Human Lung Cancer-associated Fibroblasts Enhance Motility of Non-small Cell Lung Cancer Cells in Co-culture

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Abstract. The metastatic potential of non-small cell lung cancer (NSCLC) cells has been shown to be associated with the tumor microenvironment. Cancer-associated fibroblasts (CAFs) are a major component of the tumor microenvironment, regulating tumor cell function by secreting growth factors, chemokines, and extracellular matrix (ECM). In this study, we examined the role of CAFs in the tumor progression of NSCLC. Firstly, we established primary cultures of CAFs and matched normal fibroblasts (NFs) from patients with resected NSCLC. CAFs exhibited greater expression of the pan-mesenchymal marker α-smooth muscle actin (α-SMA) than did NFs, although they displayed similar morphology. Furthermore, we employed a direct co-culture assay with human NSCLC A549 and H358 cells, and found that CAFs were more potent in inducing the epithelial-to-mesenchymal transition (EMT) phenotype than NFs, as indicated by an elongated and disseminated appearance. CAF-induced EMT led to an increase in motility and a decrease in proliferation of NSCLC cells through SMAD family number-3 (SMAD3)-dependent up-regulation of the growth inhibitory gene p21CIP1 [cyclin-dependent kinase inhibitor-1A (CDKN1A)] and α-SMA. Taken together, these findings provide evidence that lung CAFs have tumor-promoting capacity distinct from NFs and might play a significant role in the metastatic potential of NSCLC.

Lung cancer is the leading cause of cancer-related death worldwide (1, 2), and 85% of all lung cancer cases are of non-small cell lung cancer (NSCLC). Most NSCLCs are characterized by the appearance of desmoplasia at the time of diagnosis (3, 4), stromal alterations characterized by transdifferentiation of stromal fibroblasts into carcinoma-associated fibroblasts (CAFs), enhanced deposition of extracellular matrix (ECM) in tumors, and angiogenesis (5, 6). These changes have been reported to promote tumor progression, resistance to treatment, and immune reactions (7).

It has been observed decades ago that fibroblasts within an activated tumor stroma acquire a modified phenotype (8, 9). Typically, normal fibroblasts (NFs) in healthy organs appear as fusiform cells with a prominent actin cytoskeleton and are embedded within the fibrillar ECM of connective tissue. NFs interact with their surrounding microenvironment through integrins such as α1β1 integrin and constitutively express intermediate filament proteins vimentin and fibroblast-specific protein-1 (FSP1). Along with their function in healthy organs, fibroblasts are involved in a variety of pathological and physiological processes, including tumorigenesis, wound repair and organ fibrosis (10). Under these conditions, fibroblasts exhibit increased proliferative activity and enhanced secretion of ECM proteins such as type-I collagen and tenasin C, and also express α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), extra domain A-containing fibronectin (EDA-FN) isoform, and secreted protein, acidic and rich in cysteine (SPARC). Such increased activity is referred to as ‘activation’. Fibroblasts are activated by numerous stimuli including growth factors [e.g. platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), epidermal growth factor (EGF)], direct cell-to-cell contact and environmental mediators such as oxygen species, hypoxia and high glucose. Recent work has implied that epigenetic changes in response to activation stimuli cause fibroblasts to perpetuate a state that is autonomous from microenvironmental stress (11).

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It is increasingly accepted that CAFs are heterogeneous in origin and derive from various progenitor cells, including fibroblasts, mesenchymal stem cells (MSC), and normal and transformed epithelial cells (12, 13). The mechanism by which normal and transformed epithelial cells transdifferentiate into CAFs has been referred to as epithelial-to-mesenchymal transition (EMT), which represents the loss of epithelial traits such as expression of cytokeratins, and the gain of mesenchymal traits, such as expression of VIM (vimentin), FSP1, and α-SMA (12, 14, 15). In addition to its role in transdifferentiation, EMT is induced in cancer cells by CAFs. Cancer cells undergoing EMT become invasive and carry a highly metastatic potential. EMT is induced by many signal factors (e.g. PDGF, TGFβ, EGF) and is commonly mediated by the activation of transcription factors such as snail and forkhead box protein-C2 (FOXC2).

CAFs are a significant component of NSCLC tumors (16-19); however, their multifaceted role in NSCLC tumorigenesis has not been well-defined. In the current work, we tested the hypothesis that CAFs isolated from patients with lung cancer contribute to distinctive microenvironmental influences on NSCLC cells in an effort to develop an in vitro tumor model. Using direct co-culture, we found that CAFs play a major role in stimulating migratory behavior and cell proliferation by inducing an EMT phenotype.

Materials and Methods

Primary culture of fibroblasts. CAFs and NFs were isolated from lung cancer and normal skin specimens of patients who underwent surgical resection in 2012 at the Samsung Medical Center, Seoul, Korea. The protocol for the study was approved by the Institutional Review Board of Samsung Medical Center (approval ID #2011-03-025) and the study was conducted in accordance with the principles of the Declaration of Helsinki. Tissue specimens were mechanically- and enzymatically-dissociated, employing slight modifications of published methodologies (20, 21). Briefly, 30 min after surgery, fresh tissues (3 mm × 3 mm × 3 mm in size) were minced with surgical blades in 5 ml of Roswell Park Memorial Institute (RPMI)-1640 medium without serum to an approximate size of 40-60 μm in diameter, or 40-200 cells/clump. Tissue fragments were subjected to enzyme digestion in a solution containing a cocktail of collagenase I (450 U/ml; EMD, San Diego, CA, USA) and DNase I (60 U/ml; Roche, Mannheim, Germany) for 20 min and then seeded in a T25 culture flask in 5 ml of RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, N.Y., USA). Fibroblasts were separated from epithelial cells by treatment with dispase II (14 U/ml; Roche) and subsequent mechanical ablation with a pulled glass pipette tip. The culture medium was changed after 48 h to remove unattached cells and debris in suspension. Attached cells were cultured for 7-10 days to 1×10⁶ cells. All fibroblasts were used after 3-7 passages following primary culture.

Cell lines and co-culture. Human NSCLC cell lines, bronchio-alveolar carcinoma H358, and large-cell carcinoma cell line A549 were obtained from the Korean Cell Line Bank (Seoul, Korea) and transduced with a lentivirus green fluorescence protein (GFP) construct. Stable GFP-expressing H358 (GFP-H358) and A549 (GFP-A549) cells were selected and expanded in growth media (RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, N.Y., USA). Fibroblasts were separated from epithelial cells by treatment with dispase II (14 U/ml; Roche) and subsequent mechanical ablation with a pulled glass pipette tip. The culture medium was changed after 48 h to remove unattached cells and debris in suspension. Attached cells were cultured for 7-10 days to 1×10⁶ cells. All fibroblasts were used after 3-7 passages following primary culture.

2002
Figure 1. Preparation of primary cancer-associated fibroblasts (CAFs). A: Representative time lapse photographs of the isolation of CAFs. B: Representative images of immunofluorescence staining of CAFs and normal fibroblasts (NFs). Cells were stained with an antibody targeting α-smooth muscle actin (α-SMA) (green), while nuclei were counterstained blue with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI). Original magnification: ×1,500. C: Phase-contrast micrograph of cells (original magnification: ×1,000) showing the morphology of fibroblasts. D: Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of CAFs and NFs. Data shown are the means±s.e.m. from three independent experiments carried out in duplicates. The expression of each mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same sample and is presented as a fold change over that of NFs.
Time-lapse video microscopy. Time-lapse photography of GFP-expressing cells was used to measure cell proliferation rates and to observe cell morphology. Briefly, cells on a tissue culture dish were kept in a chamber at 37°C in an atmosphere with 5% CO₂. Photographs were taken daily at the same position marked by spots on the bottoms of the culture dishes with an inverted fluorescence microscope (Zeiss, Oberkochen, Germany: ×20 objective) at 30 s intervals in order to observe GFP-positive cancer cells. The images were built into a movie using MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA).

Immunocytochemistry. Fibroblasts were seeded on a sterile glass coverslip and immunocytochemical staining was performed. Briefly, sterile coverslips were fixed with 4% paraformaldehyde for 10 min and then were permeabilized with 0.5% Triton-X 100 for 5 min. Cells were then blocked for 1 h with blocking solution [1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)], followed by incubation with a primary antibody against α-SMA (1:200; Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. Subsequently, the cells were incubated with Alexa 594-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) for 60 min at room temperature.

Figure 2. Lung cancer-associated fibroblasts (CAF) in co-culture induce EMT phenotype and induce growth retardation. A: Morphology of human NSCLC H358 and A549 cell lines grown in monoculture and in co-culture with CAFs and normal fibroblasts (NFs). Original magnification: ×1,500. B: Green fluorescence protein (GFP) fluorescence intensity of H358-GFP cells. CAFs stimulate the proliferation of H358 cells. Cell proliferation was estimated by measurement of the fluorescence intensity. Error bars represent s.d. (n=3). RFU, Relative fluorescence units.
temperature. The cells were then washed with PBS, and the coverslips were mounted on a glass slide in 10% Mowiol 4-88, 1 μg/ml 4m,6-diamidine-2-phenylindole dihydrochloride (DAPI), and 25% glycerol in PBS. The cells were observed with a ZEISS FL Microscope Axiovert 200 (Zeiss, Oberkochen, Germany).

Cell sorting. After 48 h of co-culture, cells expressing GFP were trypsinized and centrifuged at 300 × g for 5 min. The cell pellet was suspended in 5 ml of PBS and cells were sorted by fluorescence-activated cell sorting (FACS) with FACS AriaIII (Becton Dickinson Biosciences, San Jose, CA, USA) analysis and segregated with a 488-nm optical filter. Sort gates were set by analyzing 50,000 cells from GFP-expressing and control cells. Cells were collected and centrifuged at 500 × g for 10 min and processed to grow, or to analyze gene expression.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted from the sorted co-cultured cells, followed by synthesis of first-strand cDNA using oligo-dT primers and M-MLV reverse transcriptase (Invitrogen). Real-time qRT-PCR was performed using an Applied Biosystems 7900HT fast real-time PCR system (Foster City, CA, USA). The real-time qPCRs were performed in a final volume of 25 μl containing 1× SYBR® Green PCR master mix (Applied Biosystems), 10 ng of cDNA, and 20 pmol of each primer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control in each reaction. The PCR condition was as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s, and then 60˚C for 1 min. To verify specific amplification, melting curve analysis was performed (55-95˚C, 0.5˚C/s). Relative expression quantification was performed by the ΔΔCT method. Genes and their primers are given in Table I. The expression of each mRNA was normalized to that of GAPDH in the same sample.

Cell proliferation analysis. The fluorescence of cells in 96 well plates was quantified in triplicate using a plate reader (Wallac 1420 Victor2™, Perkin-Elmer, Waltham, MA, USA) with filter settings of excitation at 485 nm with a bandwidth of 20 nm and emission at 530 nm with a bandwidth of 25 nm. Fluorescence intensity was calculated in relative fluorescence units (RFU). The non-specific signals of wells containing cell-free medium (blank value) was subtracted from the results to give the fluorescence signals of the GFP-expressing H358 cells.

Co-culture wound healing assay. Cells were allowed to grow to 80% confluence in a 24-well plate and were then wounded by making a single scratch in the monolayer with a pipette tip. The medium was

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Figure 3. Gene expression changes of H358 cells grown in co-culture with cancer-associated fibroblasts (CAFs). Expression profiling of mesenchymal markers (A), epithelial-to-mesenchymal transition (EMT)-inducing transcription factors (B) and cell-cycle-regulated genes (C). The mRNA expression levels were determined by quantitative real-time RT-PCR. Data shown are the mean±s.e.m. from three independent experiments carried out in duplicates. The expression of each mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same sample and presented as fold change over control H358 cells alone.
then replaced to remove floating cells and debris, and cells were subsequently allowed to grow for three days to close the wound. Photographs of the same area of the wound were taken at 24 h post-wound using a phase-contrast microscope.

**Results**

**Isolation, separation, and culture of primary fibroblasts CAFs and NFs.** To investigate the effect of primary cultured fibroblasts on lung cancer progression, we first isolated CAFs from freshly-dissected cells from a primary lung tumor and matched NFs. We used dispase to dissociate fibroblasts from epithelial cells (Figure 1A), since epithelial cells are much more sensitive than fibroblast-like cells to neutral proteases (21, 22). Epithelial cells detached within 8 min, leaving the majority of fibroblast-like cells attached to the plate (Figure 1A). The attached NFs and CAFs in culture displayed a thin and elongated (spindle-like) appearance, suggesting that the cells were fibroblasts (Figure 1B).

Figure 4. The effect of cancer-associated fibroblasts (CAFs) on motility. Representative image of H358-GFP (A) and A549-GFP (B) cells showing cell migration. In the wound-healing assay, cells were scratched and allowed to grow for three days. Cells were observed using phase-contrast fluorescence microscopy to measure the width of wound at 24 h post-wound. Original magnification: ×1,000.
However, the NFs and CAFs did not show an apparent difference in morphology (Figure 1C). We examined the expression of the mesenchymal cell markers of CAFs and NFs. CAFs exhibited a higher level of α-SMA mRNA expression (Figure 1D) and α-SMA protein (Figure 1B) than NFs, whereas CAFs exhibited a lower level of FAP, FSP, VIM, and SNAI1 and 2 mRNA expressions than NFs (Figure 1D). α-SMA, the most frequently employed myofibroblast marker, is incorporated into contractile actin/myosin-containing stress fibers, leading to augmented contractile activity of fibroblastic cells (23, 24). Taken together, these results suggest that primary cultured CAFs derived from patients with lung cancer are distinct from matched primary cultured NFs with respect to the level of contractile activity.

*Induction of EMT in human NSCLC A549 and H358 cells by CAFs.* To examine the role of CAFs in tumor progression and growth, co-culture was employed. CAFs were plated and cultured to 70-80% confluence within one or two days, followed by the addition of NSCLC A549 and H358 cells expressing green fluorescence protein (GFP) (A549-GFP and H358-GFP, respectively) on top of the primary cultures of fibroblasts. Control H358 cells formed typical epithelial cobblestone morphology with homotypic cell adhesion, but when co-cultured with primary cultured CAFs or NFs, they exhibited loss of epithelial morphology and became elongated and non-polarized in morphology, and also dispersed, which is reminiscent of the EMT phenotype (Figure 2A). A549 cells in co-culture also gave results similar to H358 cells. Of note, EMT induction of A549-GFP and H358-GFP cells by CAFs was more significant than that induced by NFs (Figure 2A). Furthermore, the proliferation of H358-GFP cells in the presence of CAFs significantly decreased by 85% and 49% compared with control H358-GFP cells alone and co-culture with NFs, respectively (Figure 2B). This result indicates that CAFs in co-culture induced cancer cells to proliferate. Many studies have shown that EMT-inducing signals attenuate proliferation, which may also compromise tumor growth (25, 26). Taken together, these results suggest that primary-cultured CAFs derived from patients with lung cancer are more potent for inducing the EMT phenotype and the proliferation of human NSCLC A549 and H358 cells.

*Expression of EMT marker genes in NSCLC cells.* To further investigate the role of EMT in cancer progression, we studied the motility of NSCLC cells in co-culture. To this end, H358-GFP and A549-GFP cells in co-culture with primary-cultured fibroblasts were scratched and allowed to migrate to close the gap. Actively migrating H358-GFP and A549-GFP cells were observed in co-culture with CAFs, but not in control and co-culture with NFs (Figure 4). This observation suggests that CAFs affect the motility of cancer cells more than NFs through the induction of the EMT phenotype.

**Discussion**

The cancer microenvironment plays a crucial role in tumor development and progression *via* reciprocal interaction between cancer and stromal cells. In the present study, we established an *in vitro* co-culture model of CAFs with NSCLC A549 and H358 cells, and assessed their functional roles. Furthermore, we demonstrated that CAFs not only regulated NSCLC motility through EMT but also inhibited cell proliferation to a greater extent than NFs.

When we examined the expression level of the mesenchymal cell markers of CAFs and NFs, we unexpectedly found that CAFs express a lower level of FAP, FSP, VIM, and SNAI1 mRNAs than NFs (Figure 1D). Fibroblasts are activated by numerous stimuli including growth factors (*e.g.* PDGF, TGFβ, EGF), direct cell-to-cell contact and environmental mediators such as oxygen species, hypoxia and high glucose (10). These observations led us to propose a model in which the CAFs used in the present study are more inducible in response to activation signals. However, there are several studies suggesting that CAFs are perpetually activated, neither reverting back to a normal phenotype, nor undergoing apoptosis, and thus they support...
tumor growth and progression through cell-to-cell interactions and by continuously secreting growth factors and cytokines (29). In light of our observation of the heterogeneous nature of primary-cultured CAFs among patients (data not shown) together with data from other investigators (30), future studies with a larger number of CAFs will be necessary to gain a comprehensive understanding of the mechanism governing the expression of mesenchymal markers in NSCLC.

In our work, although CAFs and NFs did not exhibit any apparent differences in morphology (Figure 1B), they did have differential gene expression profiling as evidenced by a higher mRNA levels of the mesenchymal marker α-SMA (Figure 1B and D). Interestingly, expression of α-SMA was significantly increased in H358 cells in co-culture with CAFs compared with control monoculture and co-culture with NFs, whereas the other well-known molecular signature of mesenchymal transition, CDH1 (E-cadherin), did not show any significant change in expression in co-culture and monoculture (Figure 3). These results suggest that the differential expression of α-SMA and E-cadherin is not necessarily followed by total phenotype change. De novo expression of α-SMA in the stroma stimulates the migration of stromal cells and cancer cells and contributes to alterations in cytoskeletal organization, which increases their contractile potential (23, 24, 31). Taken together, our findings led us to hypothesize that CAFs are more potent in inducing α-SMA which confers greater motility on NSCLC H358 and A549 cells.

The main finding of the present study is that CAFs more strongly influence the behavior of cancer cells than NFs, by activating the EMT-inducing transcription activator SMAD3, which coordinates the functions of downstream EMT-inducing signaling pathways. SMAD3 functions as an important mediator of TGFβ-induced EMT through the transcriptional regulation of target genes including CDKN1A (p21/WAF1) and α-SMA (27, 28). In agreement with this, we observed that CAFs more strongly influenced the motility of NSCLC cells than NFs (Figure 4).

In summary, we provide compelling evidence that CAFs from human lung cancer differ from NFs with respect to the levels of α-SMA mRNA and their potential to induce migration through EMT and to regulate cell proliferation. The co-culture model of CAFs with primary lung cancer cells allows for further study of the crosstalk between tumor cells and stroma, which will hopefully lead to the identification of new therapeutic targets for patients with lung cancer.

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References


