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# Pituitary follicular cells secrete an inhibitor of aortic endothelial cell growth: Identification as leukemia inhibitory factor

(aortic endothelium/capillary endothelium/angiogenesis/atherosclerosis)

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ABSTRACT **Medium conditioned by bovine pituitary follicular cells paradoxically inhibits the growth of adult bovine aortic endothelial (ABAE) cells at dilutions that are instead mitogenic to adrenal cortex capillary endothelial (ACCE) cells, suggesting that follicular cells secrete a growth inhibitor with a selectivity for ABAE cells. The ABAE cell inhibitory activity was purified to apparent homogeneity by a combination of size-exclusion chromatography, ion-exchange chromatography, and two reversed-phase steps on a $C_4$ column. Microsequencing of the purified material revealed a single $NH_2$-terminal amino acid sequence, identical to that of leukemia inhibitory factor (LIF), a glycoprotein originally identified by its ability to inhibit the growth of MT1 mouse leukemia cells and subsequently found to have numerous effects. Recombinant human LIF inhibited the growth of ABAE cells as effectively as transforming growth factor β (TGFβ$_1$). However, it failed to inhibit markedly the growth of ACCE cells, whereas TGFβ$_1$ dramatically inhibited their growth. Recombinant human LIF also failed to induce a significant angiogenic response in the chicken chorioallantoic membrane, indicating that, unlike TGFβ, LIF probably does not induce the release of direct-acting angiogenic factors from inflammatory cells. The presence of LIF in follicular cells may relate to the peculiar vascular organization of the pituitary gland, where no arteries reach the pars distalis and all of the blood supply to this area is by capillaries.**

An understanding of the mechanisms that control the proliferation and differentiation of vascular endothelial cells should provide important insight into a wide variety of physiological and pathological events such as embryonic development, wound healing, tumor growth, diabetes, and atherosclerosis (1, 2). Several angiogenic inducers have been identified, including epidermal growth factor, transforming growth factor α (TGFα), prostaglandin $E_2$, angiogenin, acidic and basic fibroblast growth factors (aFGF and bFGF), platelet-derived endothelial cell growth factor, and vascular endothelial growth factor (VEGF) (2). However, despite the almost ubiquitous distribution of at least some of those factors, endothelial cells are normally quiescent in adult tissues, except in the ovarian corpus luteum and in the endometrium (1, 2). Therefore, it appears that the expression of inhibitory factors plays an especially important regulatory role. The search for inhibitors of endothelial cell growth and angiogenesis has yielded a few candidates, such as TGFβ (3), tumor necrosis factor (TNF) (4), thrombospondin (5), and, more recently, the 16-kDa fragment of rat prolactin (6). These agents inhibit the growth of cultured endothelial cells. However, such an *in vitro* action does not necessarily have relevance to the *in vivo* situation. For example, TGFβ or TNF paradoxically potently stimulates angiogenesis in several *in vivo* models, possibly by inducing the paracrine release of direct-acting angiogenic factors from inflammatory cells (7, 8). The inhibitory control of angiogenesis is thus poorly understood.

Another important and still unresolved issue concerns the growth control of small- versus large-vessel endothelial cells. Both cell types proliferate in culture and are affected equally by ubiquitous agents such as FGF or TGFβ (9, 10). Yet their *in vivo* behavior is strikingly different. Capillary endothelial cells have a remarkable propensity to proliferate; physiological and pathological angiogenesis reflects primarily microvascular growth (1, 2). In contrast, large-vessel endothelium has a very limited regenerative capability and an injury to the intima is not repaired unless the damage involves only a few cells (11). This suggests either that *in vivo* these cell types have a different response to the same factors than in culture or that other factors exist which may preferentially affect the growth of each cell type.

In the course of the purification of VEGF from medium conditioned by bovine pituitary follicular or folliculostellate cells (12), we observed that such medium paradoxically inhibited the proliferation of aortic endothelial cells at dilutions which were strongly mitogenic to capillary endothelial cells. Initially, we even considered the possibility that a single factor might be responsible for stimulating capillary and inhibiting arterial endothelial cell growth. However, purified VEGF was mitogenic to both cell types (12, 13), indicating that a different factor was responsible for the inhibitory effect.

Here we report on the purification to apparent homogeneity of such a growth inhibitor. Microsequencing of the purified material revealed, unexpectedly, an $NH_2$-terminal sequence identical to that of leukemia inhibitory factor (LIF) or differentiation-stimulating (D) factor. This protein was originally isolated from the conditioned medium of transformed cell lines on the basis of its ability to inhibit growth and induce differentiation in mouse MT1 myeloid leukemia cells (14), and subsequently it was found to regulate a variety of highly differentiated functions in several cell types (15–17).

## MATERIALS AND METHODS

**Reagents.** Tissue culture reagents, media, and sera were obtained from GIBCO through the Genentech media facility. The Mono Q HR5/5 anion-exchange FPLC column and PD10 gel filtration columns were from Pharmacia. The Poly CAT column for cation-exchange HPLC (4.6 × 100 mm) was purchased from PolyLC (Columbia, MD). The preparative

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Abbreviations: ABAE, adult bovine aortic endothelial; ACCE, adrenal cortex capillary endothelial; bFGF, basic fibroblast growth factor; LIF, leukemia inhibitory factor; rhLIF, recombinant human LIF; TGF, transforming growth factor; rhTGF, recombinant human TGF; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; TFA, trifluoroacetic acid.

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TSK 2000 GSW HPLC sizing column (21 × 600 mm) was from Hewlett–Packard. The reversed-phase $C_4$ HPLC column (4.6 × 100 mm) was from SynChrome (Lafayette, IN). Trifluoroacetic acid (TFA) was from Pierce. HPLC-grade acetonitrile, 2-propanol, and $KH_2PO_4$ were from Fisher Scientific. Centriprep-30 concentration units were purchased from Amicon. Tissue culture plates were from Costar except for large-scale Nunc culture plates (540 $cm^2$), which were from Applied Scientific (San Francisco). Recombinant human LIF (rhLIF) was the gift of C. Schmelzer (Genentech). Recombinant human TGF$\beta_1$ (rhTGF$\beta_1$) was the gift of E. Amento (Genentech). Recombinant human bFGF was purchased from Amgen. The protein assay kit and molecular weight standards for gel chromatography were from Bio-Rad. Prestained low molecular weight standards for electrophoresis were from BRL. Fertilized chicken eggs were obtained from Feather Hill Farms (Petaluma, CA). All other reagents were from Sigma.

**Culture of Follicular Cells.** Primary cultures of follicular cells were established from bovine adenohypophyseal pars distalis as described (18). The growth medium was low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics. Confluent cultures, which consisted of homogeneous dome-forming cell monolayers, were dissociated by exposure to 0.05% trypsin/0.3% EDTA and passaged at a split ratio of 1:10. For large-scale culture, cells were seeded at 5 × $10^6$ per Nunc plate in the presence of growth medium. As soon as they reached confluence, they were washed four times with phosphate-buffered saline (PBS) to remove serum components before addition of serum-free medium consisting of low-glucose DMEM plus insulin (5 μg/ml), transferrin (10 μg/ml), selenium (10 nM, as sodium selenite), glutamine (2 mM), and antibiotics. Serum-free media were collected and changed every 3–4 days for 3–4 weeks. The collected media were centrifuged at 10,000 × $g$ for 15 min at 4°C to remove debris and particulate matter and then stored at −70°C.

***In Vitro* Bioassays.** The growth-inhibitory activity from follicular cell conditioned medium, rhLIF, or rhTGF$\beta_1$ was tested on adult bovine aortic endothelial (ABAE) cells. ABAE primary cultures were established from steer aortic arches (19). Stock cultures were maintained in the presence of low-glucose DMEM supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium). bFGF was added at 2 ng/ml. For the proliferation assay, ABAE cells were seeded at 10,000 per well in 12-well tissue culture plates with growth medium (assay volume, 2 ml). Conditioned medium or fractions were added to cells 4–6 hr after plating. After 4 days, cells were dissociated by exposure to trypsin and counted in a Coulter Counter. Selected fractions, rhLIF, or rhTGF$\beta_1$ was tested also on bovine adrenal cortex-derived capillary endothelial (ACCE) cells. ACCE cells were seeded at 10,000 per well in 12-well plates with low-glucose DMEM supplemented with 10% calf serum, and fractions were added in 5-μl/ml aliquots (12, 13). Cells were dissociated by exposure to trypsin and counted in a Coulter Counter after 4 or 5 days.

**Angiogenesis Assay.** Fertilized eggs at day 3 were cracked and embryos were placed in 10-cm Petri dishes (20) and incubated in a humidified atmosphere at 37°C in the absence of $CO_2$. On day 10, when the chorioallantoic membrane covered the entire surface of the dish, rhLIF (400 ng) in PBS, rhTGF$\beta_1$ (400 ng) in PBS, or PBS alone was mixed 1:1 (vol/vol) with 1.5% methylcellulose in PBS and allowed to dry in Teflon molds in 10-μl volumes (21). Three methylcellulose disks, containing either rhLIF, rhTGF$\beta_1$, or PBS alone, were applied on each chorioallantoic membrane ($n$ = 35). After 72 hr, the angiogenic response was evaluated (21).

**Purification of the Growth-Inhibitory Activity.** Serum-free conditioned medium (≈2.5 liters) was concentrated about 250-fold by ultrafiltration through Amicon stir cells (YM10 membranes). Aliquots (5 ml) of concentrated conditioned medium were injected into a preparative TSK column equilibrated with 100 mM $KH_2PO_4$ (pH 6.8). The column had been calibrated with thyroglobulin (650 kDa), γ-globulin (150 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and vitamin $B_{12}$ (1.35 kDa). The flow rate was 3 ml/min. Absorbance was monitored at 280 nm through a Gilson model 116 UV detector. Fractions of 3 ml were collected and aliquots were tested for bioactivity on ABAE cells.

The most bioactive fractions (nos. 21–32) from two TSK columns were pooled, concentrated about 10-fold (Centriprep-30), and reconstituted in 20 mM Tris (pH 8.0) by PD10 columns. The material was injected through a 5-ml loop into a Mono Q column that was equilibrated with 20 mM Tris (pH 8.0). Absorbance was monitored at 280 nm. Bound material was eluted with a linear gradient of NaCl (see Fig. 2*B*). The flow rate was 0.8 ml/min. Fractions of 0.8 ml were collected and tested for bioactivity.

The flowthrough of the Mono Q column (fractions 3–9) was concentrated 10-fold (Centriprep-30), diluted 4-fold with 0.1% TFA, and injected into a Synchropak $C_4$ reversed-phase column equilibrated with 25% acetonitrile/0.1% TFA. Elution was with a linear gradient of acetonitrile (see Fig. 2*C*). The flow rate was 0.5 ml/min. Fractions of 0.5 ml were collected and tested for inhibitory activity. For final purification, fractions 52–57 were pooled, diluted 4-fold with 0.1% TFA/water, and reapplied to the same column, which had been equilibrated with 25% 2-propanol. Elution was with a linear gradient of 2-propanol having the same shape as the acetonitrile gradient (see Fig. 2*D*). The flow rate was also 0.5 ml/min.

**Microsequencing.** About 90% of the pooled fractions 34 and 35 from the last step of purification was applied into a gas-phase sequenator (model 470A, Applied Biosystems). Edman degradation cycles were carried out and identification of amino acid residues was made by an on-line HPLC column (22).

## RESULTS

Medium conditioned by bovine pituitary folliculo-stellate cells inhibited ABAE cell proliferation (Fig. 1). As little as 2% (vol/vol) conditioned medium induced a detectable inhibition of cell growth. Maximal inhibition (50–60%) was observed with 10–20% conditioned medium (≈1 μg of protein per ml). In contrast, the medium was strongly mitogenic to capillary endothelial cells (Fig. 1), confirming previous studies (12). The possibility that one factor might be responsible for such

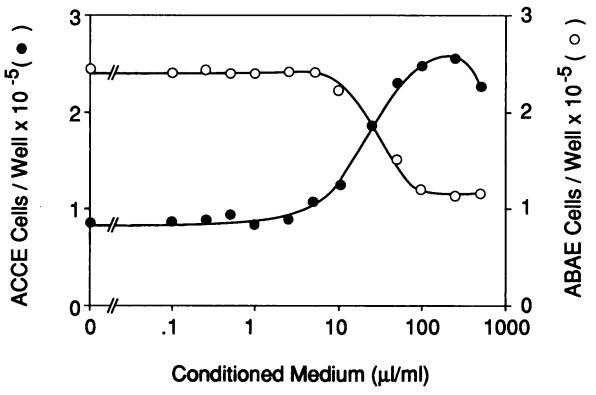


FIG. 1. Effects of serum-free follicular cell conditioned medium on ABAE (○) and ACCE (●) cell growth. Cells were seeded at 10,000 per well in 12-well plates. The indicated dilutions of conditioned medium were added about 6 hr after plating. Cells were counted in a Coulter Counter after 4 days.

opposite effects was ruled out, since purified preparations of the capillary growth-promoting activity (VEGF) were also mitogenic to ABAE cells (12, 13).

For purification of the ABAE cell inhibitory activity, ≈2.5 liters of conditioned medium (≈120 mg of protein) was used. The concentrated conditioned medium was subjected to HPLC gel filtration on a preparative TSK 2000 GSW column, a method suitable for large-scale purification of peptides in nondenaturing conditions. The growth-inhibitory activity was eluted as a single broad peak with an apparent molecular mass between 17 kDa and 45 kDa (Fig. 2*A*). This step provided a 10-fold purification. The most active fractions from two independent TSK chromatographies were applied into a Mono Q column and eluted with a gradient of NaCl. Essentially all of the inhibitory activity was unbound and was recovered in the flowthrough, except for a minor peak that was retained and eluted early in the NaCl gradient (Fig. 2*B*). Since about 90% of the proteins bound to the column, this step provided a further 10-fold purification. The Mono Q flowthrough fractions were further purified by reversed-phase chromatography on a $C_4$ column. The inhibitory activity was eluted in a single peak in the presence of ≈40% acetonitrile (Fig. 2*C*). SDS/PAGE of the most bioactive fractions showed three bands at 12, 35, and 40 kDa. For final purification, fractions 53–57 were injected into a $C_4$ column equilibrated with 25% 2-propanol/0.1% TFA. With a linear gradient of 2-propanol having the same shape as the acetonitrile gradient, the inhibitory activity was eluted in two closely apposed peaks. Most of the bioactivity (≈70%) was associated with a minor peak of absorbance at 210 nm, while a secondary peak was eluted in a region of higher absorbance (Fig. 2*D*). The most bioactive fractions in the early peak of bioactivity were pooled and further characterized. No attempts were made to characterize the secondary peak. SDS/PAGE performed on 10% of the bioactive material showed a single faint band at ≈40 kDa (data not shown). The remainder of the purified material was used for structural analysis. Gas-phase microsequencing showed unambiguously a single $NH_2$-terminal amino acid sequence: XPLPITPVVATX-ATRXP. The identified residues match those in the $NH_2$-terminal sequence of human LIF (23), except for threonine in position 14, which is instead isoleucine in human LIF. The final yield in pure protein, as estimated by sequencing, was ≈6 pmol.

To confirm that LIF is truly responsible for the inhibitory effect on ABAE cells, we compared the effect of rhLIF on ABAE and ACCE cell growth with the effect of TGF$\beta_1$. As shown in Fig. 3, rhLIF induced a dose-dependent inhibition of ABAE cell proliferation. The $ED_{50}$ was 1 ng/ml and a maximal inhibitory effect was reached at ≈5 ng/ml. The maximal inhibition (70 ± 3%) approached that induced by TGF$\beta_1$ (75 ± 4%). However, rhLIF up to 2–5 ng/ml had no

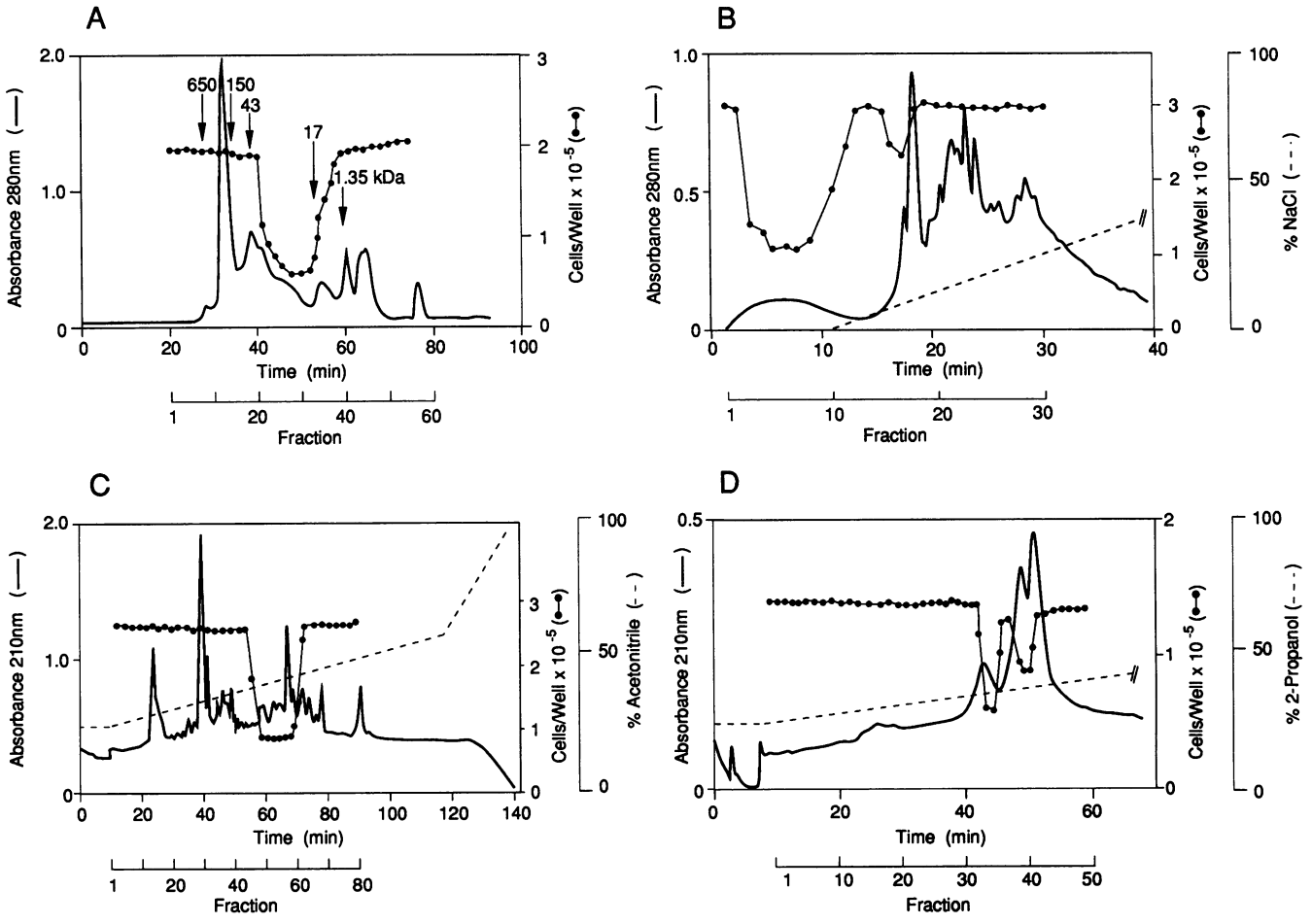


FIG. 2. Purification of the ABAE cell growth-inhibitory activity. (*A*) Concentrated conditioned medium was applied on a TSK 2000 GSW column and fractions were tested for bioactivity at a final dilution of 1:2000. (*B*) The most bioactive fractions from two independent TSK chromatographies were concentrated and equilibrated in 20 mM Tris (pH 8.0) and then injected into a Mono Q column. Fractions were tested at a dilution of 1:2000. (*C*) The flowthrough fractions from the Mono Q column were further purified by reversed-phase chromatography on a $C_4$ column with a linear gradient of acetonitrile as indicated. (*D*) For final purification, the most bioactive fractions from the previous step were applied to a $C_4$ column and eluted with a linear gradient of 2-propanol having the same shape as the acetonitrile gradient. In *C* and *D*, fractions were added to ABAE cells at a final dilution of 1:1000.

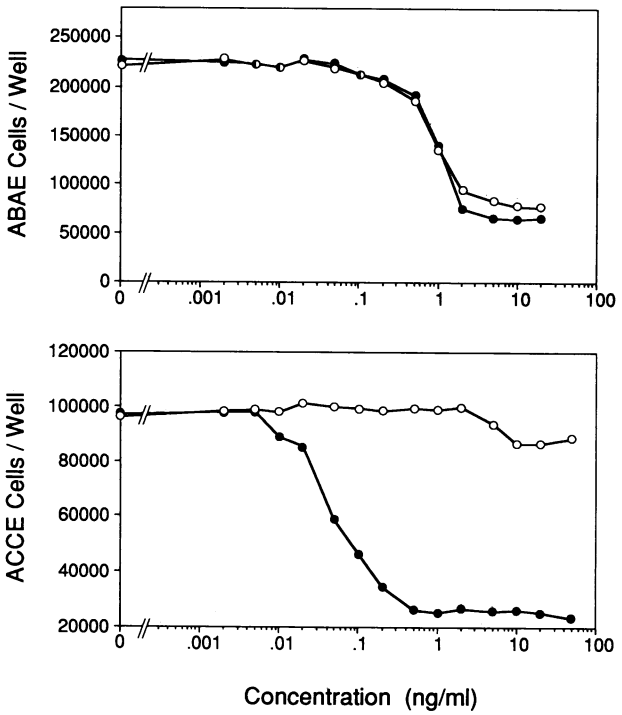


FIG. 3. Comparison of the ability of rhLIF (○) and rhTGF$\beta_1$ (●) to affect the growth of ABAE cells (*Upper*) and ACCE cells (*Lower*). Cells were seeded at 10,000 per well. The indicated amounts of rhLIF or rhTGF$\beta_1$ were added after 6 hr. Cells were counted after 4 days. The variation from the mean did not exceed 10%.

detectable effect on ACCE cell number. At higher concentrations, a small inhibition (10–30%) was observed. In contrast, TGF$\beta_1$ dramatically affected ACCE cell growth (Fig. 3), with maximal inhibition (80 ± 6%) at 0.5 ng/ml.

We also tested rhLIF in the chicken chorioallantoic membrane for its ability to affect angiogenesis *in vivo*. Methylcellulose disks containing vehicle alone (Fig. 4*A*) or 400 ng of rhLIF (Fig. 4*B*) had no appreciable effect in 90% of embryos. In contrast, rhTGF$\beta_1$ (Fig. 4*C*) induced a strong angiogenic response in 100% of the chorioallantoic membranes.

## DISCUSSION

The present results indicate that cultured pituitary follicular cells secrete an inhibitor of ABAE cell growth. Initial experiments suggested that this inhibitor was most likely different from TGF$\beta$ or TNF, since (*i*) the inhibitor did not bind to a Mono Q column at pH 8.0, whereas TNF, an acidic protein (7), strongly binds to such column; (*ii*) it bound to a poly CAT column at pH 6.5 but was eluted early in the NaCl gradient. In contrast, TGF$\beta$, a very basic protein (8), is retained and elutes at high salt concentration; (*iii*) neither TGF$\beta$ nor TNF has been reported to have a selective inhibitory effect on aortic endothelial cells.

The inhibitory activity was purified to apparent homogeneity by a combination of HPLC gel filtration, anion-exchange chromatography, and two reversed-phase HPLC steps. Microsequencing of the most bioactive fractions revealed a single $NH_2$-terminal amino acid sequence, essentially identical to that of LIF or D factor, a glycoprotein originally identified as an inhibitor of the growth of murine myeloid leukemia cells (14) and subsequently found to have diverse and apparently unrelated biological effects, including the ability to promote differentiation of cholinergic neurons (15), induce bone absorption (16), stimulate the synthesis of acute-stage plasma proteins from hepatocytes (24), inhibit embryonic stem-cell growth (17), and lead to a fatal syndrome

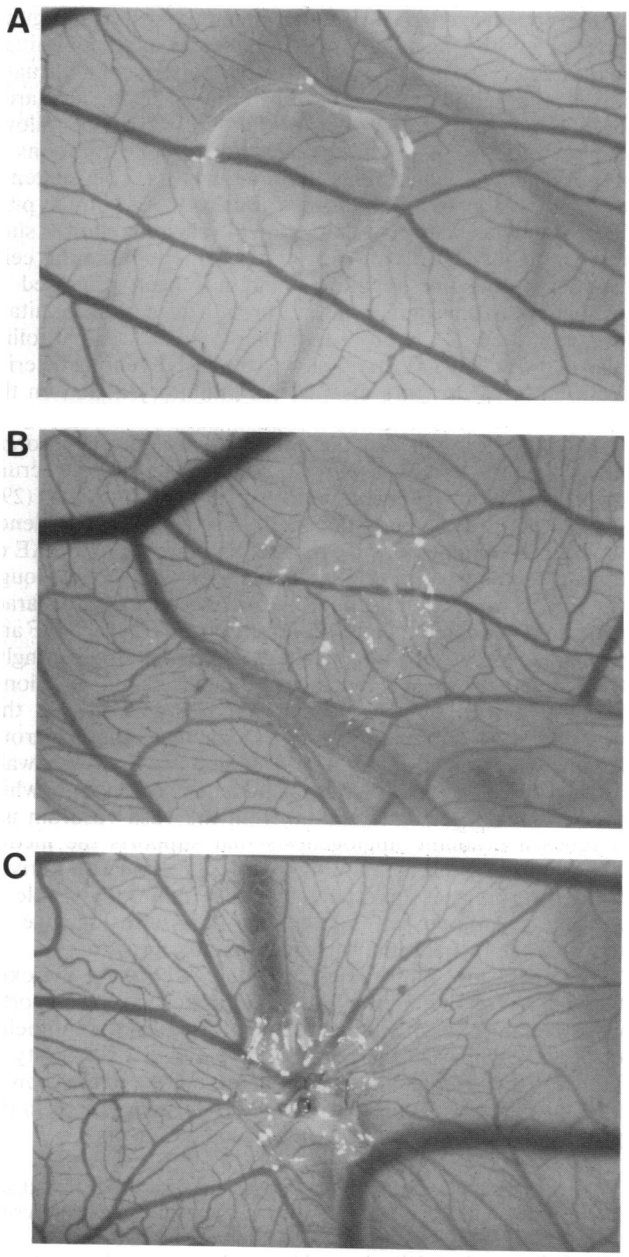


FIG. 4. Effects of rhLIF, rhTGF$\beta_1$, or vehicle on the chick chorioallantoic membrane. rhLIF (400 ng) or rhTGF$\beta_1$ (400 ng) in PBS, or PBS alone, was mixed 1:1 with 1.5% methylcellulose in PBS and allowed to dry in Teflon molds. Methylcellulose disks containing PBS alone (*A*), rhLIF (*B*), or rhTGF$\beta_1$ (*C*) were applied on 10-day chorioallantoic membranes. After 72 hr, the neovascular response was evaluated.

characterized by cachexia at high levels of LIF (25). Since rhLIF had essentially the same effects on endothelial cell growth as the material purified from follicular cell conditioned medium, we conclude that LIF is truly responsible for such effects.

The present study therefore provides evidence that LIF can act as a negative regulator of aortic endothelial cell growth, with little effect on capillary endothelial cells. That rhLIF failed to promote angiogenesis in the chorioallantoic membrane suggests that, unlike TGF$\beta$ or TNF, LIF does not induce the release of direct-acting endothelial cell mitogens from inflammatory cells, even though LIF receptors have been described in cells of the monocyte/macrophage lineage (26). Therefore, the inhibitory effects that we report here may have relevance to the *in vivo* situation.

The presence of LIF in pituitary follicular cells is intriguing in view of the peculiar angioarchitecture of the pituitary gland, where no arteries reach the pars distalis and virtually all of the blood supply to this area is from capillaries originating from the pituitary portal veins (27). This allows the secretory cells to be exposed to high concentrations of hypothalamic releasing and inhibiting factors. The existence of factors capable of inhibiting "arteriogenesis" in the pituitary gland has been postulated (6, 28). The reduction of such factors might lead to an escape of hormone-secreting cells from hypothalamic regulation and has been proposed to contribute to the induction and growth of certain pituitary tumors (28). It is therefore tempting to speculate that follicular cell-derived LIF may play a role in preventing arteries from entering the pars distalis. An inhibitory action on the endothelium alone might be sufficient, since embryological studies have shown that the endothelium is the main organizer of the arterial vessel wall, responsible for the recruitment of other key elements such as smooth muscle cells (29).

Furthermore, recent studies (30) have provided evidence that human monocytes also secrete an inhibitor of ABAE or human umbilical vein endothelial cell growth. Even though the factor was not identified, an initial biochemical characterization indicated that it is different from TGF$\beta$ or TNF and its molecular weight is consistent with LIF. Interestingly, follicular cells share several morphological and functional properties with monocytes/macrophages (31, 32). In this context, it is interesting that in advanced atherosclerotic lesions, where macrophages heavily infiltrate the vessel wall, arterial endothelium is damaged and fails to regenerate, while the microvascular endothelium from the vasa vasorum undergoes a dramatic angiogenesis that supports the medial hyperplasia (33). It is tempting to speculate that LIF derived from macrophages or other cell types may play a role in mediating large-vessel endothelium stasis after damage, in the absence of inhibition of microvascular growth.

In conclusion, our studies provide evidence for the existence of a growth inhibitor with preferential activity on aortic endothelial cells in comparison with capillary endothelial cells and emphasize the remarkable biological versatility of LIF. Further studies, like those involving the expression of LIF mRNA, are required to elucidate the role of LIF in the physiology and pathology of the vascular system.

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