Leukemia inhibitory factor (LIF) inhibits angiogenesis in vitro

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SUMMARY

Using an in vitro model in which endothelial cells can be induced to invade a three-dimensional collagen gel to form capillary-like tubular structures, we demonstrate that leukemia inhibitory factor (LIF) inhibits angiogenesis in vitro. The inhibitory effect was observed on both bovine aortic endothelial (BAE) and bovine microvascular endothelial (BME) cells, and occurred irrespective of the angiogenic stimulus, which included basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), the synergistic effect of the two in combination, or the tumor promoter phorbol myristate acetate. LIF inhibited bFGF- and VEGF-induced proliferation in BAE and BME cells. In addition, LIF inhibited BAE but not BME cell migration in a conventional two-dimensional assay. Finally, LIF decreased the proteolytic activity of BAE and BME cells and increased their expression of plasminogen activator inhibitor-1. These results demonstrate that LIF inhibits angiogenesis in vitro, an effect that can be correlated with a LIF-mediated decrease in endothelial cell proliferation, migration and extracellular proteolysis.

Key words: angiogenesis, endothelial cell, leukemia inhibitory factor, plasminogen activator inhibitor-1

INTRODUCTION

Angiogenesis is the formation of new capillary blood vessels by a process of sprouting from pre-existing vessels. In addition to its role during development and in the physiology of female reproductive function, wound healing, and collateral blood vessel formation in ischaemia, angiogenesis occurs in pathological situations such as proliferative retinopathy and hemangioma of infancy and childhood. Angiogenesis is also necessary for the continued growth of solid tumors, and contributes to the hematogenous spread of tumor cells and the formation of metastasis (Folkman and Klagsbrun, 1987; Zetter, 1988). Angiogenesis begins with localized breakdown of the basement membrane of the parent vessel. Endothelial cells then migrate into the surrounding matrix within which they form a capillary sprout. Sprout elongation occurs as a result of further migration and of endothelial cell proliferation proximal to the migrating front. Fusion with the tip of another maturing sprout produces a capillary loop. A functional capillary results once a lumen has been formed, and maturation is completed by reconstitution of the basement membrane (Folkman and Klagsbrun, 1987; Zetter, 1988). Alterations in at least three endothelial cell functions thus occur during this series of events: (1) modulation of interactions with the extracellular matrix, which requires alterations of cell matrix contacts and the production of matrix-degrading proteolytic enzymes; (2) an initial increase and subsequent decrease in locomotion (migration), which allows the cells to translocate towards the angiogenic stimulus and to stop once they reach their destination; (3) an increase in proliferation, which provides new cells for the growing and elongating new vessel, and a subsequent return to the quiescent state once the vessel is formed.

Angiogenesis is regulated by a large number of cytokines. In attempting to classify their activities, it is useful to consider the multiple cell functions that occur during angiogenesis as belonging either to a phase of activation or to a phase of resolution. The phase of activation includes: (a) basement membrane degradation; (b) cell migration and extracellular matrix invasion; (c) endothelial cell proliferation; and (d) capillary lumen formation. The phase of resolution includes: (a) inhibition of endothelial cell proliferation; (b) cessation of cell migration; and (c) basement membrane reconstitution (Pepper et al., 1993b). Cytokines that induce the activation phase either directly, or indirectly by inducing the production of other cytokines by inflammatory or stromal cells, include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1) (Klagsbrun and D’Amore, 1991; Ferrara et al., 1992a). While a great deal is known about the factors that induce the activation phase, very little is known about those involved in the phase of resolution. A potential candidate is TGF-β1 (Flaumenhaft et al., 1992), since this cytokine inhibits endothelial cell proliferation and migration, and reduces extracellular proteolysis (Klagsbrun and D’amoine, 1991). It has also been reported that TGF-β1 promotes the organization of single endothelial cells embedded in three-dimensional collagen gels into tube-like structures (Madri et al., 1988; Merwin et al., 1990). However, we have demonstrated that the in vitro effect...
of TGF-β1 is biphasic: depending on the concentration, TGF-β1 either inhibits or potentiates endothelial cell invasion and tube formation in three-dimensional collagen gels (Pepper et al., 1993b).

We have previously demonstrated that leukemia inhibitory factor (LIF) inhibits aortic endothelial cell proliferation in vitro (Ferrara et al., 1992b), and have suggested that the presence of LIF in activated macrophages in atherosclerotic plaques may indicate a role for this cytokine in promoting endothelial denudation over plaques in large blood vessels (Gillett et al., 1993). Leukemia inhibitory factor (LIF) was initially purified and cloned using a bioassay based on its ability to induce monocyte differentiation in the murine leukemic cell line, M1 (Tomida et al., 1984; Gearing et al., 1987; Hilton et al., 1988). However, LIF is one of a growing number of cytokines, characterized by pleiotropy and functional redundancy, which cannot easily be classified on the basis of their in vivo and in vitro activities (Hilton, 1992). Other properties that have been accorded to LIF include inhibition of totipotent mouse embryonic stem cell differentiation, stimulation of proliferation of haemopoietic cells and myoblasts, promotion of neuronal survival and differentiation, and stimulation of bone remodeling: LIF also stimulates acute phase protein synthesis in hepatocytes, promotes cachexia and regulates lipid metabolism (Hilton and Gough, 1991; Kurzrock et al., 1991; Hilton, 1992; Metcalf, 1992).

Using an in vitro model of angiogenesis in which endothelial cells can be induced to invade a three-dimensional collagen gel within which they form capillary-like tubes (Montesano and Orci, 1985), we have assessed the effect of LIF on the angiogenic response induced by a number of different stimuli. In addition, we have determined the effect of LIF on endothelial cell proliferation and migration using conventional two-dimensional assays. Finally, we have assessed the effect of LIF on endothelial cell extracellular proteolysis by focussing on the plasminogen activator (PA)-plasmin system (Vassalli et al., 1991).

MATERIALS AND METHODS

Reagents

Recombinant human LIF was provided by Dr C. Schmeltzer (Genentech). Endotoxin levels were less than 2 i.u./mg protein. Recombinant human VEGF (165 amino acid homodimeric species) was purified from transfected Chinese hamster ovary cells as described previously (Ferrara et al., 1991). Recombinant human bFGF (rhbFGF) was provided by Dr P. Sarmientos (Farmitalia Carlo Erba, Milan, Italy). rhbFGF used for proliferation assays was from Amgen (Thousand Oaks, CA). 4β-Phorbol 12-myristate 13-acetate (PMA) and mitomycin C were from Sigma Chemical Co. (St Louis, MI).

Cell culture

Bovine aortic endothelial (BAE) cells, isolated from scrapings of adult bovine thoracic aortas and cloned by limiting dilution as previously described (Pepper et al., 1992b), were cultured in low glucose Dulbecco’s modified minimal essential medium (DMEM, Gibco) supplemented with 10% DCS, penicillin (500 i.u./ml) and streptomycin (100 µg/ml). BAE cells used in the proliferation assays were obtained from adult bovine aortic arch, and were grown in low glucose DMEM supplemented with 10% calf serum (Gibco), 2 mM glutamine and antibiotics.

Adrenal cortical bovine microvascular endothelial (BME) cells used in all studies except proliferation assays were provided by Drs M. B. Furie and S. C. Silverstein (Columbia University, NY) (Furie et al., 1984), and were grown in minimal essential medium (MEM), alpha modification (Gibco AG, 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). BME cells used in the proliferation assays were isolated from bovine adrenal cortex according to published procedures, and were grown in low glucose DMEM supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine and antibiotics.

BAE and BME cells were subcultured at a 1:4 or 1:5 split ratio in 1.5% gelatin-coated tissue culture dishes or flasks (Falcon Labware, Becton-Dickinson Company, Lincoln Park, NJ). Culture media were changed every 2-3 days, and all experimental manipulations except for the proliferation assays were performed upon reaching confluence (5-7 days). Stock plates of BAE and BME cells used for proliferation assays were maintained in 1 ng/ml bFGF.

In vitro angiogenesis assay

BAE or BME cells were seeded onto three-dimensional collagen gels in 18 mm tissue culture wells (Nunclon, A/S Nunc, Roskilde, Denmark), at 5×10⁴ cells/well in 500 µl medium. Gels were prepared as previously described (Montesano and Orci, 1985). Briefly, 8 volumes of a solution of type I collagen from rat tail tendons (approximately 1.5 mg/ml) were quickly mixed with 1 volume of 10× minimal essential medium (MEM, Gibco) and 1 volume of sodium bicarbonate (11.76 mg/ml) on ice, dispensed into tissue culture wells and allowed to gel at 37°C for 10 minutes. Cytokines and PMA were added upon reaching confluence. Medium and cytokines/PMA were renewed every 2-3 days, and cultures were fixed in situ after 4 or 7 days with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) and photographed for quantification.

Quantification of invasion

Randomly selected fields measuring 1.0 mm × 1.4 mm were photographed in each well at a single level beneath the surface monolayer by phase-contrast microscopy, using a Nikon Diaphot TMD inverted photomicroscope. Invasion was quantified from three randomly selected fields per experiment by determining the total additive length of all cellular structures that had penetrated beneath the surface monolayer either as apparently single cells or in the form of cell cords (Pepper et al., 1992b). Results are shown as the mean length ± s.e.m. (in µm) of three randomly selected photographic fields per experiment, for each of at least 3 experiments per condition. Mean values were compared using Student’s unpaired t-test, and a significant P value was taken as <0.05.

Processing for light microscopy

Cultures that had been fixed in situ overnight were washed extensively in 100 mM sodium cacodylate buffer (pH 7.4). Collagen gels were cut into 2 mm × 2 mm fragments, post-fixed in 1% osmium tetroxide in Veronal acetate buffer for 45 minutes, and further processed as previously described (Montesano and Orci, 1985). Semi-thin sections were cut perpendicular to the culture plane with an LKB ultramicrotome, stained with 1% Toluidine Blue, and photographed under transmitted light using an Axioskop photomicroscope (Zeiss, Germany).

Proliferation assays

BAE or BME cells were seeded at 7×10³ cells per well in 12-well plates (Costar) in the presence of low glucose DMEM supplemented with 10% calf serum (Gibco), 2 mM glutamine and antibiotics. bFGF, VEGF or LIF were added at the indicated concentrations a few hours after plating in an assay volume of 2 ml. After 5 days, cells were dissociated by exposure to trypsin and counted in a Coulter counter. Medium was not changed during the assay.

To assess the effect of mitotic inhibitors on in vitro angiogenesis,
confuent monolayers of BAE or BME cells on collagen gels were treated with mitomycin C (1 and 0.1 µg/ml) for 6 hours, medium was removed, monolayers were washed and medium containing bFGF (10 ng/ml) was added. Medium and compounds were changed every 2-3 days, and invasion was quantified as described above after 4 (BAE) or 7 (BME) days.

Migration assays
Confuent monolayers of BAE or BME cells in gelatin-coated 35 mm Petri dishes were wounded with a razor blade to mark the original wound edge, washed with serum-free medium, and serum-free medium containing 0.1% gelatin, and LIF was added at the indicated concentrations. After 15 hours, monolayers were fixed and stained with 0.2% Crystal Violet in 20% ethanol for 30 minutes. Randomly selected fields measuring 1.0 mm x 1.4 mm were photographed using a Nikon Diaphot TMD inverted photomicroscope, and the total number of cells that had crossed the original wound edge was determined (Pepper et al., 1994). Values are shown as the mean ± s.e.m. of 6 randomly selected fields per condition.

PA plaque assay
BAE or BME cells were seeded at 5x10⁴ cells per gelatin-coated 35 mm Petri dish in 1.5 ml complete medium. After overnight attachment and spreading, cells were treated with LIF at the indicated concentrations in the presence or absence of bFGF (10 ng/ml). Fifteen hours 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 (Vassalli et al., 1976), incubated at 37°C for 3 hours, and photographed under dark-field illumination.

Zymography and reverse zymography
Confuent monolayers of BAE or BME cells in gelatin-coated 35 mm Petri dishes were washed twice with serum-free culture medium, and treated with either LIF alone or in combination with bFGF (10 ng/ml) in serum-free medium containing Trasylol (200 KIU/ml) (Bayer-Pharma AG, Zurich, Switzerland). Fifteen hours 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 confuent monolayers of BAE or BME cells exposed to LIF in complete medium; the last medium change was 48 hours before commencing the experiment. RNA preparation, northern blots, 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 u-PA (Krätzhammer et al., 1993), bovine u-PAr (Krätzhammer et al., 1993), human t-PA (Fisher et al., 1985) and bovine 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 time = 0 hours.

RESULTS

LIF inhibits angiogenesis in vitro
We have used an in vitro model in which endothelial cells can be induced to invade a collagen gel (Montesano and Orci, 1985), to assess the effect of LIF on the angiogenic process induced by a number of different stimuli. Since our initial observations on the antiproliferative effects of LIF indicated that this effect was most marked on BAE cells (Ferrara et al., 1992b; and see below), we initially chose to assess the effects of LIF on these cells. The in vitro angiogenic response of BAE cells grown on three-dimensional collagen gels has not previously been described. BAE cells formed a confluent monolayer on the surface of collagen gels, with minimal invasion occurring into the underlying matrix (Fig. 1a); the extent of spontaneous invasion was, however, greater than that observed with BME cells (see below). Upon addition of bFGF (Fig. 1b) or VEGF (Fig. 1c), the cells invaded the underlying gel to form an extensive network of branching and anastomosing cell cords, similar to what we have previously reported for BME cells (Montesano et al., 1986; Pepper et al., 1992a). In contrast to BME cells, however, semi-thin sections revealed that BAE cell cords induced by bFGF were usually devoid of a lumen, with lumen formation occurring slightly more frequently in response to VEGF (results not shown). Co-addition of bFGF and VEGF induced a marked synergistic effect on invasion (results not shown), similar to what we have previously described for microvascular endothelial cells (Pepper et al., 1992a). Under these conditions, the invading cell cords usually contained a clearly defined lumen, and serial sections revealed the presence of a branching network of capillary-like tubes beneath the surface monolayer (Fig. 2). Upon addition of LIF to BAE monolayers, intercellular borders became more refractile, and gaps appeared between contiguous endothelial cells (Fig. 1d). This effect was not observed with BME cells (results not shown). When co-added with bFGF or VEGF, LIF markedly inhibited BAE cell invasion (Fig. 1e,f). LIF also inhibited the synergistic effect of co-added bFGF and VEGF, although lumen formation was not inhibited in the presence of all three cytokines (results not shown). A quantitative analysis revealed a dose-dependent inhibition of bFGF-induced invasion, with values reaching statistical significance at 10 and 100 ng/ml LIF; and with a maximum of 72% inhibition at the latter concentration (Fig. 3). Addition of LIF alone did not significantly affect invasion (Fig. 3).

Since angiogenesis normally occurs in the microvasculature, we next assessed the effect of LIF on the in vitro angiogenic response of BME cells. As for BAE cells, LIF markedly inhibited bFGF-induced BME cell invasion. Significant inhibition was observed with LIF at 300 pg/ml, with maximal inhibition of approximately 80% occurring at 3 ng/ml (Fig. 3). As for BAE cells, LIF also inhibited VEGF-induced invasion, and reduced the synergistic effect of bFGF and VEGF by 73% (Fig. 4A). To determine whether LIF could modulate the angiogenic response induced by pharmacological agents, we assessed its effect on invasion induced by the tumor promoter PMA (Montesano and Orci, 1985). As for bFGF and VEGF, PMA-induced invasion was markedly inhibited in the presence of LIF (Fig. 4B).

LIF inhibits endothelial cell proliferation and migration
We have previously reported that LIF inhibits basal levels of BAE cell proliferation, with an ED50 of 1 ng/ml and a maximal inhibitory effect at 5 ng/ml (Ferrara et al., 1992b). Here we demonstrate that LIF also inhibits basal BME cell proliferation at 1 and 5 ng/ml (Fig. 5). The inhibition with 1 and 5 ng/ml LIF was 52±3% and 76±5% respectively for BAE cells, and 11±1% and 32±3% for BME cells. The greater effectiveness of LIF in inhibiting BAE cell proliferation is in agreement with previous studies (Ferrara et al., 1992b). LIF also inhibited...
bFGF- and VEGF-induced BAE and BME cell proliferation, with the inhibitory effect on BAE cells being more pronounced (Fig. 6). The concentration of LIF used in these experiments (5 ng/ml) is similar to the minimal concentration of LIF required for maximal inhibition of BME cell invasion (3 ng/ml) (Fig. 3).

Since LIF inhibits endothelial cell proliferation, and since inhibition of proliferation could conceivably contribute to its inhibitory effect on in vitro angiogenesis, we also assessed the effect of mitomycin C, a mitosis inhibitor, on collagen gel invasion. Mitomycin C inhibited bFGF-induced BAE cell invasion by 57% and 79% when added at 0.1 and 1.0 µg/ml, respectively (Fig. 7A). Similarly, bFGF-induced BME cell invasion was inhibited by 41% and 71% in the presence of 0.1

**Fig. 1.** LIF inhibits bFGF- and VEGF-induced collagen gel invasion. BAE cells grown to confluence on the surface of a three-dimensional collagen gel (a) were treated with bFGF (10 ng/ml) (b) or VEGF (30 ng/ml) (c) for 4-5 days. This results in the formation of cell cords within the gel, which can be observed by focussing beneath the surface monolayer. Co-addition of LIF (100 ng/ml) markedly inhibits BAE cell invasion induced by bFGF (e) of VEGF (f). LIF also increases refractility at the intercellular borders, and induces the formation of small gaps between contiguous endothelial cells (d). Bar, 200 µm.

**Fig. 2.** Formation of capillary-like tubes by BAE cells induced to invade three-dimensional collagen gels. BAE cells grown to confluence on the surface of a three-dimensional collagen gel (cg) were co-treated with bFGF (10 ng/ml) and VEGF (100 ng/ml) for 4 days. Serial semi-thin sections reveal the formation of a branching network of capillary-like tubes beneath the surface monolayer. Bar, 50 µm.
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and 1.0 µg/ml mitomycin C, respectively (Fig. 7B). These results demonstrate that mitomycin C, a mitosis inhibitor, reduces the total length of invading endothelial cell cords.

The effect of LIF on endothelial cell migration was assessed in a two-dimensional assay in which migration is measured as the number of cells that have moved into an artificially created wound in a confluent monolayer (Pepper et al., 1994). Under these conditions, LIF inhibited BAE cell migration, with a maximal 50% reduction in migration in response to 100 ng/ml LIF (Fig. 8). Under identical conditions, LIF had no effect on BME cell migration (results not shown).

**LIF reduces endothelial cell extracellular proteolytic activity**

To assess the effect of LIF on the proteolytic properties of BAE and BME cells, we focussed on the PA-plasmin system (Vassalli et al., 1991). Plasmin is a protease of trypsin specificity, which degrades certain matrix components and also
activates metalloproteinase zymogens. Plasmin is generated from its inactive precursor plasminogen by the activity of two PAs, urokinase-type PA (u-PA) and tissue-type PA (t-PA). u-PA activity can be localized to the cell surface through binding to a specific high-affinity receptor (u-PAr), and u-PA and t-PA are subject to inhibition by specific physiological PA inhibitors (PAIs), PAI-1 and PAI-2. A precise protease-antiprotease equilibrium is required to allow for localized pericellular matrix degradation, while at the same time protecting the extracellular matrix against inappropriate destruction (Pepper and Montesano, 1990).

Cell-associated PA activity was determined using the PA plaque assay (Vassalli et al., 1976), in which plasminogen-dependent caseinolytic activity can be detected around individual cells under dark-field illumination. The bFGF-induced increase in PA activity in BAE and BME cells was appreciably reduced in presence of LIF, although LIF had no effect on basal levels of PA activity (Fig. 9A, and results not shown). By reverse zymography, LIF was found to increase the levels of PAI-1 activity in both cell types, with a detectable increase at 1 ng/ml (Fig. 9B, and results not shown). The PAI-1 increase in response to 100 ng/ml LIF was similar in magnitude to that seen in response to a combination of bFGF and LIF, which induced an additive response (Fig. 9B). We have previously demonstrated that the PAI produced by BME cells that is detectable by reverse zymography is PAI-1 (Pepper et al., 1991a). PAI-1 mRNA was also increased in response to LIF: a kinetic analysis in BME cells with 100 ng/ml LIF revealed a 2.7-fold increase after 4 hours, a maximal 4.6-fold increase after 8 hours, with sustained elevation until the end of the 24 hour assay period (Fig. 10). (These values, which are expressed relative to controls at time = 0 hour, are likely to be an underestimate, since PAI-1 mRNA levels decreased with time in culture in controls, as seen in Fig. 10). Similar kinetics were observed in BAE cells (results not shown). A dose-response analysis performed after 15 hours exposure to LIF revealed a detectable increase in PAI-1 mRNA in response to 10 ng/ml LIF in BME and BAE cells (results not shown). Co-addition of bFGF (10 ng/ml) and LIF (100 ng/ml) induced an additive

Fig. 4. (A) LIF inhibits bFGF/VEGF synergism on BME cell invasion of collagen gels. Confluent monolayers of BME cells on three-dimensional collagen gels were treated with bFGF (10 ng/ml) (F10), VEGF (30 ng/ml) (V30) or both factors together (F10/V30) in the presence or absence of LIF (100 ng/ml), and invasion was quantified after 4 days. (B) LIF inhibits PMA-induced BME cell invasion of collagen gels. Monolayers of BME cells were treated with PMA (5, 10 or 20 ng/ml) in the presence or absence of LIF (100 ng/ml), and invasion was quantified after 4 days. Results are shown as the mean length in µm ± s.e.m. of all cells invading beneath the surface monolayer in 9 randomly selected fields (3 fields from each of 3 separate experiments) for each condition. 1P<0.025, 2P<0.005, 3P<0.001 when compared with values in the absence of LIF (Student’s unpaired t-test).

Fig. 7. Effect of mitomycin C on BAE and BME cell invasion of collagen gels. Confluent monolayers of BAE or BME cells on collagen gels were treated with mitomycin C at the indicated concentrations for 6 hours, and the effect on invasion induced by subsequent addition of bFGF (10 ng/ml) was quantified after 4 (BAE) or 7 (BME) days. Results are shown as the mean length in µm ± s.e.m. of all cells invading beneath the surface monolayer in at least 9 randomly selected fields (3 fields from each of at least 3 separate experiments) for each condition. 1P<0.005, 2P<0.001 when compared with values in the absence of mitomycin C (Student’s unpaired t-test).
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While LIF (100 ng/ml) also increased u-PA and t-PA mRNA levels in BME and BAE cells, although in contrast to PAI-1, the increase in u-PA and t-PA mRNAs was maximal after 1 hour (2.12-fold and 1.88-fold, respectively), returned to basal levels after 4 hours, and in the case of t-PA, was reduced 2.7-fold below basal levels after 24 hours (Fig. 10 and results not shown). The transient increase in u-PA and t-PA mRNA levels was not reflected in the zymographic analysis, which assayed for PA activity accumulated over a 15 hour collection period; instead, a small decrease in bFGF-induced u-PA activity was observed in the presence of LIF, which was more marked in BAE than BME cells (Fig. 9B, and results not shown). LIF (100 ng/ml) also reduced bFGF (10 ng/ml)-induced u-PA mRNA levels in BME cells (results not shown). That the cell-associated PA was u-PA was determined on the basis of inhibition of its catalytic activity by amiloride (Vassalli and Belin, 1987; Pepper et al., 1987). u-PAr mRNA expression was also increased in BME cells in response to LIF (100 ng/ml), with a detectable 1.52-fold increase after 1 hour and a maximal 1.57-fold increase after 12 hours, with levels returning to baseline by 24 hours (Fig. 10). Similar kinetics of u-PA, u-PAr and t-PA mRNA increase were observed with BAE cells (results not shown). A dose-response analysis performed with BME cells after 15 hours exposure to LIF (100 pg/ml to 100 ng/ml) did not reveal appreciable alterations in u-PA, u-PAr or t-PA mRNA levels over and above those shown at a similar time point in Fig. 10 (results not shown). Taken together, these findings reveal a net decrease in the proteolytic potential of BAE and BME cells in response to LIF. These results are in direct contrast to what we have previously observed in BME cells in response to bFGF: PAI-1 mRNA was transiently induced, while u-PA mRNA levels remained elevated throughout the entire assay period (Pepper et al., 1990).

**DISCUSSION**

In the studies reported in this paper, we have demonstrated that LIF inhibits angiogenesis in a three-dimensional in vitro model using either large vessel (aortic) or microvascular bovine endothelial cells. This inhibitory effect occurred irrespective of the angiogenic stimulus, which included bFGF, VEGF, the synergistic effect of the two cytokines in combination, or the tumor promoter PMA. In aortic endothelial cells, LIF inhibited endo-

Fig. 6. Effect of LIF (5 ng/ml) on bFGF- and VEGF-induced BAE and BME cell growth. Studies were performed in duplicate. The variation from the mean was less than 10%. A single representative experiment is shown; similar results were obtained in four separate experiments.

Fig. 5. Effect of LIF on basal growth of BAE and BME cells. This study was performed in triplicate, and data are shown as mean ± s.e.m. The variation from the mean was less than 10%. A single representative experiment is shown; very similar results were obtained in three separate experiments.
thelial cell proliferation and migration in conventional two-
dimensional assays, while in microvascular endothelial cells,
inhibition of proliferation was modest and migration was unaf-
fected. The reasons for these differences in the proliferative
and migratory responses of the two endothelial cell types are
not known. LIF decreased the proteolytic potential of both
aortic and microvascular endothelial cells by increasing their
expression of PAI-1. These results indicate that a reduction in
extracellular proteolysis and endothelial cell proliferation are
two possible mechanisms of LIF-mediated inhibition common
to the two endothelial cell types. With respect to proteolysis,
it has previously been demonstrated (Mignatti et al., 1989) that
inhibition of PA-plasmin-mediated proteolysis inhibits endo-
thelial cell invasion of a three-dimensional matrix. The exper-
iments with mitomycin C described in this paper indicate that,
following exposure to this mitosis inhibitor, the total additive
length of invading endothelial cell cords was reduced.

Is there any evidence that suggests that LIF may be anti-
angiogenic in vivo? The in vivo biological properties of LIF
have been defined in two ways. First, from pathological effects
observed upon increasing circulating levels of LIF in mice.
These effects include cachexia, excess new bone formation and
ectopic calcification, behavioral changes, thymus atrophy and
hemopoietic abnormalities, and abnormalities in the adrenal
cortex and ovarian corpora lutea (Metcalf and Gearing,
1989a,b; Metcalf et al., 1990). Many of these effects could con-
ceivably arise from atrophy of the microvasculature, which
would implicate LIF as a vessel regression factor. It is
important to recall that physiological angiogenesis is limited
in adult life to female reproductive function and wound
healing. With respect to the former, it is particularly interest-
ing to note the description in mice with elevated circulatory
levels of LIF, of a striking deficit of corpora lutea, with those
present being ill defined and containing luteal cells of small
volume (Metcalf and Gearing, 1989b). Since corpus luteum
formation is associated with neovascularization, these findings
may implicate an anti-angiogenic component in the inhibitory
effect of LIF. The second way in which the in vivo biological

Fig. 8. Effect of LIF on BAE cell migration. Confluent monolayers
of BAE cells were wounded with a razor blade and the number of
cells that had crossed the original wound edge in the presence or
absence of LIF was determined. Values are the total number of
migrating cells, and are shown as the mean ± s.e.m. of 6 randomly
selected fields per condition. A single representative experiment is
shown; very similar results were obtained in two separate
experiments. *P<0.005 when compared with values in the absence of
LIF (Student’s unpaired t-test).

properties of LIF have been defined is from gene targeting and
descriptive studies, which indicate a role for LIF during
implantation and early stages of embryonic development
(Conquet and Brûlet, 1990; Murray et al., 1990; Rathjen et al.,
1990; Bhatt et al., 1991; Conquet et al., 1992; Shen and Leder,
1992; Stewart et al., 1992). No obvious phenotype related to
the lack of a LIF-mediated inhibitory effect on angiogenesis
has been described in these reports.

Fig. 9. LIF decreases PA activity and increases PAI-1 synthesis in
BAE and BME cells. (A) Low density cultures of BAE cells were
exposed to LIF at the indicated concentrations in presence or absence
of bFGF (10 ng/ml), and 15 hours later overlaid with a thin layer of
agar containing plasminogen and casein. The bFGF-induced increase
in plasminogen-dependent caseinolytic activity observed around indi-
vidual cells by dark-field illumination was appreciably reduced in the
presence of LIF. (B) Cell extracts were prepared from BME cells
exposed to LIF at the indicated concentrations in the presence or
absence of bFGF (10 ng/ml), and subjected to zymography and
reverse zymography. Reverse zymography revealed a dose-dependent
increase in PAI-1 in response to LIF, and an additive effect when co-
added with bFGF. Zymography revealed a small decrease in bFGF-
induced u-PA activity.
LIF inhibits angiogenesis in vitro

What are the arguments against a role for LIF inhibiting angiogenesis in vivo? First, in pregnant mice lacking a functional LIF gene, uteri were found to be poorly vascularized, which is contrary to an anti-angiogenic role for LIF (Stewart et al., 1992). Second, LIF is found in high titres in the synovial fluid of patients with inflammatory arthritis, in which extensive new vessel formation occurs in the synovial pannus, and its production by synoviocytes and chondrocytes is increased in organ culture in response to inflammatory cytokines such as interleukin-1β (Lotz et al., 1992). Third, LIF is induced during inflammation in most tissues following administration of bacterial lipopolysaccharide to mice (Brown et al., 1994). Finally, although mice with elevated circulatory levels of LIF display deficit of corpora lutea, the frequency of occurrence and maturation of ovarian follicles, which are also angiogenesis-dependent, was unaltered in the same animals (Metcalf and Gearing, 1989b).

With indirect correlative evidence that argues both for and against a role for LIF in inhibiting angiogenesis in vivo, it is not easy to reconcile existing in vivo observations with our in vitro findings. Two features of complex cytokine networks that exist in vivo need, however, to be borne in mind. First, it is becoming increasingly apparent that the nature of the cellular response elicited by a specific cytokine is contextual, i.e. depends on the presence of other regulatory molecules, including other cytokines and extracellular matrix components in the pericellular environment of the responding cell (Nathan and Sporn, 1991). With respect to angiogenesis, we have previously demonstrated that the activity of one cytokine can be modulated by the presence and concentration of a second cytokine in our three-dimensional in vitro model of angiogenesis (Pepper et al., 1990, 1992a, 1993b). This may be relevant in physiological settings such as inflammation (Brown et al., 1994), in which LIF may temper the activity of high local concentrations of angiogenic cytokines, and may thus serve to prevent uncontrolled angiogenesis. Second, it is important to recall that a number of pleiotropic cytokines, including LIF, interleukin-6 (IL-6), oncostatin M and ciliary neurotrophic factor, all share a common intracellular molecular 'signal converter', namely the transmembrane glycoprotein gp130, which associates with specific receptors to form a transmembrane signalling complex (Gearing et al., 1992; Ip et al., 1992).

These findings raise the possibility that in our in vitro system, LIF might be activating a promiscuous signal converter such as gp130, and that this may serve to mimic the potential of other cytokines sharing the same converter, to inhibit angiogenesis. To our knowledge, the characterization of LIF receptors on endothelial cells, and their possible modulation by angiogenesis-regulating cytokines, has not been reported.

Finally, LIF is virtually undetectable in the tissues and circulation of normal adult mice (Bhatt et al., 1991; Shen and Leder, 1992; Brown et al., 1994). This has led to the suggestion that physiological LIF expression is normally under tight control and that it may act primarily in a local environment rather than systemically (Hilton, 1992). It has been demonstrated that LIF is induced in most tissues during inflammation (Brown et al., 1994), and it has been suggested that physiological LIF expression is under polypeptide growth factor and hormonal control (Rathjen et al., 1990; Bhatt et al., 1991; Shen and Leder, 1992; Lotz et al., 1992). In this context, it is particularly interesting to note that bFGF, TGF-β1, TGF-β2 and TGF-β3 all increase LIF expression in human and murine fibroblasts (Rathjen et al., 1990; Lotz et al., 1992). Since LIF is constitutively expressed by human umbilical endothelial cells in vitro (Lübbert et al., 1991), and since interactions between bFGF, VEGF and TGF-β are believed to be important for the control of in vitro angiogenesis (Pepper et al., 1990, 1992a, 1993b; Flaumenhaft et al., 1992), it is conceivable that some of the effects of these cytokines on endothelial cells may be indirectly mediated by LIF.

In summary, we have demonstrated that LIF inhibits angiogenesis in a three-dimensional in vitro model using large vessel and microvascular endothelial cells. We also demonstrate that key endothelial cell functions necessary for the formation of new capillary blood vessels including proliferation, migration and extracellular proteolytic activity, are inhibited by LIF, with notable variations in the proliferative and migratory responses of the two endothelial cell types. Although like LIF, TGF-β1 has also been shown to inhibit angiogenesis in vitro (Müller et al., 1987; Mignatti et al., 1989; Pepper et al., 1990, 1991b), unlike LIF the inhibitory effect of TGF-β1 in vitro is strictly
concentration dependent: at concentrations of TGF-β1 that are 10-fold lower than those required for inhibition, endothelial cell invasion of a three-dimensional collagen gel is potentiated (Pepper et al., 1993b). The studies reported in this paper, which have been performed with concentrations of LIF spanning several log orders of magnitude, provide the first demonstration of a cytokine that inhibits angiogenesis, and lacks biphasic effects in vitro. A large number of diverse functions have been attributed to LIF, and our findings add to the list of biological properties that have been defined in vitro. It will therefore be important to determine whether our in vitro observations can be confirmed in vivo.

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REFERENCES


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