Paracrine induction of angiogenesis in vitro by Swiss 3T3 fibroblasts

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SUMMARY

During angiogenesis, normally quiescent endothelial cells that line existing microvessels are induced to invade the surrounding extracellular matrix and to form capillary sprouts in response to paracrine factors released by neighboring cell types. In an attempt to mimic the physiological conditions under which angiogenesis is triggered in vivo, we have designed two co-culture systems that are suitable for the study of paracrine interactions between microvascular endothelial cells and cell types that might produce angiogenic factors. In the first model, cells to be co-cultured with endothelial cells were suspended within a collagen gel and overlaid with an additional collagen gel devoid of cells, onto which bovine microvascular endothelial cells were subsequently seeded and grown to confluence. In the second model, a small collagen gel disc containing a suspension of endothelial cells was embedded into a larger, cell-free collagen gel disc, which in turn was surrounded by an annular collagen gel containing other cell types. We show that Swiss 3T3 fibroblasts and their 3T3-L1 substrain induce the formation of capillary-like tubes by endothelial cells in these co-culture systems, whereas Balb 3T3 cells, as well as a number of other fibroblasts and epithelial cells, lack this ability. Thus, endothelial cells grown on a collagen gel containing Swiss 3T3 or 3T3-L1 cells invaded the underlying matrix to form a network of interconnecting tubules. In addition, Swiss 3T3 and 3T3-L1 cells stimulated extensive radial outgrowth of tubular sprouts from the periphery of endothelial-cell-containing collagen discs. Conditioned medium from Swiss 3T3 cells mimicked the effect of co-culture by inducing formation of capillary-like tubes, and also increased plasminogen activator activity in endothelial cells. Conditioned medium from Balb 3T3 cells, in contrast, lacked these activities. Preliminary evidence suggests that the factor(s) in Swiss 3T3 conditioned medium that induces tubule formation by endothelial cells may be different from a number of well-characterized angiogenic cytokines. The co-culture systems described here should prove to be useful for the identification of physiological regulators of angiogenesis produced by various cell types.

Key words: endothelial cells, extracellular matrix, plasminogen activators, cytokines

INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels, plays a crucial role in many physiological and pathological settings, including embryonic development, wound healing, ocular diseases, and tumor growth and metastasis (Folkman and Klagsbrun, 1987; Zetter, 1988; Klagsbrun and Folkman, 1990; Folkman and Shing, 1992). During angiogenesis, new capillaries are formed by a process of sprouting from existing microvessels: in response to locally released angiogenic factors (for a recent review, see Klagsbrun and D’Amore, 1991), microvascular endothelial cells degrade their basement membrane and subsequently invade the surrounding interstitial matrix, in which they form tubular capillary sprouts (Auspunk and Folkman, 1977; Paku and Paweletz, 1991).

To investigate the mechanisms of angiogenesis under well-defined in vitro conditions, we previously developed an experimental model in which microvascular endothelial cells grown as a monolayer on a three-dimensional collagen gel can be stimulated to invade the underlying matrix, within which they form capillary-like tubular structures (Montesano and Orci, 1985). Agents that induce this phenomenon include phorbol esters (Montesano and Orci, 1985), sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases (Montesano et al., 1988), and the angiogenic polypeptides, basic fibroblast growth factor (bFGF) (Montesano et al., 1986) and vascular endothelial growth factor (VEGF) (Montesano et al., 1986) and vascular endothelial growth factor (VEGF) (Bikfalvi et al., 1991; Pepper et al., 1992).

The ability of bFGF and VEGF to induce morphogenesis of capillary-like tubules in collagen gels indicates that, in an appropriate extracellular matrix environment, physiological messengers can stimulate endothelial cells to express a latent ‘angiogenic program’ that recapitulates some of the early events of the neovascularization process. However, one potentially relevant difference between the in vitro settings described above and the physiological conditions of in vivo induction of angiogenesis is that in the collagen gel invasion assay angiogenic factors are added to the culture medium above the cell monolayer and thereby
interact primarily with the apical (luminal) surface of endothelial cells, whereas in most in vivo situations angiogenic factors are likely to interact with the basal (abluminal) endothelial cell surface.

To approximate the in vivo situation as closely as possible, in vitro models of angiogenesis should ideally be designed so as to expose the basal surface of endothelial cells to angiogenic molecules released by neighboring cell types. In an attempt to identify novel physiological regulators of angiogenesis produced by normal or tumoral cell types, we have developed two co-culture systems that meet these criteria. In this report, we describe the construction of these in vitro models, and show that, among the cell types examined, Swiss mouse embryo 3T3 fibroblasts induce a prominent angiogenic response when co-cultured with endothelial cells.

MATERIALS AND METHODS

Cell culture

Cloned bovine microvascular endothelial (BME) cells from the adrenal cortex (Furie et al., 1984), generously provided by Drs M. B. Furie and S. C. Silverstein (Columbia University, New York), were subcultured in 1.5% gelatin-coated tissue culture flasks (Falcon, Becton Dickinson Labware, Lincoln Park, New Jersey, USA) in minimal essential medium, alpha modification (alpha-MEM) (Gibco, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (DCS) (Flow Laboratories, Baar, Switzerland), penicillin (500 i.u/ml) and streptomycin (100 µg/ml). Cells were used between passages 14 and 22. Cultured pulmonary artery endothelial (CPAE) cells, purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA), and human umbilical vein endothelial (HUVE) cells, generously provided by Dr N. Maggiano (University of Rome, Italy), were cultured as described (Montesano and Orci, 1987).

Swiss 3T3 (CCL 92), Balb 3T3 clone A 31 (CCL 163) and NIH 3T3 (CRL 1658) mouse embryo fibroblasts (purchased from the ATCC) were routinely subcultured before reaching confluence at a ratio of 1:1000 every 7-10 d according to the indications provided in the ATCC Catalogue of Cell Lines and Hybridomas (7th edition, Rockville, 1992). This transfer schedule keeps the cells in continuous exponential growth phase, thus minimizing the chances of spontaneous transformation. 3T3-L1 (CCL 92.1), Balb 3T12-3 (CCL 164), and C3H/10T1/2 (CCL 226) mouse embryo fibroblasts, WI-38 (CCL 75) human embryonic lung fibroblasts, Detroit 550 (CCL 109) human foreskin fibroblasts, Intestine 407 (CCL 6), Chang liver (CCL 13), and NMuMG mammary gland (CRL 1636) epithelial cells were also purchased from the ATCC and grown according to the indications provided. MDCK cells (strain II, clone 3B5) were grown as described (Montesano et al., 1991a,b).

Suspension of cells within collagen gels

Cells were harvested using EDTA-trypsin, counted, adjusted to the desired concentration, and centrifuged in a plastic tube. The tube was placed on ice, and the cell pellet was resuspended in a gelling collagen solution, which was prepared essentially as previously described (Montesano et al., 1983). In brief, 8 volumes of collagen stock solution (approximately 1.5 mg/ml) were mixed with 1 volume of 10X concentrated minimal essential medium (MEM, Gibco) and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile flask kept on ice to prevent premature collagen gelation. Cells were resuspended in the cold mixture, which was then dispensed into plastic culture dishes (see below) and allowed to gel for about 10 min at 37°C.

Three-dimensional co-culture systems

Endothelial cells were co-cultured in collagen gels with fibroblasts or epithelial cells using either of the two procedures described below.

The ‘collagen bilayer’ co-culture system (see Fig. 1)

Fibroblasts or epithelial cells were suspended in a gelling collagen solution (see above) at concentrations ranging from 5×10³ to 2×10⁶ cells per ml of collagen, and 1000-1500 µl aliquots of the suspension were dispensed either into 35 mm plastic tissue culture dishes (Nunc, Kamstrup, Roskilde, Denmark) or into high-walled 35 mm wells of 6-well Falcon plates (catalog number 3046). After the collagen solution had gelled, a cell-free collagen gel layer (700-1000 µl) was applied onto the cell-containing bottom collagen layer. Finally, endothelial cells were seeded at a density of approximately 2×10⁴ cells/cm² on top of the cell-free collagen layer, and the cultures incubated at 37°C for 10-20 d in 2-3 ml of a 1:1 mixture of the media used to grow each of the co-cultured cell types. Culture media were renewed every 2-3 d. In some experiments, co-cultures were established in 22 mm wells of 12-well plates (tissue culture Cluster, catalog number 3512, Costar, Cambridge, Massachusetts, USA), using a bottom collagen gel layer of 1000-1200 µl and a top layer of 500-800 µl. In control cultures, fibroblasts or epithelial cells were omitted from the bottom collagen gel layer.

The ‘collagen disc’ co-culture system (see Fig. 4a,b)

Fibroblasts or epithelial cells were suspended in a gelling collagen solution (see above) at concentrations ranging from 2×10³ to 1×10⁶ cells per ml of collagen, and 2.5 ml aliquots of the suspension were cast either into 35 mm dishes (Nunc) or into 35 mm wells of 6-well Falcon plates, in the external compartment delimited by a centrally placed stainless steel ring (outer diameter, 15 mm; inner diameter, 13 mm; height, 5 mm). After the solution had gelled, 2-3 ml of complete culture medium were added on top of the collagen gel.

BME cells were suspended in a gelling collagen solution at a concentration of 1×10⁶ cells per ml, and 40 µl aliquots of the suspension were cast into each of several stainless steel cylindrical molds (outer diameter, 6 mm; inner diameter, 5 mm; height, 4 mm) placed in 35 mm dishes. After the collagen had gelled, 3 ml of complete culture medium were added to the dishes to cover the cylindrical molds. About 24 h later, the 15 mm metallic ring was gently removed from the inner circumference of the annular collagen gels containing fibroblasts or epithelial cells, and the well thereby created in the center of the dishes was coated with 150 µl of gelling collagen solution. The endothelial-cell-containing collagen gels cast into the 6 mm cylindrical molds were then carefully detached from the stainless steel wall using a sterile metal spatula. The resulting collagen discs (diameter, 5 mm; height, approximately 1.6 mm) were transferred into the central well of the 35 mm dishes, positioned concentrically to the peripheral collagen gel containing non-endothelial cells, and embedded in 500-600 µl of gelling collagen solution. After an additional 600 µl collagen layer was applied on top of the embedded collagen disc to completely fill the central well, 2-3 ml of complete culture medium was added to the co-cultures, and the dishes incubated at 37°C for 10-15 d.

In control cultures, the peripheral cell-containing collagen gel was omitted: endothelial-cell-populated collagen discs were embedded in the center of a larger collagen gel in a 35 mm dish. In some experiments, control gels were constructed exactly as in co-cultures, except that fibroblasts or epithelial cells were omit-
ted from the peripheral collagen gel. The cultures were photographed under phase-contrast using a Nikon Diaphot TMD inverted photomicroscope.

Collagen gel invasion assay
A collagen gel invasion assay previously developed in our laboratory (Montesano and Orci, 1985; Montesano et al., 1986) was slightly modified in order to improve the access of culture medium components to the basal surface of endothelial cells. For this purpose, a 10 µl drop of a concentrated suspension of BME cells (1x10^6 cells/ml) was applied to a 700 µl collagen gel in the center of 22 mm wells of 12-well plates (Costar). After a 3 h incubation at 37°C to allow cell attachment, the drop of culture medium with unattached cells was carefully aspirated, fresh culture medium (2 ml) was added, and the culture was incubated overnight. This procedure yields a disc-shaped confluent monolayer of endothelial cells (about 6 mm in diameter) surrounded by a cell-free collagen gel surface, through which substances added to the culture medium can diffuse into the underlying gel matrix to reach the basal surface of endothelial cells.

Preparation and treatment of conditioned media
To prepare conditioned media, cells were seeded into 150 mm Intergrid Tissue Culture Dishes (Falcon no. 3025) in 30 ml culture medium and grown to confluence, at which time medium was aspirated and 10 ml fresh complete medium added to each dish. After a further 2-3 d incubation, the conditioned medium (CM) was collected, centrifuged at low speed to remove floating cells and cell debris, and stored at −20°C.

For partial characterization of the in vitro angiogenic activity in Swiss 3T3 CM, the following treatments were carried out and the treated CM were then tested in the collagen gel invasion assay described above:

Dialysis
CM was dialysed through a 5-8x10^4 M_o cut-off membrane against 40 vol. of MEM (three changes) for 3 d at 4°C.

Trypsin treatment
To avoid inhibition of trypsin by serum protease inhibitors, serum-free CM was used for these experiments. Confluent cultures of Swiss 3T3 cells in 150 mm dishes were washed in serum-free MEM and further incubated in 10 ml serum-free MEM containing 1% CR-ITS supplement (insulin, transferrin, selenious acid, bovine serum albumin and linoleic acid; Collaborative Research, Bedford, Massachusetts, USA). After 2 or 3 d, the CM was collected as described above. For trypsin treatment, the CM was incubated with 100 µg/ml trypsin for 3 h at 37°C, and the digestion stopped by addition of 10% DCS. As a control, CM was incubated for 3 h at 37°C with a mixture of trypsin and DCS.

Heat treatment
CM was heated to 70°C for 30 min.

Acid treatment
CM was acidified to pH 3.0 with 0.5 M hydrochloric acid for 3 h at room temperature and then neutralized by the addition of 0.1 M sodium hydroxide.

Dithiothreitol (DTT) treatment
CM was incubated with 50 mM DTT for 90 min at room temperature, and then dialysed against three changes of MEM as described above.

Incubation with anti-bFGF antibodies
Two different polyclonal antibodies to bFGF were tested for their ability to neutralize the in vitro angiogenic activity of Swiss 3T3 CM. A rabbit antiserum raised against a synthetic fragment of bFGF (1-24) conjugated to BSA was generously provided by Dr A. Baird (The Whittier Institute, La Jolla, California, USA). An Ig fraction from a rabbit serum against a synthetic bFGF (1-24) peptide conjugated to keyhole limpet hemocyanin (KLH) was purchased from Sigma (Product no. F-3393, Sigma, St. Louis, Missouri, USA). Swiss 3T3 CM was mixed with an equal volume of BME complete medium and preincubated with either anti-bFGF antiserum (1:100 dilution) or anti-bFGF Ig fraction (1:50 dilution) for 4 h at 4°C before being added to BME cells in the collagen gel invasion assay described above.

Plasminogen activator (PA) plaque assay, zymography and reverse zymography
Plaque assay: BME cells were seeded at 2x10^4 cells per gelatin-coated 35 mm dish in 1.5 ml complete medium. After overnight attachment and spreading, cells were exposed to Swiss 3T3 CM or Balb 3T3 CM prepared as described above, with the exception that both cell lines were grown in Balb 3T3 medium containing serum. CM was mixed with an equal volume of spent BME medium. At 15 h later, cells were washed with PBS containing acid-treated BSA (1 mg/ml), overlaid with a thin layer of agar containing casein and plasminogen as previously described (Vasali et al., 1976), incubated at 37°C for 5 h, and photographed under dark-field illumination.

Zymography and reverse zymography: Swiss 3T3 CM or Balb 3T3 CM prepared as above was added to confluent monolayers of BME cells in gelatin-coated 35 mm dishes. CM was mixed with an equal volume of spent BME medium; the last medium change was 24 h before commencing the experiment. Trasylol (200 kiu/ml) (Bayer-Pharma AG, Zurich, Switzerland) was added to the medium; 15 h later, cell extracts were prepared, and analyzed by zymography and reverse zymography as previously described (Pepper et al., 1990).

RNA preparation, in vitro transcription and northern blot hybridization
Total cellular RNA was prepared from confluent monolayers of BME cells exposed to Swiss 3T3 CM or Balb 3T3 CM prepared as described above for the PA plaque assay. CM was mixed with an equal volume of spent BME medium; the last medium change was 24 h before commencing the experiment. RNA preparation, northern blotting, UV cross-linking and methylene blue staining of filters, in vitro transcription, hybridization and post-hybridization washes were as previously described (Pepper et al., 1990).

32P-labelled cRNA probes were prepared from bovine urokinase-type PA (u-PA) (Krätzschmar et al., 1993), bovine u-PA receptor (u-PAR) (Krätzschmar et al., 1993), human tissue-type PA (t-PA) (Fisher et al., 1985) and bovine PA inhibitor-1 (PAI-1) (Pepper et al., 1990) cDNAs as previously described (Pepper et al., 1990, 1993a). Autoradiographs were scanned with a GenoScan laser scanner (Genofit, Geneva, Switzerland). Results are expressed relative to control cultures at the corresponding time points.

Processing for light and electron microscopy
Cultures were fixed in situ overnight with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After washing in cacodylate buffer, the collagen gels were cut to 2 mm x 2 mm fragments, which were post-fixed in 1% osmium tetroxide in Veronal acetate buffer for 45 min, and further processed as previously described (Montesano et al., 1990). Semi-thin (1 µm) and thin sections were cut perpendicular to the culture plane with an LKB ultramicrotome. Semi-thin sections were stained with 1% toluidine blue and photographed under transmitted light using a Zeiss Photomicroscope II. Thin sections were stained with uranyl acetate.
and lead citrate and examined in a Philips CM 10 electron microscope.

RESULTS AND DISCUSSION

The initial objective of the studies reported in this paper was to determine whether co-culture of endothelial cells with tumor cells in collagen gels might induce an ‘angiogenic’ response (i.e. invasion of the collagen matrix and formation of capillary-like tubes) similar to that which we observed previously in endothelial cell cultures incubated with bFGF (Montesano et al., 1986) or VEGF (Pepper et al., 1992). To this aim, tumor-derived cells (including MCF7 and MDA-MB-231 breast adenocarcinoma cells, A431 epidermoid carcinoma cells, and C6 glioma cells) were suspended within a collagen gel and overlaid with a cell-free gel onto which BME cells were subsequently seeded and grown as a monolayer (see Fig. 1). These attempts, however, were hampered by the rapid proliferation of tumor cells within collagen gels, resulting in acidification of the culture medium with ensuing toxicity to BME cells. This problem could not be overcome by the use of Mitomycin C because of its toxicity in collagen gel cultures. Owing to this shortcoming associated with the use of tumor cells, in subsequent studies we chose to examine the interaction between endothelial cells and non-tumoral cell types that might produce angiogenic factors.

On the basis of previous reports showing that mouse embryo 3T3 fibroblasts produce factors that induce angiogenesis in vivo (Klagsbrun et al., 1976) and stimulate endothelial cell proliferation in vitro (Birdwell and Gosподarowicz, 1977; McAuslan et al., 1980), we wished to determine whether these cells might also be able to induce an angiogenic response in our collagen gel co-culture system. For this purpose, BME cells were grown as a monolayer on a cell-free collagen gel cast on top of a bottom gel layer containing Swiss 3T3 cells. As soon as BME cells attained confluence, numerous endothelial cell cords began to extend from the surface monolayer into the underlying collagen matrix. Over the next few days of co-culture, the endothelial cords elongated, branched progressively and developed widely patent lumina, whereas new sprouts continued to form from the surface monolayer (Figs 2B and 3A). After about 10 d, many tubules had traversed the thickness of the upper cell-free collagen gel and invaded the fibroblast-containing bottom layer. Induction of endothelial tube formation was observed with concentrations of Swiss 3T3 cells ranging from about $1 \times 10^5$ to $2 \times 10^6$ cells per ml of collagen. Decreasing fibroblast concentration below $1 \times 10^5$ cells/ml resulted in a progressively weaker effect and tubules were not formed with less than $1 \times 10^4$ cells per ml. Control BME cells (grown for the same time period on a collagen gel bilayer of equivalent thickness but devoid of Swiss 3T3 cells) remained confined to the surface of the gel and did not form tubular sprouts (Fig. 2A). Semi-thin (Fig. 3C,D) and thin sections (not shown) of BME/Swiss 3T3 co-cultures confirmed the capillary-like tubular nature of the endothelial sprouts seen by phase-contrast microscopy.

To assess whether the ability to invade a collagen matrix as vessel-like structures in response to co-culture with Swiss 3T3 cells was confined to microvascular endothelial cells, we next examined the behavior of endothelial cells isolated from the human umbilical vein (HUVE cells) or the calf pulmonary artery (CPAE cells). When grown on collagen gels containing Swiss 3T3 cells, both types of large vessel endothelial cells invaded the underlying collagen gel, within which they formed tubular structures (shown in Fig. 3E for CPAE cells). These findings support our previous conclusion (Montesano and Orci, 1987; Pepper et al., 1990) that endothelial cells not usually involved in neovascularization processes can express a latent angiogenic phenotype in response to appropriate signals.

Since fibroblasts are thought to represent a population of functionally heterogeneous cells (reviewed by Schor and Schor, 1987), we subsequently questioned whether or not the ability to induce tubule formation by endothelial cells could be shared by fibroblasts other than Swiss 3T3 cells. To answer this question, BME cells were co-cultured in collagen gels with the following fibroblast strains: Balb 3T3, NIH 3T3, Balb 3T12-3 and C3H/10T\(\text{I}\)\(\text{f}\) mouse embryonic lung fibroblasts, WI-38 human embryonic lung fibroblasts, and Detroit 550 human foreskin fibroblasts. None of these fibroblast strains induced BME cells to invade the underlying collagen gel and to form capillary-like tubules (Fig. 2D shows the absence of tubules in a co-culture of BME and Balb 3T3 cells). In contrast, a clear-cut angiogenic response was observed in co-cultures with 3T3-L1 cells, a clonally derived substrain of Swiss 3T3 cells (Green and Kehinde, 1974) (Fig. 2C). Co-culture with a number of non-transformed epithelial cells, including kidney MDCK, Intestine 407, Chang liver and NMuMG mammary gland epithelial cells also failed to induce formation of tubular sprouts by BME cells (results not shown). Thus, among the cell types examined, only parental Swiss 3T3 cells and their clonally derived 3T3-L1 substrain were able to induce an angiogenic response when co-cultured with BME cells in collagen gels (Table 1).

In the ‘collagen bilayer’ co-culture system described above, Swiss 3T3 cells induce endothelial cells grown as a monolayer on the surface of a collagen gel to invade the underlying matrix, within which they form tubular sprouts. To assess whether Swiss 3T3 cells could also stimulate formation of capillary-like tubules from endothelial cells already embedded within a collagen matrix, we designed...
an additional co-culture system. A collagen gel disc containing a suspension of BME cells was included in a larger cell-free collagen gel, which was in turn surrounded by a ring-shaped collagen gel containing Swiss 3T3 cells (Fig. 4A,B; see Materials and Methods for details of preparation). In both control cultures (i.e. in the absence of Swiss 3T3 cells in the peripheral collagen gel ring), and in co-cultures with Swiss 3T3 cells, BME cells suspended in the central collagen disc formed a network of tubules within 24-48 h (Fig. 4C). However, in 4- to 8-d control cultures, only rare short tubular sprouts had extended from the periphery of the disc into the surrounding cell-free collagen gel (Fig. 5A). In striking contrast, in co-cultures with either Swiss 3T3 or 3T3-L1 cells, an extensive outgrowth of branching tubules occurred from the periphery of the endothelial-cell-containing collagen disc, resulting in the formation of a thick ‘corona’ of vessel-like channels extending out into the surrounding cell-free gel (Fig. 5B). The radially disposed endothelial sprouts typically comprised a proximal segment containing a widely patent lumen, and a distal, blind-ending advancing tip with cytoplasmic processes extending into the collagen matrix (Fig. 5C-E). Comparison of micrographs of the same culture fields taken at various time intervals revealed that contiguous tubular sprouts often joined and anastomosed to form capillary-like loops (Fig. 5D,E). The tubular nature of the endothelial sprouts was confirmed by the examination of semi-thin and thin sections (not shown). In agreement with the results obtained in the ‘collagen bilayer’ system, radial outgrowth of capillary-like tubules was not stimulated by co-culture with other fibroblast strains, or by co-culture with epithelial cells (Table 1).

The co-culture experiments described above (using either the collagen bilayer or the collagen disc invasion assay) suggested that diffusible factors released by Swiss 3T3 cells were responsible for the induction of tubule formation by BME cells. To test this possibility directly, we initially examined the effect of conditioned medium (CM) from Swiss 3T3 cells in a collagen gel invasion assay that we had previously developed (Montesano and Orci, 1985; Montesano et al., 1986). Addition of Swiss 3T3 CM (50%, v/v) above a confluent monolayer of BME cells on colla-
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Fig. 3. Details of capillary-like tubules formed by endothelial cells co-cultured with Swiss 3T3 or 3T3-L1 cells. (A,B) Tubular structures with widely patent lumina (arrows) formed by BME cells grown on a cell-free collagen gel cast on top of a gel layer containing Swiss 3T3 cells (A) or 3T3-L1 cells (B) (phase-contrast microscopy). (C-E) Semithin sections of collagen gel co-cultures of BME (C,D) and CPAE (E) cells with Swiss 3T3 cells. The endothelial cells have invaded the underlying collagen gel (Cg) and have formed tubular structures with large lumina. Bars, 100 µm in A and B; 25 µm in C,D,E.

Table 1. Ability of a variety of cell lines to stimulate formation of capillary-like tubules by BME cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Co-culture system</th>
<th>Collagen bilayer</th>
<th>Collagen disc</th>
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<td>Fibroblasts</td>
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<td>Swiss 3T3</td>
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<td>Balb 3T3</td>
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<td>3T3-L1</td>
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<td>Balb 3T12-3</td>
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<td>C3H/10T1</td>
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<tr>
<td>WI-38</td>
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<tr>
<td>Detroit 550</td>
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<tr>
<td>Epithelial cells</td>
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<tr>
<td>MDCK</td>
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<td>Intestine 407</td>
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<td>Chang liver</td>
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<td>NMuMG</td>
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Fibroblasts or epithelial cells were cocultured with BME cells as described in Materials and Methods, using either the collagen bilayer or the collagen disc system. A plus sign indicates either invasion of BME cells into the underlying collagen matrix and formation of capillary-like tubes (in the bilayer assay), or a markedly increased radial outgrowth of tubular sprouts (in the disc assay). A minus sign indicates lack of stimulation of tube formation.
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Since it is believed that an increase in extracellular proteolysis, which is precisely regulated in time and space, is necessary for endothelial cell invasion and capillary sprout formation (reviewed by Pepper and Montesano, 1990), we next assessed the effect of Swiss 3T3 CM on endothelial cell proteolytic properties by focussing on the PA-plasmin system (reviewed by Moscatelli and Rifkin, 1988; Vassalli et al., 1991). Using the PA plaque assay, which detects plaques of plasminogen-dependent casein lysis around individual cells, we found that Swiss 3T3 CM markedly increased BME cell-associated PA activity, while Balb 3T3 CM had a minor effect (Fig. 7A). The nature of the cell-associated PA activity was determined by zymography. This revealed an increase in u-PA activity in response to Swiss 3T3 CM but not Balb 3T3 CM (Fig. 7B). The nature of the cell-associated PA activity was established on the basis of its sensitivity to amiloride (Vassalli and Belin, 1987) (results not shown). Northern blot hybridization revealed a 2.7-fold increase in BME u-PA mRNA after 4 h of exposure to either Swiss 3T3 CM or Balb 3T3 CM; the effect of Balb 3T3 CM was however transient, while at a later time point (20 h), Swiss 3T3 CM induced a further increase which was 5.2-fold greater than controls (Fig. 8). Swiss 3T3 CM but not Balb 3T3 CM also increased u-PA receptor (u-PAr) mRNA levels, with a maximal 8.7-fold increase after 4 h (Fig. 8). Similarly, Swiss 3T3 CM but not Balb 3T3 CM increased the levels of t-PA mRNA, with a maximal 6.2-fold increase after 4 h (Fig. 8). Finally, in two separate experiments neither Swiss 3T3 CM nor Balb 3T3 CM increased levels of mRNA for the physiological PA inhibitor type-1 (PAI-1); in one experiment (shown in Fig. 8), Swiss 3T3 CM decreased PAI-1 mRNA 3.1-fold after 4 h. When compared with previously described effects of in vitro-angiogenic cytokines on the PA-plasmin system, some similarities and differences were noted. Firstly, like bFGF and VEGF (Montesano et al., 1986; Moscatelli et al., 1986; Pepper et al., 1991; Mignatti et al., 1991; Pepper, 1992), Swiss 3T3 CM increased BME cell u-PA and u-PAr expression. Similarly, like VEGF (Pepper et al., 1991), Swiss 3T3 CM increased t-PA expression in BME cells. However, unlike bFGF and VEGF (Saksela et al., 1987; Pepper et al., 1990; Pepper et al., 1991), Swiss 3T3 CM did not increase PAI-1 expression in BME cells. Taken together, these results indicate that the net overall balance of extracellular proteolysis is increased in BME cells following exposure to Swiss 3T3 CM, similar to what has previously been described for bFGF and VEGF (Pepper et al., 1990, 1991). However, unlike bFGF and VEGF, Swiss 3T3 CM did not increase the levels of PAI-1 mRNA in microvascular endothelial cells. These findings suggest that

Fig. 4. The ‘collagen disc’ co-culture system. (A,B) Coculture system shown in a schematic cross-sectional diagram (A) or viewed from above (B). The system consists of two concentric cell-containing collagen gels separated by a ring-shaped gel devoid of cells (for procedures of preparation, see Materials and Methods). The peripheral collagen gel contains a suspension of fibroblasts or epithelial cells, whereas the central collagen disc contains a suspension of endothelial cells. The black bars in (A) represent cells suspended in collagen gels. The co-culture shown in (B) consists of a peripheral collagen gel containing a suspension of Swiss 3T3 cells (1×10⁶ cells/ml) and a central collagen gel disc containing a suspension of BME cells (1×10⁶ cells/ml). The co-culture was photographed 24 h after assembly. (C) Low-power micrograph of the outer portion of a BME-cell-containing collagen disc in a control culture (i.e. fibroblasts were omitted from the peripheral collagen gel), 24 h after commencing the experiment. The endothelial cells have formed a network of interconnecting tubules. The culture was photographed under transmitted light using a Nikon Diaphot TMD inverted photomicroscope. Bar, 500 µm.
if a single factor in Swiss 3T3 CM is responsible for the changes observed in the PA-plasmin system, it is unlikely to be either bFGF or VEGF. Our observations do not, however, exclude the possibility that more than one factor in Swiss 3T3 CM is responsible for these modulations.

In order to characterize the physicochemical properties of the Swiss 3T3-derived factor(s) that induce tubule formation by BME cells, we evaluated the effect of various treatments on the angiogenic activity of Swiss 3T3 CM in the modified collagen gel invasion assay described above. The biological activity of the conditioned medium was nondialysable through a 5-8×10^3 M_r cut-off membrane, and was abolished by incubation with trypsin, heating to 70°C, acidification at pH 3, or treatment with DTT. These findings (which are summarized in Table 2) suggest that the molecule responsible for the induction of endothelial cell invasiveness and tube formation is a protein, probably containing disulfide bonds essential for its biological activity.

bFGF is a well-characterized angiogenic polypeptide (reviewed by Baird and Böhlen, 1990), which induces formation of capillary-like tubules by BME cells (Montesano et al., 1986). Although bFGF lacks a signal sequence required to enter the classical secretory pathway and is normally released from cells in very limited amounts (reviewed by D’Amore, 1990), certain cells have the ability to export it by unknown mechanisms (Kandel et al., 1991). The possibility that bFGF is the factor in Swiss 3T3 CM that induces tubule formation by BME cells was addressed using two different polyclonal antibodies prepared against bFGF (see Materials and Methods). Although both antibodies fully neutralized the in vitro angiogenic activity of recombinant bFGF (5-10 ng/ml) (results not shown), neither anti-

Fig. 5. Swiss 3T3 and 3T3-L1 cells stimulate radial outgrowth of capillary-like tubules from endothelial cell-containing collagen discs (phase-contrast microscopy). (A) 8-d-old control culture: only a few short endothelial sprouts have extended from the periphery of the central collagen disc into the surrounding cell-free collagen gel. (B) 8-d-old co-culture of BME cells and Swiss 3T3 cells: a dense network of long tubular sprouts has extended from the periphery of the endothelial-cell-containing collagen disc (indicated by the broken line) into the surrounding cell-free gel. (C) Higher magnification of branching tubular structures radiating out from a collagen disc (limits indicated by the broken line) in a 7-d-old co-culture of BME cells and 3T3-L1 cells. (D,E) The same field from a co-culture of BME and 3T3-L1 cells was photographed on day 12 (D) and day 14 (E). Comparison of the two micrographs reveals that two adjacent endothelial tubules have joined and anastomosed (arrows in E) to form a loop with an apparently continuous lumen. Bars, 500 µm in A,B; 100 µm in C-E.
10213T3 cells induce angiogenesis in vitro

The body was able to abrogate the activity of Swiss 3T3 CM (Table 2). The fact that bFGF is unlikely to be responsible for the in vitro angiogenic activity of Swiss 3T3 CM is also indicated by the finding that DTT treatment, which does not inactivate bFGF (Rosenthal et al., 1990), abolishes the tubule-inducing activity of Swiss 3T3 CM (Table 2), as well as by the finding that Swiss 3T3 CM, in contrast to bFGF (Togari et al., 1985; Rydel and Greene, 1987), does not induce neurite outgrowth from rat pheochromocytoma PC12 cells (results not shown).

VEGF (for a recent review, see Ferrara et al., 1992) is another angiogenic polypeptide that we have found induces formation of capillary-like tubules by BME cells (Pepper et al., 1992). Since VEGF is secreted by a variety of cell types, it appeared to be a potential candidate for mediating the in vitro angiogenic activity of Swiss 3T3 CM. However, this possibility seems unlikely because VEGF is heat- and acid-stable (Rosenthal et al., 1990), whereas the Swiss 3T3-derived activity is not (see Table 2). In addition, the finding that Swiss 3T3 CM markedly potentiates the tubule-inducing activity of optimal concentrations of either VEGF or bFGF (R. Montesano, unpublished observations) further suggests that the factor(s) responsible for the induction of tubule formation by BME cells is neither bFGF nor VEGF.

A number of other cytokines that have been reported to be angiogenic in vivo (Klagsbrun and D’Amore, 1991; Folkman and Shing, 1992; Koch et al., 1992; Risau et al., 1992) are also unlikely to mediate the effect of Swiss 3T3

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**Table 2. Effect of various treatments on the in vitro angiogenic activity of Swiss 3T3 CM**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>In vitro angiogenic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Dialysis (5-8×10^3 M, cut-off)</td>
<td>+</td>
</tr>
<tr>
<td>Trypsin</td>
<td>–</td>
</tr>
<tr>
<td>Heat (70°C, 30 min)</td>
<td>–</td>
</tr>
<tr>
<td>Acid (pH 3)</td>
<td>–</td>
</tr>
<tr>
<td>DTT (50 mM)</td>
<td>–</td>
</tr>
<tr>
<td>Anti-bFGF antibodies</td>
<td>+</td>
</tr>
</tbody>
</table>

Treated Swiss 3T3 CM was added to BME cells in the modified collagen gel invasion assay as described in Materials and Methods. A plus sign indicates formation of capillary-like tubules invading the underlying collagen matrix; a minus sign indicates the absence of tubule formation.

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**Fig. 6.** Conditioned medium from Swiss 3T3 cells induces formation of capillary-like tubules by BME cells. (A) BME cells were seeded at high density in the center of a collagen gel as described in Materials and Methods and grown under control conditions for 4 d. The cells have formed a confluent monolayer on the surface of the gel without invading the underlying matrix. (B) BME cells were seeded at high density in the center of a collagen gel. After overnight incubation, Swiss 3T3 CM (50%, v/v) was added and the culture incubated for a further 3 d. The endothelial cells have invaded the underlying collagen matrix to form a network of branching tube-like structures. Focus is beneath the surface monolayer. Bars, 200 μm.

**Fig. 7.** Swiss 3T3 CM increases PA activity in BME cells. (A) Low-density cultures of BME cells were exposed to Swiss 3T3 CM or Balb 3T3 CM, and 15 h later overlaid with a thin layer of agar containing plasminogen and casein. Swiss 3T3 CM markedly increased plasminogen-dependent caseinolytic activity around individual cells as observed by dark-field illumination, while Balb 3T3 CM had a minor effect. (B) Cell extracts were prepared from BME cells exposed to Swiss 3T3 CM or Balb 3T3 CM and subjected to zymography. This revealed an increase in u-PA activity in cells exposed to Swiss 3T3 CM (S) but not to Balb 3T3 CM (B).
CM, because they do not induce formation of capillary-like tubules in our collagen gel invasion assay. Specifically, polypeptides that lack 'angiogenic' activity in our system include angiogenin (R. Montesano, M. Moenner and J. Badet, unpublished observations), platelet-derived growth factor AA (PDGF-AA) and platelet-derived growth factor BB (PDGF-BB), epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), and interleukin-8 (IL-8) (R. Montesano, unpublished observations). Recently, Okamura et al. (1992) reported that tubule formation by microvascular endothelial cells co-cultured with oesophageal cancer cells was inhibited by antibodies against transforming growth factor alpha (TGF-α), and Sakuda et al. (1992) proposed that the in vitro angiogenic activity of CM from hypoxic smooth cells was due, at least in part, to transforming growth factor beta-1 (TGF-β1). Since, however, neither TGF-α (R. Montesano and R. Derynck, unpublished observations) nor TGF-β1 (Pepper et al., 1993b; R. Montesano, unpublished observations) is able to induce endothelial tubule formation in our system, these two cytokines are unlikely to account for the in vitro angiogenic activity of Swiss 3T3 CM. Finally, fibroblasts are known to produce the multifunctional cytokine, hepatocyte growth factor-scatter factor (HGF-SF) (for recent reviews, see Matsumoto and Nakamura, 1992; Furlong, 1992; Goldberg and Rosen, 1993), which has been reported to be angiogenic in vivo and to induce endothelial cell proliferation and motility in vitro (Bussolino et al., 1992; Grant et al., 1993). In our experimental conditions, however, we have not observed invasion of collagen gels and tubule formation by microvascular or large vessel endothelial cells in response to HGF-SF (R. Montesano, M.S. Pepper, T. Nakamura, and L. Orci, unpublished observations). In addition, among a variety of fibroblast strains that we have found possess HGF-SF activity (as inferred by their ability to induce tubulogenesis by MDCK epithelial cells; cf. Montesano et al., 1991a,b) only Swiss 3T3 cells were capable of eliciting an angiogenic response in our culture systems. Taken together, the observations described above suggest that the activity produced by Swiss 3T3 cells may be distinct from a number of well-characterized angiogenic cytokines, and that it may represent either a novel molecule(s) or a hitherto unsuspected function of a known molecule(s).

A remarkable finding in this study is that, among the cell lines we examined, only Swiss 3T3 cells (as well as their subclone 3T3-L1) are able to induce an angiogenic response by BME cells. Particularly striking in this respect was the difference between Swiss 3T3 and Balb 3T3 cells, since these two strains were established from mouse embryo cultures according to the same experimental protocol and possess very similar in vitro properties (Todaro and Green, 1963; Aaronson and Todaro, 1968). The reasons why the ability to induce formation of capillary-like tubules by endothelial cells appears to be restricted to Swiss 3T3 cells are at present unclear. Swiss 3T3 cells were maintained in our laboratory under conditions minimizing the chances of spontaneous transformation, and exhibited properties of 'normal' cells (e.g. contact inhibition of growth and low saturation density). It is nevertheless conceivable that Swiss 3T3 cells have acquired the ability to induce in vitro angiogenesis as an early event in the multistep process of transformation (Ziche and Gullino, 1982; Polverini and Solt, 1988), well before the occurrence of overt alterations in growth behavior. While we cannot dismiss this possibility,
it is noteworthy, however, that both Balb 3T12-3 cells, which are insensitive to contact inhibition in vitro and tumorigenic in vivo (Aaronson and Todaro, 1968), and a transformed variant of Balb 3T3 cells developed in our laboratory (R. Montesano, unpublished data) lack angiogenic activity in our in vitro systems (see Table 1; and R. Montesano, unpublished data), which indicates that transformation per se is not necessarily associated with the ability to induce in vitro angiogenesis.

A possible mechanism that might be responsible for the peculiar properties of Swiss 3T3 cells is the activation of a dormant gene coding for an angiogenic polypeptide. More specifically, it can be suggested that Swiss 3T3 cells constitutively express an ‘angiogenic’ gene, which in mesenchymal-like cells is only transcribed in response to specific environmental signals, e.g. during embryonic development or wound healing. Additional mechanisms that might account for the in vitro angiogenic activity of Swiss 3T3 cells include the mutation (or deletion) of a gene encoding an angiogenesis inhibitor (Rustinejad et al., 1989), the ability to export a signal-less angiogenic polypeptide (Kandel et al., 1991; Jackson et al., 1992), or the ability to activate extracellularly a latent angiogenic cytokine (Flaumenhaft and Rifkin, 1992).

In conclusion, we have developed two co-culture systems that are suitable for the study of interactions between endothelial cells and cell types that might produce angiogenic factors. We have found that, among a variety of non-transformed cells, Swiss 3T3 fibroblasts have the unique ability to induce formation of capillary-like tubules by endothelial cells, and that this effect is mediated by a paracrine-acting diffusible factor(s) that may be different from a number of well-characterized angiogenic cytokines. The collagen gel co-culture systems described here provide an attractive alternative to the widely used endothelial cell proliferation assays for the identification of additional angiogenesis factors. Indeed, endothelial cell proliferation represents only one component of the angiogenic response, and probably not the most crucial, as suggested by the finding that certain polypeptides that induce angiogenesis in vivo (e.g. TGF-β, tumor necrosis factor-α and angiogenin) are not endothelial mitogens (reviewed by Klagsbrun and D’Amore, 1991; Folkman and Shing, 1992). In contrast, collagen gel invasion assays rely on the detection of biological activities that induce both endothelial cell migration into a three-dimensional extracellular matrix substrate and morphogenesis of capillary tubules, which are two essential components of the angiogenic process.

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