Pulmonary Fibroblasts Induce Epithelial Mesenchymal Transition and Some Characteristics of Stem Cells in Non-Small Cell Lung Cancer

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Background. Fibroblasts are key components of the tumor microenvironment. The purpose of this study was to clarify the role of fibroblasts in tumor progression in non-small cell lung cancer (NSCLC).

Methods. Fibroblasts isolated from surgical exploration were co-cultured with human lung adenocarcinoma cell lines. We defined fibroblasts obtained from tumors as cancer associated fibroblasts (CAFs) and those from normal lung tissue as lung normal fibroblasts (LNFs).

Results. Expression levels of myofibroblast markers were higher in CAFs than LNFs within 5 passages in the absence of continuing interaction with carcinoma cells. Thus, we used at least 2 pairs of these CAFs and LNFs in the following experiments; conditioned medium (CM) from fibroblast-induced epithelial mesenchymal transition and acquisition of cancer stem cell-like qualities in lung cancer cells (A549 and NCI-H358), indicating that CM from fibroblasts was biologically active. Furthermore, the concentration of the transforming growth factor (TGF)-β1 was higher in CM from CAFs as compared with that from LNFs, and phenotypic changes of cancer cells by CM from CAFs were greater than those induced by CM from LNFs. These CAF-induced changes were inhibited by addition of the TGF-β inhibitor SB431542. Subcutaneous co-injection of lung cancer cells and CAFs in mice enhanced tumor growth when compared with cancer cells alone, which was attenuated by administration of SB431542.

Conclusions. Fibroblasts were associated with increased malignant potential and the acquisition of stem cell-like properties in NSCLC tumors. Targeting CAFs as a therapeutic strategy against cancer is an intriguing concept that would benefit from further study.

Lung cancer is the leading cause of cancer death worldwide [1]. Overall prognosis for affected patients is poor due to metastatic disease and lack of curative systemic therapy, underscoring the need for a better understanding of the biologic changes that promote the aggressive neoplastic phenotype [2].

Epithelial to mesenchymal transition (EMT) is a fundamental biologic process during which epithelial cells lose their polarity and change to a mesenchymal phenotype [3]. When cancer cells invade or metastasize, they use a mechanism similar to EMT [4]. Furthermore, some studies have reported a role for EMT in the development of cancer cell resistance to anti non-small cell lung cancer (NSCLC) agents [5–7]. The cancer stem cells (CSC) theory proposes that cancers are maintained by subpopulations of tumor cells that possess progenitor cell characteristics. These cells can initiate tumor formation, differentiate along multipotent pathways, and are relatively resistant to conventional chemotherapy [8–10]. The EMT generates cells with many of the properties of epithelial stem cells [11]. The induction of EMT in primary lung cancer cell line results in the acquisition of mesenchymal profile and in the expression of stem cell markers [12]. Thus, EMT-driven gain of cancer stem cell properties may be associated with aggressiveness and metastatic spread [13].

The tumor microenvironment is a key component of tumor progression, and the invasion of cancer cells into and through the stroma requires EMT [14]. A specific subset of stromal cells, termed cancer associated fibroblasts (CAFs), show morphologic characteristics of both fibroblasts and smooth muscle cells [15]. The CAFs modulate the behavior of adjacent cancer cells by secreting various growth factors and cytokines, thereby promoting tumorigenesis [16]. Tumor-localized CAFs can comprise up to more than half of the tumor mass, and there is active multi-directional communications...
between these coevolving cell types within cancer tissue [17–19].

In order to identify new targets for prevention of metastasis, it is important to understand the molecular mechanisms that drive EMT. Therefore, the goal of the present study was to clarify the role of CAFs in the induction of EMT and CSC of NSCLC cells.

Material and Methods

Cell Culture and Materials

A549 cells and NCI-H358 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Roswell Park Memorial Institute 1640 with 10% fetal bovine serum (FBS). Antibodies for western blotting and immunofluorescence were as follows: anti-alpha-smooth muscle actin (α-SMA) pAb (catalog number ab5694; Abcam, Tokyo, Japan), anti-fibroblast activating protein pAb (Abcam, ab53066), anti-glyceraldehyde-3-phosphate dehydrogenase mAb (G9545; Sigma-Aldrich, St. Louis, MO), anti-N-cadherin (sc-59987; Santa Cruz Biotechnology, Inc, Dallas, TX), anti-E-cadherin mAb (M106; Takara, Otsu, Shiga, Japan), and anti-CD44 mAb (156-3C11; Cell Signaling Technology, Beverly, MA). Antibodies for the immunohistochemistry experiments were as follows: anti-E-cadherin mAb (M3612; Dako, Carpinteria, CA), anti-CD44 mAb (156-3C11; Cell Signaling), and Ki67 (M7240; Dako). Cisplatin was purchased from Sigma (catalog# 479306, St. Louis, MO), and trans-

Detergent Extraction, SDS-PAGE, and Immunoblots

Total RNA was isolated from cells treated with or without CM from fibroblasts for 24 hours using RNaseasy Mini Kit (Qiagen, Tokyo, Japan). Real-time RT-PCR (E-cadherin, Hs00170423_m1; N-cadherin, Hs00169953_m1 [Applied Biosystems, Tokyo, Japan]) was performed using a CFX96 system (BioRad, Tokyo, Japan) and relative expression levels were calculated by the comparative Ct method.

Spheroids Culture

Cells were cultured in low adherent 35-mm dishes (Corning, Corning, NY) under serum-free condition for 21 days [21]. Briefly, 1 × 10^5 cells were suspended in 6 mL of DMEM or CM from CAFs or LNFs, supplemented with 10 ng/mL basic fibroblast growth factor. Spheroid bodies were harvested every 5 days and re-cultured with fresh media. To inhibit TGF-β signaling, SB431542 was added to CM. Spheres with a diameter over 50 μm were counted on an inverted microscope.

Cell Viability Assay

For quantitative viability assays, Cell Proliferation Kit I (MTT; 3-[4.5 dimethylthiazol 2-yl]-2,5-diphenyltetrazolium
bromide) was obtained from Roche Applied Science (Mannheim, Germany). Cells were plated in 96-well plates (1 × 10^4 cells/20 μL serum free media for each well). Fifty μL of serum free media, CM from CAFs, or CM from LNFs were added to each well. The cells were treated according to the manufacturer’s protocol and absorbance was measured at 490 nm using a microplate reader (BioRad) after indicated days. Cisplatin or SB431542 was added at various concentrations.

Enzyme-Linked Immunosorbent Assay (ELISA) for TGF-β1

Around 1 × 10^6 cells were plated in 10-cm dishes, and cells were fed with serum-free DMEM (6 mL) next day.
FIBROBLASTS INDUCE EMT AND STEMNESS IN NSCLC

A

Tumor Volume

\[
\begin{array}{c|ccc}
 & A549 & H358 \\
\hline
\text{alone} & +CAFs & +LNFs & +CAFs \\
n=6 & n=8 & n=8 & n=5 \\
\end{array}
\]

B

HE

E-cadherin

CD44

Ki67

A549 A549+CAFs H358 H358+CAFs

C

E-cadherin score

\[
\begin{array}{c|ccc|ccc}
 & A549 & H358 & A549 & H358 & A549 & H358 \\
\hline
\text{CAF} & \text{CAF} & \text{CAF} & \text{CAF} & \text{CAF} & \text{CAF} & \text{CAF} \\
0.028 & 0.047 & 0.028 & 0.022 & 0.028 & 0.012 \\
\end{array}
\]

CD44 score

Ki67 labeling index

0 0.028 0.012
The CM was collected after 48 hours and quantification of TGF-β1 was assessed by ELISA according to the manufacturer's protocol (human Quantikine; R&D System, Minneapolis, MN).

**Animal Studies**

All manipulations were done in accordance with protocols approved by the Osaka University Institute Animal Care Committee. The $1 \times 10^6$ tumor cells (A549 or NCI-H358 cells) mixed with or without $1 \times 10^5$ CAFs or LNFs (2 pairs of primary cultured CAFs and LNFs for A549 cells and 1 pair of those for H358 cells [A549 alone, n = 6; A549 + CAFs, n = 8; A549 + LNFs, n = 8; H358 alone, n = 4; H358 + CAFs, n = 4; H358 + LNFs, n = 4]) were injected subcutaneously into the back of 4-week-old nude mice. Tumor volume was calculated according to the formula: tumor volume (mm$^3$) = $d^2 \times D/2$, where $d$ and $D$ were the shortest and the longest diameters, respectively. The SB431542 was injected intraperitoneally at 10 mg/kg (once per day, 5 times a week for 2 weeks) 1 week after co-injection ($1 \times 10^6$ A549 cells with or without $1 \times 10^5$ CAFs, n = 5 for each group) according to protocols described in previous reports [22, 23].

**Immunohistochemistry**

Immunohistochemistry was performed as previously described [7]. All sections stained with E-cadherin and CD44 were scored in a semiquantitative manner classified as 0 (no staining), +1 (weak staining), +2 (moderate staining), or +3 (strong staining) in intracellular compartments of carcinoma cells [24]. The Ki-67 labeling index (labeling frequency %) was calculated by the following formula: [number of positive nuclei/total number of represented cells] \times 100. All immunohistochemistry results were quantified by counting at least more than 200 tumor cells in randomly selected 5 areas in each specimen.

**Statistical Design and Data Analysis**

A $\chi^2$ test, Mann-Whitney U test, or repeated measures analysis of variance was used to compare the results. All statistical analyses were performed using StatView version 5.0 for Windows (Abacus Concepts, Berkeley, CA).

**Results**

**NSCLC Cell Lines Undergo EMT in Response to Conditioned Medium From Fibroblasts**

Primary cultured CAFs and LNFs each expressed α-SMA and fibroblast activating protein, with the levels greater in CAFs than LNFs even after 5 passages (Fig 1A, 1B). Conditioned medium from both also induced EMT in A549 and NCI-H358 cells, which became more spindle shaped (Fig 1C). Furthermore, the expression of E-cadherin decreased and that of N-cadherin increased in cancer cells (Fig 1D, 1E). These changes in EMT markers were significantly greater in A549 cells treated with CM from CAFs as compared with those treated with CM from LNFs, while migration induced by CAFs was significantly greater than that induced by LNFs in A549 cells (Fig 1F). These findings suggest that paracrine interplay between fibroblasts and cancer cells leads to EMT in cancer cells. In addition, they indicate that CAFs can maintain the phenotypic properties of myofibroblasts even in the absence of continuing interaction with carcinoma cells.

**Spheroid Formation Ability and Resistance to Treatment by NSCLC Cell Lines in Response to CM From Fibroblasts**

When cultured with CM from fibroblasts, both A549 and NCI-H358 cells gave rise to significantly greater spheroid body formation as compared with cells cultured without CM (Fig 2A, 2B). Furthermore, CM-treated cells showed a significant increase in cell viability in response to cisplatin when compared with untreated control cells (Fig 2C).

**CAF-Mediated EMT Promoted Generation of Cancer Stem Cells**

We evaluated changes in the CSC markers CD44 and CD133. The CM from CAFs increased the expression of CD44 to a greater degree than that seen after treatment with CM from LNFs (Fig 2D). The SDS-PAGE results also showed that the expression of CD44 was increased to a greater degree in cancer cells treated with CM from CAFs than in those treated with CM from LNFs (Fig 1E).

**Blocking TGF-β Signaling Inhibited Phenotypic Changes Associated With EMT and Stem Cell-Like Qualities**

Transforming growth factor-β is a strong inducer of EMT in epithelial cells. The ELISA findings showed that the concentration of TGF-β1 was greater in CM from CAFs as compared with that from LNFs (Fig 2E). Thus, we blocked TGF-β signaling to clarify the role of TGF-β in CAF-induced EMT. The CAF CM-driven phenotypic changes were inhibited by addition of the TGF-β inhibitor SB431542 to CM (Fig 2F), while down-regulation of E-cadherin and increased CD44 expression (Fig 2G, 2H), and spheroid formation ability induced by CM from CAFs (Fig 2I) were significantly inhibited by SB431542. Although TGF-β inhibition did not change the growth of cancer cells after 2 days, it attenuated the resistance to chemotherapy in vitro (Fig 2J). Taken together, these results suggest that TGF-β signaling may mediate EMT and maintenance of stem cell-like qualities induced by CAFs.

**CAF-Mediated Tumor Formation of Human Lung Adenocarcinoma Cell Lines**

Although cancer cells co-cultured with fibroblasts did not show an increase in cell proliferation (Fig 2), compare left panel with middle panel, p = 0.74), subcutaneous co-injection of each lung cancer cell line with human CAFs or LNFs into nude mice resulted in a high rate of tumor formation, as compared with injection of cancer cells alone (Fig 3A). There was no difference between co-injection with cancer cells and CAFs and that with LNFs. We also compared specimens obtained from mice injected with cancer cells alone with those co-injected with cancer cells and CAFs. Hematoxylin and eosin
staining revealed both necrosis and apoptotic cell death in the middle of tumors from mice injected with A549 or H358 cells alone (Fig 3B). On the other hand, E-cadherin and CD44 expressions were changed in tumors from mice that underwent co-injection with cancer cells and CAFs as compared with those with cancer cells alone. We compared these findings using a semiquantification technique, which showed that the expression of E-cadherin was lower and that of CD44 higher in cancer cells injected with CAFs as compared with cancer cells alone (Fig 3C). Furthermore, the Ki-67 labeling index was also higher in tumors from mice injected cancer cells and CAFs as compared with cancer cells alone. These results suggest that co-injection with A549 and CAFs induced EMT and stem cell-like qualities in cancer cells, resulting in rapid tumor growth in vivo.

When compound SB431542 was injected in an intraperitoneal manner, tumor formation enhanced by co-injection with CAFs was attenuated, whereas that compound did not affect tumor growth in mice injected with A549 alone (Fig 4A). Furthermore, the expression of E-cadherin was increased and Ki-67 labeling index was decreased in tumors obtained from mice treated with SB431542 (Fig 4B, 4C).

Comment
In the present study we found that CM from fibroblasts induced EMT in NSCLC cells, while those cells also acquired characteristics of stem cell-like qualities. The EMT changes induced by CAFs in A549 cells were greater than those induced by LNFs, indicating that CAFs may have been more activated in our experimental system as compared with LNFs. Navab and colleagues [25] reported that CAFs were activated to a greater degree than LNFs by showing α-SMA expression and collagen gel contraction. Together, these findings suggest that CAFs play a more crucial role in tumor homeostasis, progression, and maintenance of stem cell-like qualities in cancer cells as compared with normal fibroblasts. However, it is difficult to compare CAFs with LNFs in a rigorous manner. One reason is that the present fibroblasts were isolated from heterogeneous tissues obtained from patients with different backgrounds. Furthermore, while fibroblasts are in an inactive and quiescent state in normal tissues, isolated fibroblasts may become activated and release mediators when cultured with FBS or co-cultured with cancer cells. We found that primary cultured CAFs and LNFs each expressed α-SMA and fibroblast activating protein,
though those levels were greater in CAFs than LNFs after 5 passages. Therefore, the cells used in the present in vitro experiments were collected within the limit of 5 passages. We found that co-injection of each lung cancer cell line with human fibroblasts into mice resulted in a high rate of tumor formation. A previous report [25] noted that CAFs demonstrated a greater ability to enhance the tumorigenicity of lung cancer cell lines in vivo, while co-injection with CAFs increased tumor formation in a manner similar to that seen when co-injected with LNFs in the present experimental system. It is possible that co-injection of LNFs with cancer cells may stimulate LNFs in vivo and change their characteristics in a manner similar to CAFs. Although the marker profile of lung cancer stem cells remains to be fully elucidated, recent findings suggest that CD44 or CD133 may be an effective lung cancer stem cell marker [26, 27]. In addition, CD44 has been associated with a number of signaling cascades that mediate tumor progression and resistance to chemotherapy [28, 29]. Cancer cells in the present study showed increased levels of these CSC markers, supporting the notion that fibroblasts in the tumor stroma maintain cancer stem cell-like properties.

Phenotypic changes associated with EMT and stem cell-like qualities were inhibited by addition of the TGF-β inhibitor SB431542, indicating that TGF-β signaling plays a major role in fibroblast-induced changes. The TGF-β is expressed by both cancer and stromal cells, and establishes interactive pathways in the cross-talk between them [2]. In the efferent pathway cancer cells trigger a reactive response in the stroma, while in the afferent pathway cells responding to modified stromal cells in the surrounding microenvironment have effects on cancer cell response [30, 31]. These interactive pathways, which become established during cross-talk between cancer and stromal cells, may form a malignant signaling cycle for cancer progression. Because CAFs are genetically stable and less likely to develop drug resistance [15], targeting them as a therapeutic strategy against cancer is an intriguing concept that would benefit from further study.


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ABTS Requirements for the 10-Year Milestone for Maintenance of Certification

Diplomates of the American Board of Thoracic Surgery (ABTS) who plan to participate in the 10-Year Milestone for the Maintenance of Certification (MOC) process as Certified-Active must hold an unrestricted medical license in the locale of their practice and privileges in a hospital accredited by the JCAHO (or other organization recognized by the ABTS). In addition, a valid ABTS certificate is an absolute requirement for entrance into the MOC process. If your certificate has expired, the only pathway for renewal of a certificate is to take and pass the Part I (written) and the Part II (oral) certifying examinations.

The CME requirements are 150 Category I credits over a five-year period. At least half of these CME hours need to be in the broad area of thoracic surgery. Category II credits are not accepted. Interested individuals should refer to the Board’s website (www.abts.org) for a complete description of acceptable CME credits.

Diplomates will be required to take and pass a secured exam after their application has been approved. Taking SESATS in lieu of the secured exam is not an option. The secured exam is administered over a two-week period in September of every year at Pearson Vue Testing Centers, which are located nationwide. Diplomates will have the opportunity to select the day and location of their exam. For the dates of the next MOC exam, visit the Board’s website at www.abts.org.

Starting on July 1, 2014, the ABTS will require its Diplomates to participate in an outcomes database as fulfillment of Part IV (Performance in Practice) for the 10-year Milestone of Maintenance of Certification (MOC). For a list of approved outcomes databases or for more information on how to have a database approved by the Board, visit the Board’s website at www.abts.org. Participation in the Professional Portfolio will no longer be accepted as fulfillment of MOC Part IV after July 1, 2014.

Diplomates may apply for MOC in the year their certificate expires or, if they wish to do so, they may apply up to two years before it expires. However, the new certificate will be dated 10 years from the date of expiration of their original certificate or most recent MOC certificate. In other words, going through the MOC process early does not alter the 10-year validation. Diplomates certified prior to 1976 (the year that time-limited certificates were initiated) are also required to participate in MOC if they wish to maintain valid certificates.

The deadline for submitting an application for 10-year Milestone of MOC is March 15 of every year. Information outlining the rules, requirements, and dates for MOC in thoracic surgery is available on the Board’s website at www.abts.org. For additional information, please contact the American Board of Thoracic Surgery, 633 N St. Clair St, Ste 2320, Chicago, IL 60611; telephone (312) 202-5900; fax (312) 202-5960; e-mail: info@abts.org.