for the observed effects. The up-regulation of IL-6 and the involvement of the IL-6 pathway by the p50 subunit of nuclear factor-κB or the involvement of the IL-6 pathway trough SDF-1A (CXCL12) in the progress of angiogenesis was recently shown (59, 60). There could be a direct effect of IL-6 on the enhanced migration of endothelial cells via up-regulation of angiogenic relevant factors in the endothelial cells. Another explanation could involve an autocrine up-regulation of certain receptors on the surface of CAFs, such as vascular endothelial growth factor receptor, which is involved in angiogenesis. Another possibility could be that the proliferation of endothelial cells was enhanced by IL-6; thus, more cells could migrate, an effect recently described by Karst et al. (59). A similar growth-inhibiting effect could be responsible for the decreased migration of endothelial cells toward NAFs or CAFs treated with TNFα. It is known that TNFα impairs endothelial cell growth and angiogenesis by modulating the vascular endothelial growth factor–specific (61) or the hepatocyte growth factor-specific angiogenic pathways (62). Further experiments have to be conducted to gain more insight into the exact mechanism of IL-6–induced migration of endothelial cells and the inhibitory effect of TNFα.

It is well accepted that CAFs contribute to the progression of cancer by enhancing proliferation and angiogenesis. Less is known about the role that normal stromal cells play in preventing tumors to develop. Our results suggest that premalignant cells can be protected from transforming into tumors by the secretion of TNFα and the possible induction of cell death from the surrounding stromal fibroblasts. Furthermore, it would be of major interest to elucidate the mechanism in which premalignant cells alter the microenvironment so that tumor-hostile NAFs will turn into tumor-friendly CAFs. A broader understanding of how the stromal-epithelial interactions are regulated and how they are altered at very early stages of tumorigenesis could provide an important tool for an early intervention in prostate cancer progression.

Materials and Methods

Cell Culture and Media

Epithelial cells and stromal fibroblasts were immortalized with hTERT as previously described. Shortly, epithelial EP156T cells, NAFs, and CAFs were obtained after telomerase (hTERT)–induced immortalization of cells isolated from benign prostate tissues of elderly men of the age of 68 that underwent radical prostatectomy due to prostate cancer as described in ref. 42. Epithelial cells were maintained in MCDB medium supplemented with 1% FCS, glutamine, bovine pituitary extract, and epidermal growth factor. Fibroblasts were maintained in MEM supplemented with 10% FCS, 0.5% glutamine, and 1% pyruvate. Mouse bone marrow endothelial cells were maintained in a low-glucose DMEM with 10% FCS and 1% glutamine. PC-3 cells were maintained in DMEM with 10% FCS. All media and media supplements were obtained from Beit Haemek, Biological Industries Co. Cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Cocultivation of Epithelial Cells and Fibroblasts

To test the effect of stromal fibroblasts on the growth of prostate stromal epithelial cells, we established a cocultivation model in which each cell type was labeled either with firefly-luciferase or GFP so that each cell type could be individually assayed for the cell number when cocultivated in one tissue culture plate. To this end, human immortalized epithelial cells EP156T were stably infected with a firefly-luciferase vector generating $EP156T^{-luc}$ cells. Human immortalized NAFs and CAFs were stably infected with GFP, generating NAF^{-GFP} or CAF^{-GFP} cells. Subsequently, confluent epithelial cells $EP156T^{-luc}$ and fibroblasts were trypsinized and counted in a Neubauer's chamber. Epithelial cells (1×10^3 or 2×10^3) were seeded in a 12-well microplate, and increasing numbers of fibroblasts, such as 1×10^3, 2×10^3, 4×10^3, or 8×10^3 fibroblasts, or no fibroblast

Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

was added accordingly in epithelial cell medium. Furthermore, 50 ng/mL TNFα (PeproTech, Inc.) were added to CAFs or 5 ng/mL IL-6 (PeproTech, Inc.) were added to NAFs when cocultivated or cells were left untreated. Four or 8 d later, cocultivated cells were lysed with passive lysis buffer (Promega). Then the GFP fluorescence and the activation of luciferase were measured in the Luminometer Victor^2 (Perkin-Elmer). The intensity of the fluorescence or the luciferase activity was considered as a measurement for the growth of the cells in cocultivation.

Collection of Conditioned Media

Fresh epithelial cell medium was added to confluent NAFs, CAFs, or SMCs for 1 day. The supernatants were collected and sterile filtered through a 0.22 μmol/L syringe–driven filter unit (Millipore) to dispose dead cells and stored at −20°C. Epithelial cells (2,500; EP156T^{-luc} or EP153T) were seeded in 96 wells. Semiconfluent cells were serum-starved for 24 h before treatment with conditioned media from NAFs, CAFs, or SMCs. For complementing CAF conditioned medium and NAF or SMC conditioned media, 50 ng/mL TNFα and 5 ng/mL IL-6 were, respectively, added to the conditioned media. Proliferation of the cells was determined by measuring mitochondrial dehydrogenase enzyme activity using the WST-1 assay (Roche Applied Bioscience). Absorption was measured in an ELISA reader (TECAN Spectra) using the Delta Soft 3 software at a wavelength of 450 nm. Data were analyzed using Microsoft Excel 2000 for Windows XP.

Cell Death Assay

Epithelial cells EP156T were seeded in 96-well plates and treated with conditioned medium from NAFs or 2 ng/mL TNFα or left untreated. To test the importance of TNFα for the growth-decreasing effect of NAFs, 5 μmol/L BAY117082 (Alexis Biochemicals), an inhibitor against nuclear factor-κB, were added additionally. At 24 h later, 0.4% trypan blue solution (Sigma-Aldrich) was added to the medium in a final 1:1 dilution for 10 min. The cells were washed once with PBS, and the number of cells, which had incorporated trypan blue, was measured in an ELISA reader (TECAN Spectra) using the Delta Soft 3 software at a wavelength of 620 nm. Data were analyzed using Microsoft Excel 2000 for Windows XP.

Cytokine Array

Fresh medium without FCS was added to confluent NAFs or CAFs for 1 day. Supernatants were collected and stored at −20°C until further processing. Secreted proteins were determined using the ChemiArray Human Cytokine Antibody Array III (Chemicon International) according to manufacturer's instructions. Membranes were exposed to an X-ray film (Fujifilm), which was developed in a Medical X-ray Processor (Kodak).

TNFα and IL-6 ELISA

Fresh medium was added to confluent NAFs or CAFs at early (p14-p16), middle (p18-p20), or late passages (p22-p24). One day later, supernatants were collected for the measurement of the secretion of TNFα and IL-6 by ELISA. ELISAs were done according to manufacturer's instructions (eBioscience). ELISA plates were read at 450 nm in an ELISA reader (TECAN Spectra) using the Delta Soft 3 software and analyzed with Microsoft Excel 2000 for Windows XP.

Quantification of mRNAs by Real-time Reverse Transcription-PCR

RNA was isolated from NAFs and CAFs from early (p14-p16), middle (p18-p20), or late passages (p22-p24) using the NucleoSpin RNA isolation kit (Machery-Nagel) according to the manufacturer's instructions. RNA concentrations were determined with the NanoDrop 1000 Spectrophotometer (ThermoScientific). Same concentrations of isolated RNA were transcribed into cDNA using BioRT (BioLabs). The relative amount of specified mRNA was determined by real-time PCR using the Platinum SYBR Green qPCR SuperMix-UDG with ROX kit (Invitrogen) according to manufacturer's instructions. The expression level of mRNA was normalized against the internal standard glyceraldehyde-3-phosphate dehydrogenase. The following primers were used: glyceraldehyde- 3-phosphate dehydrogenase, forward primer GGTATCGTGGAAGGACTCATGAC and reverse primer ATGCCAGTGAGCTTCCCGTTCAG; TNFα, forward primer GCCCATGTTGTAGCAAACCCT and reverse primer ATGAGGTACAGGCCCTCTGATG; IL-6, forward primer TGAACTCCTTCTCCACAAGCG and reverse primer GGCGGCTACATCTTTGGAATC. Primers were designed using Primer Express 2.0 for the 7000 System SDS Software (Applied Biosystems).

Migration of Endothelial Cells

Mouse bone marrow endothelial cells were allowed to migrate to NAFs or CAFs through a Transwell® permeable support 8.0 μm polycarbonate membrane (Costar, Corning, Inc.) for 24 h toward 1×10^3, 2×10^3, or 3×10^3 NAFs or CAFs in 24 wells. TNFα (50 ng/mL) was added to CAFs, or IL-6 (5 ng/mL) was added to NAFs. As a control, endothelial cells were also allowed to migrate toward fibroblast culture medium only. Medium was aspirated from the transwells 24 h later, and nonmigrated cells from the top of the transwell were mechanically removed with a cotton stick. Migrated cells were washed with PBS and fixed with 4% PFA (Merck) for 15 min and then washed once in PBS to remove residual fixing solution. Cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 5 min, washed with PBS, and then stained with crystal violet (Merck) for 20 min at room temperature. Afterwards, they were washed in water and dried. The membranes were removed and subjected to 100 μL of 10% acetic acid to dissolve crystal violet of the cells. The amount of absorbed crystal violet to the cells was measured in an ELISA reader (TECAN Spectra) using the Delta Soft 3 software at 540 nm.

Statistics

For statistical analysis and graphs, Microsoft Excel 2002 for Windows XP Home was used. For determination of the statistical significance, the Student's t test was done. P values of <0.05 were considered as statistically significant.

Disclosure of Potential Conflicts of Interest

This publication reflects the authors' views and not necessarily those of the European Community. The European Community is not liable for any use that may be made of the information contained herein. V. Rotter is the incumbent of the Norman and Helen Asher Professorial Chair Cancer Research at the Weizmann Institute.

Published OnlineFirst August 20, 2009; DOI: 10.1158/1541-7786.MCR-09-0073

Acknowledgments

We cordially thank Tsvee Labidot of Weizmann Institute of Science for providing mouse bone marrow–derived endothelial cells.

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Differential Influence of Normal and Cancer-Associated Fibroblasts on the Growth of Human Epithelial Cells in an *In vitro* Cocultivation Model of Prostate Cancer

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Mol Cancer Res 2009;7:1212-1223. Published OnlineFirst August 20, 2009.