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ORIGINAL ARTICLE

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Myofibroblasts enable invasion of endothelial cells into three-dimensional tumor cell clusters: a novel in vitro tumor model

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Abstract *Purpose*: In an effort to study the importance of stromal involvement in angiogenesis, we developed a novel, multicellular model that utilizes three of the primary cell types involved in tumor angiogenesis. *Methods*: Fluorescently labeled human microvascular endothelial cells (HMVECs), 10T1/2 cells and myofibroblasts were incubated in the presence of a three-dimensional tumor cell cluster resuspended in collagen and embedded in Matrigel. *Results*: HMVECs cultured in the presence of a human SKOV-3 ovarian carcinoma tumor cell cluster, surrounded the tumor cell cluster, while myofibroblasts invaded the cluster, localizing within the tumor cell mass. In contrast, 10T1/2 cells, a pluripotent mouse mesenchymal cell line with pericyte-like properties, did not demonstrate the same invasive phenotype. HMVECs cultured in the presence of myofibroblasts invaded the tumor cell cluster and colocalized with the myofibroblasts as demonstrated by fluorescent microscopy and immunohistochemistry. The angiogenesis inhibitors SU6668 and paclitaxel inhibited stromal invasion, while a broad-spectrum matrix metalloproteinase inhibitor did not. *Conclusions*: This model emphasizes the critical interaction between endothelial cells and myofibroblasts and provides a more complete in vitro model for studying angiogenesis and tumor progression.

Keywords Tumor stroma · Invasion · Angiogenesis · Inhibitors · In vitro model

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Introduction

The role of the tumor stroma in tumor progression is an important area of current research and has become a potential target for therapeutic intervention. The cellular compartment of the tumor stroma is composed of immune cells, inflammatory cells, smooth muscle cells, pericytes, myofibroblasts, and vascular endothelial cells [19]. These cells are recruited by the tumor cells and infiltrate the tumor microenvironment to support tumor growth and progression by the secretion of growth factors and extracellular matrix proteins, and by stimulating angiogenesis [2, 6, 14, 19, 29].

The most prominent stromal cell type, the myofibroblast [22, 24], is primarily responsible for host desmoplasia observed in carcinomas as these cells secrete large amounts of extracellular matrix proteins [27]. Myofibroblasts are defined immunohistochemically by the presence of α-smooth muscle actin, vimentin, smooth muscle myosin heavy chain, desmin, calponin and α1-integrin [16]. These cells are believed to be derived from normal fibroblasts during times of tissue stress or altered homeostasis [17]. Myofibroblast differentiation is provoked by growth factor signaling from tumor cells and degradation of the basement membrane that separates epithelial and stromal compartments resulting in the juxtaposition of epithelial and fibroblast cells. Transforming growth factor-β, which is often highly expressed in tumors [8], is believed to be one of the key factors in this differentiation [4, 31].

Another prominent cell type of tumor stroma is the endothelial cell. Angiogenesis, the development of blood vessels from pre-existing vasculature, plays a major role in tumor growth. Cytokines and growth factors, secreted by the tumor and stromal cells, stimulate endothelial cells to proliferate and migrate to form new vessels throughout the tumor. Blocking angiogenesis by targeting endothelial cells is a valid therapeutic target, but has yet to produce dramatic results in human clinical trials.

To study the multicellular aspects of angiogenesis, we developed a novel model involving endothelial cells, myofibroblasts, and tumor cells. This model demonstrated the importance of myofibroblasts in tumor angiogenesis and provided a tool for studying inhibitors of stromal cell invasion.

Materials and methods

Materials

Human adult dermal microvascular endothelial cells (HMVECs), EGM2-MV medium and EBM2/5% FBS medium were purchased from Clonetics (Walkersville, Md.). 10T1/2 cells (ATCC no. CCL-226; a pluripotent mouse mesenchymal cell line with pericyte characteristics), and SKOV-3 cells (ATCC no. HTB-77; a human ovarian carcinoma cell line) were purchased from American Type Culture Collection (ATCC, Manassas, Va.). Human adult dermal fibroblasts were a gift from Dr. James Gailit (SUNY, Stony Brook, NY). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and F12/Ham's medium was purchased from Gibco/BRL (Gaithersburg, Md.). Falcon tissue culture flasks, 24-well plates and Matrigel were purchased from Becton Dickinson (Franklin Lakes, N.J.). The fluorescent labels, PKH26 and PKH67, FITC-conjugated anti-rabbit IgG and the α-smooth muscle actin antibody were purchased from Sigma Chemical Company (St. Louis, Mo.). Antigen unmasking solution was purchased from Vector Laboratories (Burlingame, Calif.). Hematoxylin and eosin stain was purchased from Surgipath (Richmond, Ill.). Collagen I (Vitrogen) was supplied by Cohesion Technologies (Palo Alto, Calif.). MMP Inhibitor III was obtained from Calbiochem (San Diego, Calif.). Paclitaxel was purchased from Sigma Chemical Company. SU6668 was synthesized by Andrew Janjigian (Genzyme Corporation, Cambridge, Mass.).

Cell culture

All cells were grown in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. HMVECs (up to passage nine) were cultured in EGM2-MV. SKOV-3 cells were cultured in F12/Ham's/10% FBS. Human dermal fibroblasts and 10T1/2 cells were grown in DMEM/10% FBS. Fibroblast cultures at high passage number were confirmed to be substantially comprised of myofibroblasts as judged by α-smooth muscle actin expression analyzed by FACS (data not shown) [21].

In vitro model

A schematic of the experimental design is shown in Fig. 1. A thick layer of Matrigel (300 μl) was added to each well of a 24-well plate and allowed to polymerize. A plug of Matrigel of approximately 1 mm diameter was removed using a glass pipette under light vacuum. The resulting space was filled with SKOV-3 cells resuspended in a 2.4 mg/ml collagen I solution prepared according to the manufacturer's suggestions, at a concentration of approximately 1×10^6 cells in approximately 5 μl. The collagen was allowed to polymerize for 30 min to form a tumor cell cluster. HMVECs, 10T1/2 cells and myofibroblasts were fluorescently labeled with PKH67 (green) and PKH26 (red) according to the manufacturer's suggested protocol. Briefly, cells were harvested and resuspended in serum-free medium. Cells were incubated in the presence of 2.5 μ*M* dye diluted in the diluent provided by the manufacturer for 5 min. The labeling was terminated with 1 ml FBS for 1 min followed by three washes in serum-containing medium. Following the washes, cells were resuspended in EGM2-MV or EBM2/5% FBS and counted. A total of 30,000 endothelial cells, 10T1/2 cells and/or myofibroblasts, or combinations of endothelial cells with 10T1/2 cells or myofibroblasts, were added to each well in EGM2-MV or EBM2/5% FBS with or without inhibitors (SU6668, paclitaxel, MMP inhibitor) in a volume of 1 ml. After 48 h, the cells in the wells were visualized using a fluorescein (PKH67) or rhodamine (PKH26) filter. Endothelial cells in the presence of SU6668 could not be visualized due to the inherent fluorescence of the small molecule. Fluorescent and brightfield images were captured with a 4× objective on a Sony DXC-390 digital camera using Scion Image version 1.62c.

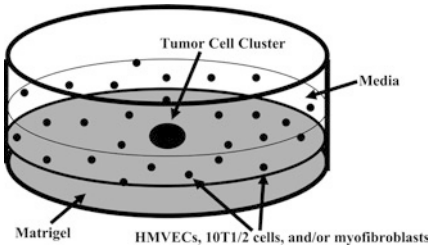


Fig. 1 Schematic of multicellular in vitro model. A tumor cell cluster of approximately 1 mm in diameter containing SKOV-3 cells (1×10^6) resuspended in 1% collagen I was implanted into a thick layer (1 mm) of Matrigel. Endothelial cells and myofibroblasts/10T1/2 cells were added to the medium and allowed to adhere and migrate around the tumor cell cluster for 48 h. See Methods for more details

Immunohistochemistry

SKOV-3 tumor cell clusters were fixed in 4% paraformaldehyde, removed from the Matrigel and embedded in paraffin. Sections (5 μm) were deparaffinized and unmasked for 5 min, followed by blocking with 1% BSA for 1 h. The primary antibody for α-smooth muscle actin was diluted in 0.5% saponin (1:100) and allowed to incubate on the sections for 1 h. Following three washes, sections were incubated for 1 h in the presence of FITC-conjugated anti-mouse IgG (1:100 dilution). For cellular localization studies not using immunohistochemistry, the original fluorescent PKH label of the cells in culture remained after fixation and sectioning.

Results

Many cell types, including HMVECs, rapidly form cellular networks when cultured on Matrigel within 5 h (Fig. 2A). In the presence of an SKOV-3 human ovarian carcinoma cell cluster, the HMVECs arranged in a distinctive pattern around the tumor cell cluster that was visible at 5 h after cells were plated (Fig. 2B) and was strongly apparent after 24 h (Fig. 2C). The HMVECs formed long, linear projections around the condensed tumor cell cluster suggesting cellular interactions between the HMVECs and the tumor cells.

Fluorescent labels were used to visualize the localization of the HMVECs and myofibroblasts or 10T1/2 cells (a mouse pluripotent cell line with pericyte-like properties) in the presence of the SKOV-3 tumor cell cluster (Fig. 3Aa–f) or a collagen plug without tumor cells (Fig. 3Ag–i). Endothelial cells (Fig. 3Aa, d—green) and 10T1/2 cells (Fig. 3Ab, e—red) adhered to the outside of the tumor cell cluster or collagen plug, most likely because of its firmer consistency as compared to

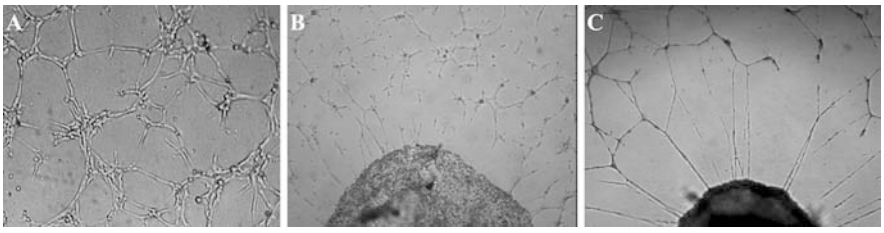


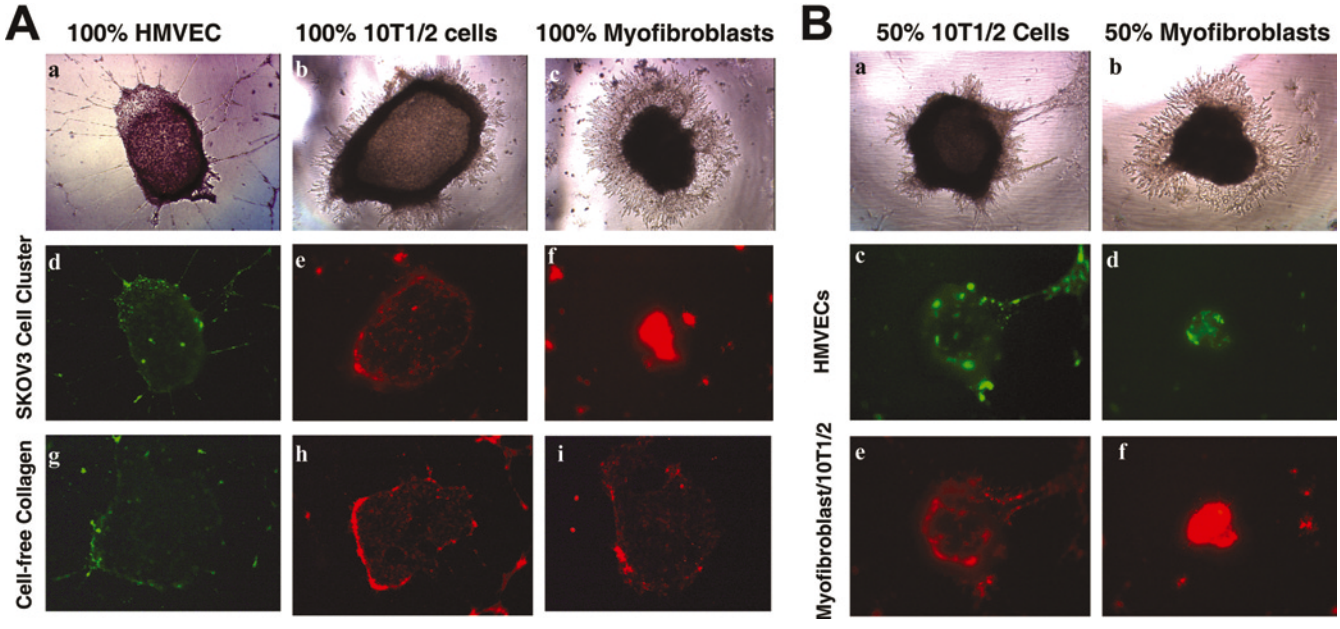
Fig. 2A–C The presence of a tumor cell cluster changes the morphology of the endothelial cell network. HMVECS form a cellular network when cultured on Matrigel within 5 h (**A**). When HMVECs are seeded on a Matrigel layer in the presence of an SKOV-3 tumor cluster, the HMVECs migrate to form a network around the cluster within 5 h (**B**). After an overnight incubation (**C**), the network formation becomes more condensed with thin projections protruding toward the tumor cell mass with a zone of projections surrounding the cluster. Brightfield images were taken using a 4× objective on an inverted phase microscope

the Matrigel monolayer. In comparison, the myofibroblasts (red) localized in the middle of the tumor cell cluster, as identified by comparing the fluorescent image to the brightfield image of the same field (Fig. 3Ac, f).

Fig. 3A, B Myofibroblasts invade the tumor cell cluster and enable the invasion of endothelial cells. Independent cultures of HMVECs (**A** *a, d, g*), 10T1/2 cells (**A** *b, e, h*) and myofibroblasts (**A** *c, f, i*) were incubated in the presence of an SKOV-3 tumor cell cluster for 48 h. HMVECs and 10T1/2 cells form a shell around the spheroid (**A** *d, e*), while myofibroblasts invade the spheroid (**A** *f*). This was not seen in a cell-free collagen plug (**A** *g, h, i*). Fluorescently labeled HMVECs at 50% HMVECs and 50% 10T1/2 cells (**B** *a, c, e*) or myofibroblasts (**B** *b, d, f*) were cultured in the presence of an SKOV-3 tumor cell cluster. The myofibroblasts and HMVECs colocalize within the SKOV-3 tumor cell cluster and the 10T1/2 cells and HMVECs cultured in the presence of 10T1/2 cells remain on the outside. HMVECs were imaged using a fluorescein filter (**A** *d, g*; **B** *c, d*) and myofibroblasts and 10T1/2 cells (*red*) were imaged using a rhodamine filter (**A** *e, f, h, i*; **B** *e, f*). Brightfield and fluorescent images were captured on an inverted phase microscope using a 4× objective

This invasion did not occur in a cell-free collagen plug (Fig. 3Ai). The invasion of the myofibroblasts caused a conformational change of the tumor cell cluster, presumably from the contraction of collagen induced by the myofibroblasts [5]. As the collagen contracted, the tumor cells migrated and spread into the Matrigel layer in the presence of myofibroblasts, as demonstrated by the cellular projections from the tumor cell cluster (Fig. 3Ac).

Since the stromal compartment of the tumor microenvironment included both endothelial cells and mesenchymal cells, different combinations of fluorescently labeled HMVECs with 10T1/2 cells or myofibroblasts were incubated in the presence of the SKOV-3 tumor cell cluster. The combinations included 100% HMVECs (Fig. 3Aa, d), 25% myofibroblasts or 10T1/2 cells with 75% HMVECs (data not shown), and 50% HMVECs with 50% 10T1/2 cells (Fig. 3Ba, c, e) or myofibroblasts (Fig. 3Bb, d, f). In the presence of 25% or 50% myofibroblasts, the endothelial cells colocalized with the myofibroblasts in the center of the tumor cell cluster. In contrast, when cultured in the presence of either 25% or 50% 10T1/2 cells, the endothelial cells remained outside the tumor cell cluster. It appeared that the myofibroblasts were enabling the endothelial cells to invade the SKOV-3 cell cluster. The findings were similar when human vascular smooth muscle cells were incubated with endothelial cells in the presence of an SKOV-3 tumor cell cluster (data not shown).



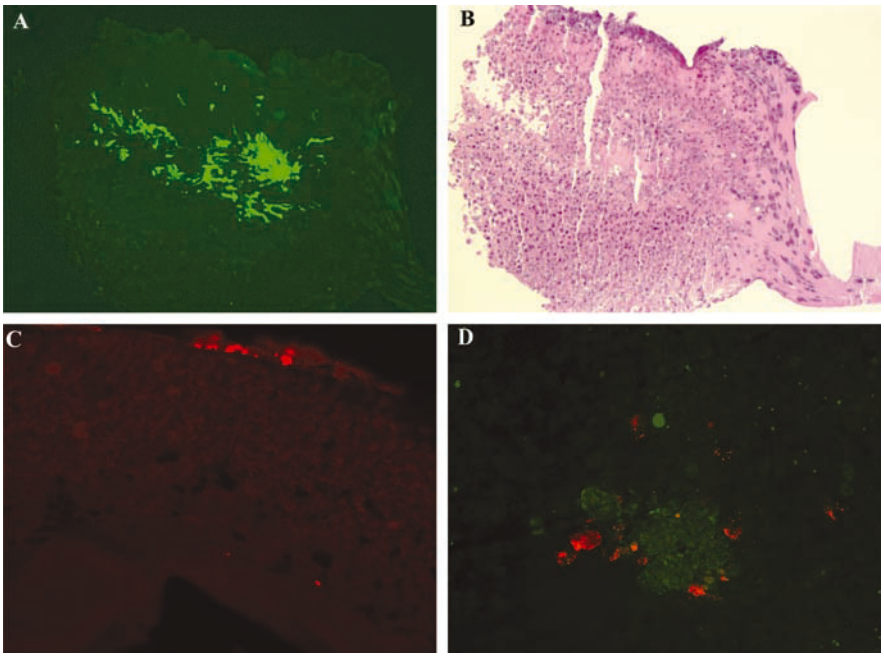


Fig. 4A–D Myofibroblasts and HMVECs colocalized in the middle of the tumor cell cluster. SKOV-3 tumor cell clusters cultured in the presence of 100% myofibroblasts (**A**, **B**), 100% 10T1/2 cells (**C**), and 50% HMVECs and 50% myofibroblasts (**D**), were fixed, paraffin-embedded and sectioned for immunohistochemistry studies. Immunodetection with α-smooth muscle actin, followed by an FITC-conjugated secondary antibody (**A**) localizes the fibroblasts within the cluster when compared to a serial hematoxylin and eosin-stained section (**B**). The prelabeled PKH dyes were used to identify the 10T1/2 cells on the outside of the tumor cell cluster (**C**), and the myofibroblasts (*red* **D**) and endothelial cells (*green* **D**) within the SKOV-3 tumor cell cluster. Brightfield and fluorescent images were captured on a microscope using a 4× objective

Immunohistochemical analysis and fluorescent prelabeling of the cells in culture (PKH dyes) were used to verify the localization of the myofibroblasts, 10T1/2 cells and HMVECs, within or around the SKOV-3 cell cluster. An immunohistochemical α-smooth muscle actin stain of a portion of a tumor cell cluster that was incubated in the presence of 100% myofibroblasts (Fig. 4A) localized the myofibroblasts within the tumor cell cluster, when evaluated against a hematoxylin and eosin stained serial section (Fig. 4B). In contrast, a section of an SKOV-3 tumor cell cluster that had been incubated in the presence of 100% 10T1/2 cells localized the 10T1/2 cells (red) on the outside of the tumor cell cluster (Fig. 4C). Figure 4D shows myofibroblasts (red) and endothelial cells (green) colocalized within an SKOV-3 tumor cell cluster that had been cultured in the presence of 50% HMVECs and 50% myofibroblasts.

To determine the effects of known angiogenesis inhibitors on the invasion of endothelial cells and myofibroblasts into an SKOV-3 tumor cell cluster, the aforementioned model, using a mixture of 50% myofibroblasts and 50% HMVECs, was incubated with SU6668 (50 μ*M*) (Fig. 5A, E), paclitaxel (1 μ*M*) (Fig. 5B, D, F) or a MMP inhibitor (1 μ*M*) (Fig. 5C, E, G) for 48 h. In the presence of SU6668 (Fig. 5E) or paclitaxel (Fig. 5F), the myofibroblasts were prevented from migrating into the tumor cell cluster, as compared to untreated cells (Fig. 3Bb, d, f). Likewise, the HMVECs did not invade the tumor cell cluster in the presence of paclitaxel (Fig. 5D). The fluorescently labeled HMVECs in the presence of SU6668 were difficult to image due to the inherent fluorescence of the small molecule (data not shown). In contrast, the broad-spectrum MMP inhibitor against MMPs 1, 2, 3, 7 and 13 did not inhibit myofibroblast and HMVEC invasion into the tumor cell mass (Fig. 5E, G). Notably, there were few tumor cell projections from the cluster as compared to tumor clusters incubated without the presence of the MMP inhibitor (Fig. 3Bb). Similar results were seen in tumor cell clusters incubated with 25% myofibroblasts and 100% myofibroblasts, while clusters incubated with 100% HMVECs were not affected by the angiogenesis inhibitors and remained on the outside of the tumor cell cluster (data not shown). Likewise, inhibition of stromal invasion by paclitaxel and SU6668 was observed at lower concentrations in a concentration-dependent manner (data not shown).

Discussion

These studies demonstrate the importance of myofibroblasts in the endothelial cell invasion of a tumor cell cluster using a novel heterogeneous three-dimensional in vitro model. Endothelial cells cultured in the presence myofibroblasts invaded a cluster composed of SKOV-3 tumor cells and collagen, while endothelial cells alone did not. The endothelial cells and myofibroblasts colocalized within the tumor cluster, suggesting the possibility of cell-to-cell communication between the

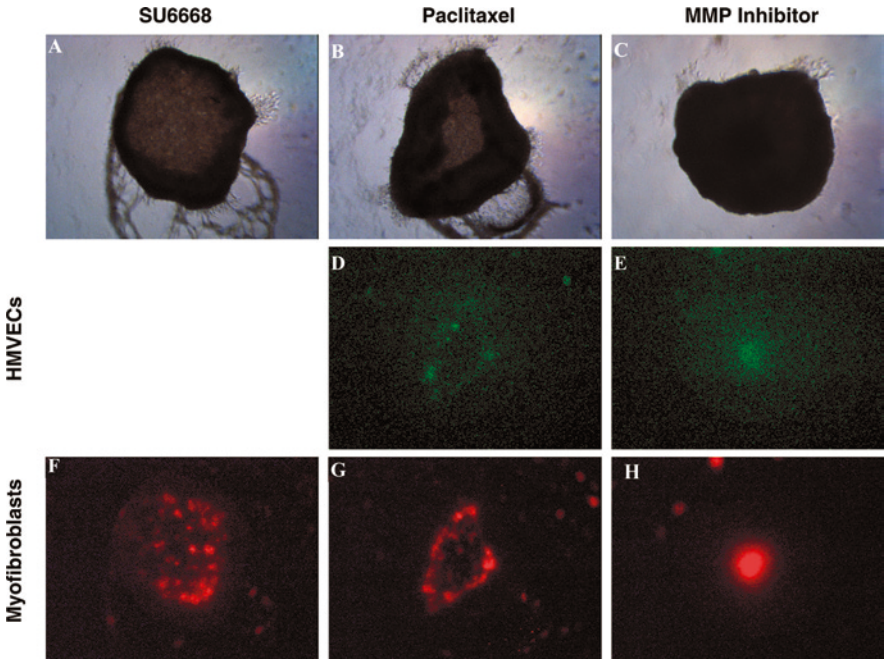


Fig. 5A–G Angiogenesis inhibitors have an effect on myofibroblast invasion into the tumor cell cluster. A combination of 50% HMVECs (*green* **D**, **E**) and 50% myofibroblasts (*red* **F**, **G**, **H**) were incubated in the presence of an SKOV-3 tumor cell cluster. SU6668 (50 μ*M*; **A**, **F**), paclitaxel (1 μ*M*; **B**, **D**, **G**), and a broad-spectrum MMP inhibitor (1 μ*M*; **C**, **E**, **H**) were added to the medium. After 48 h, brightfield and fluorescent images were taken using an inverted phase microscope and a 4× objective

endothelial cells and myofibroblasts, since myofibroblast-conditioned medium present for the duration of the experiment did not promote this phenotype (data not shown). Similarly, human vascular smooth muscle cells invaded the tumor cell cluster and aided in the invasion of endothelial cells.

In contrast, endothelial cells cultured in the presence of 10T1/2 cells did not invade the tumor cell cluster. The mouse 10T1/2 cells were used as a presumptive mural cell precursor since they are known to differentiate toward a smooth muscle cell lineage expressing α-smooth muscle actin, calponin and SM22a when exposed to endothelial cells in the presence of Matrigel [11]. This trans-differentiation has been attributed to exposure to TGF-β and is prevented by TGF-β antagonists [10]. When 10T1/2 cells resuspended in a collagen matrix are injected subcutaneously into mice, these cells become incorporated into the medial layer of developing vessels where they express smooth muscle cell markers [11]. The observation that 10T1/2 cells failed to invade the tumor cell cluster may be attributed to differences in the functional and signaling phenotype between the myofibroblasts and 10T1/2 cells. Although both cell types were exposed to the same signals from the SKOV-3 cells, only the myofibroblasts responded with directed migration into the tumor cell cluster and the concomitant migration of the HMVECs. Alternatively, the lack of migration by the 10T1/2 cells could be attributed to a species difference between the cells, i.e., protein signals secreted by the human tumor cells may not have been recognized by the murine 10T1/2 cells.

In the presence of the angiogenesis inhibitor, SU6668, an inhibitor of bFGF, VEGF, and PDGF receptor tyrosine kinase activity [13], and the cytotoxic and antiangiogenic agent [15] paclitaxel, a tubulin inhibitor [23], the myofibroblasts alone, and the endothelial cells cultured with the myofibroblasts, did not invade the tumor cell cluster. This observation suggests that endothelial cell migration into the tumor cell cluster is driven by a signaling cascade from tumor cell to myofibroblast to endothelial cells, and that paclitaxel and SU6668 interrupt signal transduction between tumor cell and myofibroblast. The possibility of such a multicellular cascade of angiogenic stimuli has been suggested by work in other in vitro angiogenesis models, although previous studies have not been done with a combination of three cell types. The migration of either endothelial cells or myofibroblasts is apparently not significantly mediated by matrix metalloproteinases since a broad-spectrum MMP inhibitor did not change the invasion pattern of the cells.

The importance of myofibroblasts and stromal invasion in tumor progression is well established. Stromal invasion is dependent on a balance between growth factor secretion, matrix degradation, and cellular motility. Stromal cells have been found to comprise the majority of the tumor mass in many common carcinomas including breast, colon, stomach, and pancreas [5, 26]. Many xenograft tumor models have demonstrated the importance of myofibroblasts in tumor progression. Transformed human mammary epithelial cells coinjected with tumor-derived fibroblasts greatly stimulate tumor formation in nude mice [7]. SKOV-3 human ovarian carcinoma cells injected into nude mice in the presence of normal fibroblasts result in early inhibition of tumor growth which is overcome by the infiltration of stromal cells from the host [20]. In similar studies, conditioned medium from stromal cells derived from tumors had a stimulatory effect on MCF-7 human breast carcinoma cell proliferation while stromal cells from cnormal tissue

had an inhibitory effect [1]. Once stromal cells are recruited to the tumor, they secrete growth factors, cytokines and other factors and provide a stimulatory environment for tumor growth [18]. The model described above supports this observation since tumor cell migration and spreading from the tumor cell cluster occurred in the presence of myofibroblasts in culture.

While the function of myofibroblasts in tumor progression has been well characterized, the role of myofibroblasts in angiogenesis is less well understood. Myofibroblasts have been found to express many growth factors including VEGF, bFGF, and TGF-β as well as many extracellular matrix proteins, which, in concert, stimulate endothelial cell migration, invasion, proliferation and vessel stability [25, 32]. Ultrastructural investigations have shown that sprouting endothelial cells establish frequent cell-cell contacts to pericapillary fibroblasts [17], suggesting a dependence on cell-cell communication between the two cell types. Our observation that myofibroblasts and endothelial cells colocalize within the tumor cell cluster supports this hypothesis. Similarly, Tuxhorn et al. have recently identified lacZ-expressing 3T3 cells surrounding the vessel wall within a xenograft tumor after injecting a combination of the lacZ-expressing 3T3 cells with the tumor cells and allowing the tumor to grow [30]. Fibroblasts have also been found to support vasculature in systems other than tumors. Using electron microscopy, Hansen-Smith et al. have identified activated fibroblasts embedded in the capillary basement membrane of skeletal muscle vessels, suggesting that the fibroblasts will transform into pericytes and possibly smooth muscle cells [9]. Similarly, in a model of pulmonary hypertension, fibroblasts are recruited to form several layers of the pulmonary microvessel walls where they express α-smooth muscle actin [14].

Even though there is little information surrounding the role of myofibroblasts in angiogenesis, there are significant data supporting the role of pericytes and smooth muscle cells in angiogenesis [3, 33]. However, the primary marker used to identify pericytes, smooth muscle cells and myofibroblasts is α-smooth muscle actin. We would suggest that the uncertainties in identifying the distinct phenotypes and functions of these three cell populations leads to ambiguities in defining the specific roles of these cells in angiogenesis. The use of this model with additional well-defined α-smooth muscle-positive cell types may help to clarify this issue.

There are a few in vitro models that have been used to study the interaction between stromal cells and tumor cells. Janvier et al. have cultured a heterogeneous mixture of fibroblasts, endothelial cells and PC3 human prostate carcinoma cells resuspended in a monolayer of collagen or fibrin matrices and have demonstrated the importance of cellular interactions of endothelial cells with fibroblasts in the promotion of capillary formation [12]. Similarly, Shekhar et al. have shown that breast carcinoma-derived fibroblasts, breast carcinoma epithelial cells, and human umbilical vein endothelial cells cultured together on a layer of Matrigel develop a compartmentalized spheroid with a stromal core including fibroblasts and endothelial cells surrounded by epithelial cells. These heterogeneous spheroids show increased proliferation and invasion, degradation of extracellular matrix, and expression of MMP9 [28]. These models support our observations that stromal cells play an important role in the process of angiogenesis. However, since these models do not contain a three-dimensional tumor cell cluster, they are not able simulate the invasive nature of angiogenesis and stromal infiltration.

Recognizing the importance of myofibroblasts in angiogenesis and tumor progression reinforces the need to study these heterogeneous cellular interactions in vitro. Currently, many in vitro angiogenesis assays are highly focused on an aspect of the angiogenic process using primarily endothelial cells. These models are less than ideal for simulating in vivo tumor angiogenesis and could therefore be less than optimal for the identification of possible therapeutic inhibitors. Utilizing a number of multiple cell types involved in angiogenesis permits the study of the cell-cell interactions in a more complex and hopefully representative environment. The model described here provides an opportunity to characterize the functional interactions between myofibroblasts, endothelial cells and tumor cells and our findings suggest that the myofibroblast is a valid target for anticancer therapeutic agents.

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