The Essential Role of Fibroblasts in Esophageal Squamous Cell Carcinoma–Induced Angiogenesis

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**Background & Aims:** Esophageal squamous cell carcinoma (ESCC) is known to be a highly angiogenic tumor. Here, we investigated the role of the stromal fibroblasts in the ESCC-induced angiogenic response using a novel 3-dimensional model. **Methods:** A novel assay was developed where cocultures of ESCC and esophageal fibroblasts induced human microvascular endothelial cell (HMVEC) vascular network formation in a 3-dimensional collagen gel. Biochemical studies showed that the ESCC-induced activation of the fibroblasts was required to induce vascular network formation via a transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF)-dependent pathway. **Results:** Conditioned media from a panel of 4 ESCC lines transdifferentiated normal esophageal fibroblasts into myofibroblasts via TGF-β signaling. The presence of fibroblasts was essential for efficient HMVEC network formation, and the addition of ESCC cells to these cultures greatly enhanced the angiogenic process. The role of TGF-β in this process was shown by the complete inhibition of network formation following TGF-β inhibitor treatment. Finally, we showed that ESCC-derived TGF-β regulates angiogenesis through the release of VEGF from the fibroblasts and that the VEGF release was blocked following TGF-β inhibition. **Conclusions:** This study shows the essential role of fibroblasts in the ESCC angiogenic-induced response and suggests that the pharmacologic targeting of the TGF-β signaling axis could be of therapeutic benefit in this deadly disease.

The tumor “organ” consists of a dynamic mixture of tumor cells, fibroblasts, endothelial cells, and immune cells that all work together to drive tumor progression.1 Activated fibroblasts, also known as carcinoma-associated fibroblasts (CAFs),2 have been identified at the leading edges of many solid tumors, including breast, colon, and melanoma.3–5 The presence of CAFs within the tumor microenvironment is preceded by the chemotraction and migration of precursor cells, which can either arise from the surrounding host fibroblasts or from circulating mesenchymal precursors/stem cells.6,7 Once recruited, paracrine tumor-derived growth factors activate the CAFs, which undergo a myofibroblastic transdifferentiation, defined by an elongated spindle shape, and the expression of contractile α-smooth muscle actin (α-SMA) and vimentin.8 CAFs are hypothesized to drive tumor progression through the deposition of extracellular matrix proteins, the secretion of growth factors, and the stimulation of invasion.9

One area that has been little explored is the potential role of CAFs in tumor angiogenesis. Much of the growth of solid tumors is dependent on the ready supply of nutrients and oxygen from a local blood supply. As tumors grow beyond a few millimeters in size, they readily outstrip the local supply of nutrients available through simple diffusion and stimulate the formation of their own tumor vasculature. Although it has been shown that stromal fibroblasts are an important source of the proangiogenic factor vascular endothelial cell growth factor (VEGF),10 it has been difficult to study the interaction of carcinoma cells, fibroblasts, and endothelial cells in a physiologically relevant model. In the present study, we used a novel 3-dimensional (3D) in vitro model in which the interaction of esophageal squamous cell carcinoma (ESCC) cells with fibroblasts drives vascular network formation in a 3D collagen gel. We show that ESCC cells require the presence of stromal fibroblasts to stimulate vascular network formation, thereby suggesting that fibroblasts are the critical mediators of angiogenesis in this system. Mechanistic studies reveal that paracrine transforming growth factor (TGF)-β from the ESCC cells leads to activation of the fibroblasts and that pharmacologic

***Abbreviations used in this paper:*** CAF, carcinoma–associated fibroblast; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; ESCC, esophageal squamous cell carcinoma; FBS, fetal bovine serum; GFP, green fluorescent protein; HMVEC, human microvascular endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; α-SMA, α-smooth muscle actin; TGF, transforming growth factor; 3D, 3-dimensional; VEGF, vascular endothelial growth factor.

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0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.02.061
inhibitors of TGF-β signaling can reverse both fibroblast activation and vascular network formation.

Materials and Methods

Cell Lines

Esophageal cancer cells, TE cell lines (TE1, TE8, TE10, TE11, TE12), were cultured as previously described. Human esophageal keratinocytes EPC2 have been described previously. Human microvascular endothelial cells (HMVECs) are available commercially through Cascade Biologies, Inc (Portland, OR). Primary human esophageal fibroblasts designated as FEF3 were isolated from human fetal esophagus as described previously. FEF3 cells were stably transduced using the ViralPower Lentiviral Expression System (Invitrogen, Carlsbad, CA) containing the gene for green fluorescent protein (GFP). GFP lentivirus was raised in our laboratory, and the FEF3 cells were transduced in the presence of 6 μg/mL polybrene. Forty-eight hours after transduction, cells were selected in the presence of 10 μg/mL blasticidin for 14 days.

Antibodies and Reagents

The following antibodies were used in this study: anti-human CD31 (Dako, Carpinteria, CA), anti-α-SMA (Sigma-Aldrich, St Louis, MO), anti-β-tubulin (Novo RC, Fremont, CA), anti–fibroblast activation protein (EMD Biosciences, San Diego, CA), anti-TGFβRII (Santa Cruz Biotechnology, Santa Cruz, CA), and phalloidin/Texas Red (Molecular Probes, Eugene, OR). The anti-smad2, anti–phospho-smad2 (Ser465/467), anti–phospho-smad3 (Ser423/425), and Smad1 (Ser463/465) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant human TGF-β1 was purchased by R&D Systems, Inc (Minneapolis, MN). SB-505124, GW788388, and GW654652 were provided by GlaxoSmithKline (Collegeville, PA). Bevacizumab (Avastin; Genentech, South San Francisco, CA) containing the gene for green fluorescent protein (GFP). GFP lentivirus was raised in our laboratory, and the FEF3 cells were transduced in the presence of 6 μg/mL polybrene. Forty-eight hours after transduction, cells were selected in the presence of 10 μg/mL blasticidin for 14 days.

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In Vitro 3D Network Formation Assay and Fluorescence Imaging

Reconstruction of a vessel-like structure in 3D collagen gels and subsequent fluorescent staining of networks/cords in whole-mount gels were performed as previously described. Briefly, HMVECs were cultured as monolayers on bovine type I collagen-coated 24-well plates at 1.5 × 10^5 cells/well for 24 hours and overlaid with acellular collagen mixed in 10% Medium 199 (Invitrogen) with heparin (100 U/mL), vitamin C (50 μg/mL), and fetal bovine serum (FBS; 1%). After polymerization of the collagen gels, the cells were further overlaid with a second collagen layer containing each 1.0–2.5 × 10^5 cells/mL FEF3, 0.5 × 10^5 cells/mL TE cells, or both cells. Cells were then filled with EBM-2 medium containing EGM-2MV. The reconstructions were incubated at 37°C for 7 days. To prepare for staining, medium was removed and the collagen gels were fixed in Prefer (Anatech Ltd, Battle Creek, MI) for 4 hours at room temperature. Gels were processed as whole mounts. After blocking with 1% bovine serum albumin, gels were stained with monoclonal anti-Cd31 antibody followed by Texas Red–conjugated secondary antibody. Gels were treated with VectaShield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA), and the stained endothelial cell networks were photographed under a Nikon E600 fluorescent microscope (Nikon, Melville, NY).

The capillary-like networks were scored by counting the number of CD31-stained branches. One branch was counted to be 3 cells thick or less (to discount disorganized masses of HMVECs) and at least 3 whole cells long. At least 5 randomly chosen low-power fields were counted per sample (10× magnification). Each figure shows one representative experiment. Data show the mean of at least 3 independent experiments.

Immunofluorescence Microscope

For cocultures, FEF3 cells and TE cells mixed in a 1:1 ratio (2.5 × 10^5 cells per each) were seeded onto glass coverslips in 6-well plates and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS for 48 hours. Cells were then fixed and stained as described previously.

Western Blotting Analysis

Subconfluent cells were lysed and separated on a 4%–12% sodium dodecyl sulfate/polyacrylamide gel before being blotted as described previously.

Treatment of FEF3 Cells With Conditioned Media

For preparation of conditioned medium, TE cells were cultured with DMEM containing 10% FBS overnight. Supernatants were removed, and cells were washed with DMEM. Cells were cultured for 48 hours with fresh medium DMEM containing 2% FBS. FEF3 cells were cultured overnight with DMEM containing 10% FBS. Supernatants were replaced and cultured with fresh culture medium DMEM containing 2% FBS with or without TGF-β1 or cultured with conditioned medium for each time.

TGF-β1/2 Enzyme-Linked Immunosorbent Assay

Cells were cultured overnight with DMEM containing 10% FBS. Supernatants of these cells were removed, and cells were washed with DMEM basal medium. Cells were cultured for 48 hours with fresh culture medium DMEM containing 2% FBS. These supernatants were measured as samples using each enzyme-linked im-
Figure 1. The interaction of ESCC cells and fibroblasts drives efficient vascular network formation in 3D culture. Immunofluorescence staining shows GFP-tagged FEF3 esophageal fibroblasts (green), HMVECs (CD31; red), and total nuclei (DAPI; blue). (Inset) CD31 staining. (A) Incubation of the HMVECs with each of 3 ESCC lines (TE1, TE8, TE11) was not associated with any vascular network formation. (B) Coculture of the HMVECs with human esophageal fibroblasts (FEF3) led to increased vascular network formation. (C) The addition of ESCC cells to the fibroblast/endothelial cell coculture markedly increased the organization of the vascular networks. Increasing the culture time to 14 days dramatically enhanced the organization of the vascular network. (D) ESCC number was also found to increase the degree of vascular network formation. (E) The extent of network formation under each of the culture conditions was scored, with the addition of increasing numbers of ESCC cells found to significantly increase the level of vascular network formation. Scale bar = 200 μm.
**Figure A**

- **A1**: GFP-DAPI images showing fibroblasts labeled with FEF3.
- **A2**: α-SMA images showing fibroblasts labeled with TE1.
- **A3**: Merge images showing fibroblasts labeled with FEF3 and TE1.

**Figure B**

- **B**: Co-cultured with TE1, showing images of fibroblasts labeled with FEF3 and TE1.

**Figure C**

- **C1**: GFP-DAPI images showing fibroblasts labeled with FEF3 and TE11.
- **C2**: α-SMA images showing fibroblasts labeled with TE11.
- **C3**: Merge images showing fibroblasts labeled with FEF3 and TE11.

**Figure D**

- **D1**: GFP-DAPI images showing fibroblasts labeled with FEF3 and TE8.
- **D2**: GFP-DAPI images showing fibroblasts labeled with FEF3 and TE10.
- **D3**: GFP-DAPI images showing fibroblasts labeled with FEF3 and TE12.

**Figure E**

- **E1**: Western blotting showing α-SMA expression with control (cont) and different conditions (TE1, TE8, TE10, TE11, TE12).
- **E2**: Western blotting showing β-actin expression with control (cont) and different conditions (TE1, TE8, TE10, TE11, TE12).

**Figure F**

- **F1**: Western blotting showing α-SMA expression with control (cont) and EPC2 conditions.
- **F2**: Western blotting showing β-actin expression with control (cont) and EPC2 conditions.
phenyltetrazolium bromide (MTT) assay as previously analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-
SB-505124, or GW788388 in triplicate. Proliferation was
were activated by adding 0.1 mL of 1 mol/L HCl for 10
minutes and neutralized with 100 μL of 1.2 mol/L
NaOH/0.5 mol/L HEPES before assay to measure the
total amount of TGF-β.

VEGF ELISA

For 2-dimensional cocultures, fibroblasts and TE1
cells at a 1:1 ratio were seeded onto plates and cultured
with or without SB-505124 compound overnight. Su-
pernatants were removed, and cells were washed with
DMEM. Cells were cultured for 48 hours with fresh culture
medium DMEM containing 2% FBS with or without the
compound. These supernatants were measured as sam-
ple using VEGF ELISA. For 3D culture, each sample was
cultured with or without SB-505124 compound for 48
hours, and these supernatants were measured using the
VEGF ELISA kit (R&D Systems).

Cell Proliferation Analysis

Cells were plated into a 96-well plate at a density
of 2.5 × 10^4 cells/mL and left to grow overnight. Cells
were treated with increasing concentrations of TGF-β,
SB-505124, or GW788388 in triplicate. Proliferation was
analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-
phenyltetrazolium bromide (MTT) assay as previously
described.14

Statistics

Unless otherwise stated, all experiments show the
mean ± SD of at least 3 independent experiments. Sta-
tistical significance was measured using the Student t
test, where P < .05 was judged to be significant.

Results

The Presence of Fibroblasts Is Required for
ESCC-Induced Angiogenesis

Studying the role of fibroblasts in ESCC-induced
network formation in vivo is technically challenging. To
overcome some of these issues, we developed a novel 3D
model of ESCC-induced angiogenesis, allowing us to
study the role of CAFs in vascular network formation13
(see Supplementary Figure 1 for scheme; see supplemen-
tal material online at www.gastrojournal.org). In the
model, factors derived from either fibroblasts or a mix-
ture of fibroblasts and ESCC cells cause HMVECs to
detach from the tissue culture plate and migrate upward
into the 3D collagen gel, where they organize and form
vascular networks. In an initial series of studies, it was
found that coculture of the ESCC lines with the HMVECs
(TE1, TE8, and TE11) did not lead to vascular network
formation (Figure 1A). However, coculture of the esoph-
ageal fibroblasts (FEF3) with the HMVECs led to endo-
thelial cell migration and formation of moderately well-
differentiated vascular networks as shown by CD31 and
von Willebrand factor staining (Figure 1B). Addition of ESCC cells to the fibroblast/
HMVEC cocultures had the most striking effects on
vascular network formation and led to the establish-
ment of very organized capillary-like structures (Figure
1C). Increasing the numbers of both the fibroblasts
and ESCC cells was associated with significantly more
organized capillary networks (Figure 1B, D, and E),
showing that the interaction of both the ESCC cells
and fibroblasts was critical for efficient vascular net-
work formation.

Coculture of Esophageal Fibroblasts With
ESCC Cells Leads to Their Activation and
Transdifferentiation Into Myofibroblasts

CAFs are typically in an activated state, having
undergone transdifferentiation to a myofibroblast phe-
notype. Growing the human esophageal fibroblasts with
the ESCC line TE1 for 48 hours led to a change in
phenotype associated with the increased cytoplasmic ex-
pression of the myofibroblast marker α-SMA and fibro-
blast activation protein (Figure 2A and B and not shown).
Three additional esophageal carcinoma lines were also
noted to induce a similar degree of myofibroblast trans-
differentiation (TE11, TE8, and TE12; Figure 2C and D).
The induction of the myofibroblast phenotype was in-
duced through soluble factors derived from the ESCC
cells, as shown by the ability of conditioned media from
a panel of ESCC lines to induce α-SMA expression in the
fibroblasts (Figure 2F). In contrast, conditioned media
from primary human esophageal keratinocytes (EPC2)
were unable to induce α-SMA expression in the fibro-
blasts (Figure 2F).
**Paracrine TGF-β Secreted From ESCC Cells Is Responsible for Esophageal Fibroblast Transactivation**

ELISA experiments showed that all 4 ESCC lines secreted high levels of both TGF-β1 and TGF-β2 (Figure 3A). To rule out the role of other possible growth factors, further studies were performed showing that the ESCC cells secreted very little platelet-derived growth factor or insulin-like growth factor (data not shown). In a similar manner to that of ESCC-conditioned media, exogenous TGF-β was also shown to induce esophageal fibroblast transdifferentiation associated with increased α-SMA expression (Figure 3B). Stimulation of the fibroblasts with exogenous TGF-β was also accompanied by increased...
SMAD signaling, as shown by the rapid increase in phospho-SMAD2 (Figure 3B). Evidence for TGF-β1 being the ESCC-derived fibroblast-activating factor came from the similar induction of nuclear SMAD3 accumulation seen in both exogenous TGF-β1 and ESCC conditioned media (Figure 3C). Next, we investigated whether exogenous TGF-β would stimulate the fibroblasts to drive vascular network formation. Treatment of esophageal fibroblast/HMVEC cocultures with 1 ng/mL TGF-β for 7 days led to a statistically significant increase in capillary network formation (Figure 3D) and showed the essential role of TGF-β-induced fibroblast differentiation in the angiogenic process. To show the essential role of the fibroblasts in TGF-β-induced vascular network formation, we treated 3D monolayers of endothelial cells with TGF-β1 (1 ng/mL) and observed very little vascular network formation. Indeed, the endothelial cells remained attached to the plates and did not migrate upward into the collagen (Supplementary Figure 2; see supplemental material online at www.gastrojournal.org).

TGF-β plays a complex role in tumor progression and is known to be growth inhibitory to most epithelial cell types. To overcome the effects of TGF-β, most carcinoma cells escape by down-regulating their expression of TGF-β receptors and instead secrete autocrine/paracrine TGF-β that recruits the surrounding stromal cells. In agreement with this idea, the ESCC lines tested were not found to express any TGF-β receptor subtype II protein, whereas esophageal fibroblasts and the parent esophageal keratinocyte line EPC2 maintained receptor expression (Figure 3E). The ESCC lines were shown to have escaped the inhibitory effects of TGF-β and proliferated normally in the presence of increasing concentrations of TGF-β (0.1–10 ng/mL), whereas the primary human esophageal keratinocyte line EPC2 was growth arrested (Figure 3F).

**The TGF-β-Specific Inhibitor SB-505124 Inhibits ESCC-Induced Fibroblast Transdifferentiation**

Pharmacologic approaches that block tumor neoangiogenesis are an attractive therapeutic option. We next tested whether the specific inhibitor of TGF-β receptor kinase SB-505124 blocked fibroblast transdifferentiation. Pretreating the esophageal fibroblasts with increasing concentrations of SB-505124 (0.01–10 μmol/L) successfully blocked the TGF-β-induced increases in α-SMA expression in the esophageal fibroblasts (Figure 4A). Maximal inhibition of the TGF-β-induced fibroblast activation was seen at 1 μmol/L SB-505124, and this concentration was selected for all subsequent experiments. Treatment of the cells with SB-505124 also blocked α-SMA induction and SMAD2 activation following treatment with ESCC conditioned media (Figure 4B and C). To investigate whether TGF-β inhibition was growth inhibitory on any of the cell types used in the neoangiogenesis assay, endothelial cells (HMVEC), fibroblasts (FEF3), and 3 ESCC lines (TE1, TE8, and TE12) were treated with increasing concentrations of each of 2 structurally distinct small molecule inhibitors of TGF-β (SB-505124 and GW788388) for 72 hours. Measuring levels of proliferation using the MTT assay showed that neither TGF-β inhibitor affected the growth of any of the cell types tested (Figure 4D and E).

**The TGF-β Inhibitors SB-505124 and GW788388 Inhibit ESCC-Induced Vascular Network Formation**

We next investigated whether inhibition of fibroblast activation blocked ESCC-induced vascular network formation. Coculture of fibroblasts with the HMVECs led to endothelial cell migration but very little differentiated vascular network formation (Figure 5A). Treatment of the fibroblast/HMVEC cocultures with the TGF-β inhibitor GW788388 (1 μmol/L) did not significantly alter the level of vascular network organization (Figure 5A), showing that there was only limited fibroblast activation in the absence of ESCC cells. As before, addition of the ESCC lines TE1 and TE8 to the fibroblast/HMVEC coculture dramatically increased the level of vascular network organization (Figure 5A). In contrast, treatment of the ESCC/fibroblast/HMVEC cocultures with either SB-505124 (1 μmol/L) or GW788388 (1 μmol/L) led to a complete reversal of vascular network formation (Figure 5B and C). These results show that inhibition of fibroblast activation by small molecule...
TGFβ inhibitors blocks ESCC-induced neoangiogenesis (Figure 5D).

**Pharmacologic Inhibition of TGF-β Inhibits VEGF Secretion From Both Esophageal Fibroblasts and ESCC Cells**

VEGF is one of the most potent inducers of angiogenesis. To investigate the possible role of paracrine VEGF release from esophageal fibroblasts in ESCC-induced neoangiogenesis, we tested VEGF release from cocultured and TGFβ–stimulated esophageal fibroblasts using an ELISA. Unstimulated esophageal fibroblasts produce a basal level of VEGF under 2-dimensional cell culture conditions, and this was significantly increased when the fibroblasts were grown under 3D conditions (Figure 6A and B). Treatment of the fibroblasts with the TGFβ inhibitor SB-505124 did not reduce basal levels of VEGF under 2-dimensional cell culture conditions (data not shown); however, it did inhibit basal VEGF release when the fibroblasts were grown under 3D cell culture conditions (Figure 6B). This showed that there was some level of fibroblast activation when the cells were switched from the 2-dimensional to 3D cell culture conditions. Stimulation of the fibroblasts with exogenous TGFβ (1 ng/mL) led to a significant increase in the level of VEGF secreted (Figure 6A). In a similar manner, coculture of the fibroblasts with the ESCC line TE1 also led to a significant increase in VEGF secretion under both 2-dimensional and 3D cell culture conditions (Figure 6A and B). Treatment of the ESCC/fibroblast cocultures with SB-505124 (1 μmol/L) led to a significant inhibition in the level of VEGF, showing a possible mechanism by which ESCC-activated fibroblasts can induce vascular network formation.

As a final test, we determined whether inhibition of VEGFR2 signaling via the selective inhibitor GW654652 could also block ESCC-induced vascular network formation. Coculture of the HMVECs and fibroblasts led to some limited vascular network formation after 7 days of culture, and this was not affected by the presence of GW654652 (1 μmol/L) (Figure 6C). Treatment of the ESCC, fibroblast, and HMVEC cultures with GW654652 (1 μmol/L) did not completely inhibit vascular network formation but did lead to a reduction in the number of endothelial cell tubes formed (Figure 6C). Analysis of these results showed a significant reduction (P < .005) in the extent of ESCC-induced vascular network formation following GW654652 treatment (Figure 6D). Similar results were also seen upon ESCC-induced vascular network formation following treatment with an antibody directed against VEGF receptor (bevacizumab) (Supplemental Figure 3; see supplemental material online at www.gastrojournal.org). A sample scheme showing how ESCC cells stimulate fibroblasts to drive vascular network formation is shown in Figure 7.

**Discussion**

Here we show for the first time, using a novel 3D in vitro model of the tumor microenvironment, the critical role of the host fibroblasts in directing ESCC-induced vascular network formation. This model differs
Figure 5. Pharmacologic inhibitors of TGF-β signaling block ESCC-induced vascular network formation. (A) TGF-β inhibition has little effect on fibroblast-induced vascular network formation. Fibroblasts (FEF3; 1.5 × 10^5 cells/mL) were incubated with HMVECs in the absence or presence of 1 of 2 TGF-β inhibitors (SB-505124 or GW788388, both 1 μmol/L) for 7 days. Cultures were stained for HMVECs (CD31; red), fibroblasts (GFP; green), and nuclei (DAPI; blue). (B and C) TGF-β inhibition completely blocks ESCC-induced vascular network formation. Addition of either SB-505124 or GW788388 (both 1 μmol/L) led to complete inhibition of vascular network formation. (D) Bar graph shows mean data for vascular network formation in the absence and presence of either SB-505124 or GW788388. All representative images are shown as 3-color merges, and original monochrome CD31 (white) images are inset. Scale bar = 200 μm.
Figure 6. Activated fibroblasts secrete VEGF, leading to increased vascular network formation. (A) Stimulation of esophageal fibroblasts with either TGF-β through coculture with ESCC cells leads to enhanced VEGF release in 2-dimensional adherent culture. Fibroblasts (FEF3) in monoculture or FEF3 and ESCC cells (TE1) at a ratio of 1:1 in coculture were cultured in the absence or presence of SB-505124 (1 μmol/L) for 48 hours. Supernatants were harvested and quantified for VEGF expression using a specific ELISA. (B) Stimulation of esophageal fibroblasts with either TGF-β or ESCC conditioned media leads to enhanced VEGF release in 3D culture. Monocultures and cocultures of esophageal fibroblasts were grown in a 3D collagen in the absence or presence of SB-505124 for 48 hours. (C) The VEGF inhibitor GW654652 (1 μmol/L) inhibits vascular network formation. (D) The bar graph shows mean data for vascular network formation in the absence and presence of GW654652. All representative images are shown as 3-color merges; monochrome images of CD31 are inset. Scale bar = 200 μm.
from that in previous studies because it contains cancer cells, fibroblasts, and endothelial cells. Coculture of ESCC cells with HMVECs did not stimulate vascular network formation in the absence of fibroblasts. Whereas the coculture of fibroblasts with HMVECs induced some endothelial cell migration into the collagen, it was associated with large, poorly defined vascular networks with little organized branching. Truly organized vascular network formation was only seen when ESCC cells were added to the fibroblast/HMVEC cultures. This suggested both that the fibroblasts were absolutely required for endothelial tube formation and that a prior fibroblast activation step, via factor(s) released from the ESCC cells, was required for efficient network formation. Activated fibroblasts are often observed in the stroma associated with growing tumors.\textsuperscript{15,16} In agreement with this observation, we noted that coculture of ESCC cells with esophageal fibroblasts led to their transdifferentiation into myofibroblasts. Myofibroblasts are known to be a good model for CAFs because the activated fibroblasts within the tumor milieu exhibit a phenotype that is virtually indistinguishable from that of myofibroblasts.\textsuperscript{17}

One of the best known factors responsible for the phenotypic switch of fibroblast to myofibroblast is TGF-\(\beta\).\textsuperscript{18} Loss of TGF-\(\beta\) receptor function is a common event in cancer,\textsuperscript{18} and ESCC cells are no exception. They typically become insensitive to the growth inhibitory effects of TGF-\(\beta\) through either the loss of TGF-\(\beta\) receptor expression or the acquisition of missense mutations in TGF\(\beta\)RI.\textsuperscript{19–21} In agreement with these published studies, we found that our panel of ESCC lines was refractory to the growth inhibitory effects of exogenous TGF-\(\beta\), whereas primary human esophageal keratinocytes were strongly growth inhibited. Loss of TGF\(\beta\)RII expression in the ESCC lines was also accompanied by the autocrine/paracrine production of both TGF-\(\beta\)1 and TGF-\(\beta\)2. Both ESCC conditioned media and exogenous TGF-\(\beta\) were similarly able to induce the myofibroblast phenotype and stimulate SMAD signaling, suggesting that TGF-\(\beta\) was the factor likely for the fibroblast activation observed. The requirement for TGF-\(\beta\)–induced fibroblast activation in neoangiogenesis was shown by the ability of exogenous TGF-\(\beta\) to enhance vascular network formation in the absence of the ESCC cells. Further evidence for the role of TGF-\(\beta\) in ESCC-induced fibroblast activation came from studies showing that a pharmacologic inhibitor of TGF-\(\beta\) blocked the ability of ESCC-conditioned media induction of the myofibroblast phenotype in esophageal fibroblasts.

Having shown that ESCC-secreted TGF-\(\beta\) is essential for fibroblast-induced activation, we next tested whether pharmacologic inhibition of TGF-\(\beta\) signaling could inhibit vascular network formation. For these studies we used SB-505124, a selective TGF-\(\beta\) receptor antagonist with very little activity against any other kinase tested.\textsuperscript{22} Increasing concentrations of SB-505124 were found to inhibit myofibroblast transdifferentiation induced both by TGF-\(\beta\) and ESCC conditioned media. The essential role of TGF-\(\beta\)–induced fibroblast activation in vascular

**Figure 7.** Schematic illustration showing the role of ESCC cells and fibroblasts in vascular network formation. Esophageal cancer cells produce TGF-\(\beta\) to activate stromal normal fibroblasts. Tumor stromal fibroblasts become transdifferentiated into myofibroblasts that secrete VEGF, which in turn induces endothelial cell migration and the formation of a microvascular network.
network formation was indicated by the fact that SB-505124 and the structurally unrelated TGF-β inhibitor GW788388 significantly inhibited ESCC-induced neangiogenesis. The requirement for both ESCC cells and fibroblasts in the angiogenic process was shown by the fact that neither of the TGF-β inhibitors tested blocked the limited amount of vascular network formation induced by fibroblasts alone.

ESCC-induced fibroblast activation was shown to drive vascular network formation through the stimulation of VEGF release. VEGF is a potent proangiogenic factor that stimulates endothelial cell migration and proliferation and regulates microvascular permeability. There is compelling evidence that fibroblasts can be a major source of growth factors that may contribute to tumor progression and possibly angiogenesis. Studies using transgenic mice expressing GFP under the control of VEGF promoter showed strong GFP staining in the stroma of spontaneously arising mammary tumors. Here we show that fibroblasts activated by exogenous TGF-β through coculture with ESCC cells secrete significantly higher VEGF levels through a mechanism involving SMAD signaling. The fact that a selective inhibitor of VEGF receptor signaling blocks efficient ESCC-induced vascular network formation provides the link between ESCC-induced fibroblast activation and angiogenesis in this model. In agreement with this, others have also shown that fibroblasts can release VEGF in response to hypoxic conditions and exogenous TGF-β. It is unclear whether the ESCC cells are releasing latent VEGF from the fibroblasts, as has been suggested by others, or are instead driving de novo protein expression.

Here, we have shown that the stromal fibroblasts are essential for the angiogenic response in ESCC, opening up an intriguing new possibility for therapy. There is a growing body of evidence suggesting that therapies targeting angiogenesis can lead to dramatic responses in diseases such as colorectal cancer, particularly when combined with established chemotherapeutic regimens. Because ESCC cells are typically detected at relatively advanced stages, optimized antiangiogenic/chemotherapy combinations are ideal novel therapeutic candidates. The postulated role of CAFs in tumor invasion also makes targeting the stromal fibroblast activation through the inhibition of TGF-β signaling an appealing therapeutic option.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.02.061.

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