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Cell Biology

Basic fibroblast growth factor induces angiogenesis *in vitro*

(endothelial cells/collagen matrix/plasminogen activator/urokinase)

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ABSTRACT **Fibroblast growth factors (FGFs) are potent mitogens for vascular and capillary endothelial cells *in vitro* and can stimulate the formation of blood capillaries (angiogenesis) *in vivo*. A crucial event in this process is the invasion of the perivascular extracellular matrix by sprouting endothelial cells. Using a recently developed *in vitro* model of angiogenesis, we show here that highly purified basic pituitary FGF can induce capillary endothelial cells to invade a three-dimensional collagen matrix and to organize themselves to form characteristic tubules that resemble blood capillaries. We also show that basic FGF concomitantly stimulates endothelial cells to produce a urokinase-type plasminogen activator, a protease that has been implicated in the neovascular response. The results demonstrate that basic FGF can stimulate processes that are characteristic of angiogenesis *in vivo*, including endothelial cell migration, invasion, and production of plasminogen activator.**

The formation of new blood capillaries (angiogenesis) occurs in a wide range of important biological processes in response to angiogenic factors released by either normal or tumoral cells (1). A crucial step in the sequence of events that leads to the angiogenic response is the invasion of the perivascular extracellular matrix by sprouting endothelial cells (2). The process includes endothelial cell migration, proliferation, and production of enzymes capable of modifying the extracellular matrix. We have recently shown that the invasiveness of capillary endothelial cells can be induced experimentally *in vitro* by well-defined chemical signals (3). Cells grown on three-dimensional collagen gels and treated with the tumor promoter 4β-phorbol 12-myristate 13-acetate (PMA) infiltrate the underlying collagen matrix and organize into vessel-like tubular structures (3). Although phorbol esters are not physiologically occurring substances, they have been shown to mimic, in many instances, the effects of endogenous mediators, such as hormones or growth factors (4–7). It was therefore important to establish whether phenomena similar to those induced by PMA could also be triggered by physiological angiogenic factors.

In this study, we have examined the effect of highly purified basic fibroblast growth factor (FGF) on the invasive and proteolytic properties of cultured capillary endothelial cells. Basic and acidic FGFs are the best-characterized angiogenic substances. They are potent mitogens for several cell types, including vascular and capillary endothelial cells, and are capable of inducing an angiogenic response *in vivo* (8–13). In this report, we demonstrate that basic FGF induces cultured endothelial cells to produce urokinase-type plasminogen activator (u-PA) and stimulates their migration into collagen matrices to form capillary-like tubules. The phenomenon, which mimics some of the events that occur during neovascularization *in vivo*, demonstrates that the angiogenic response to FGF *in vivo* is a direct effect of the growth factor and not secondary to an inflammatory response.

MATERIALS AND METHODS

Isolation of FGF. Basic FGF was purified to homogeneity from bovine pituitaries by successive steps of ammonium sulfate precipitation, ion exchange chromatography, and heparin-Sepharose affinity chromatography (8). Purity of the growth factor was established by reverse-phase high-performance liquid chromatography, amino acid analyses, $NaDodSO_4$/polyacrylamide gel electrophoresis, and amino-terminal sequence analyses (10).

Cell Culture. Three-dimensional gels of reconstituted collagen fibrils were prepared as described (3). Cloned capillary endothelial cells derived from the bovine adrenal cortex (14) were a generous gift of M. B. Furie and S. C. Silverstein (Columbia University, New York). The cells were routinely subcultured in gelatin-coated tissue culture flasks (Falcon, Becton Dickinson Labware, Oxnard, CA) in minimal essential medium, alpha modification (GIBCO) supplemented with 15% heat-inactivated donor calf serum (Flow Laboratories, Ayrshire, Scotland), penicillin (500 units/ml), and streptomycin (100 μg/ml). The endothelial cells were used between passages 15 and 23 and were seeded and grown to confluency in 35-mm collagen-coated dishes. Morphological changes induced by FGF were observed and photographed in phase contrast using a Zeiss ICM 405 inverted photomicroscope.

Processing for Light and Electron Microscopy. The endothelial cell cultures were fixed *in situ* with 2.5% glutaraldehyde/1% tannic acid (Mallinckrodt) in 0.1 M sodium cacodylate buffer (pH 7.4) and further processed as described (3). Semi-thin and thin sections were cut perpendicular to the culture plane with an LKB ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 410 LS electron microscope.

PA Plaque Assay. Low density cultures (1.2×10^4 cells per 35-mm dish) were grown on plastic dishes and were incubated for 24 hr in the presence or absence of FGF (3 ng/ml) or PMA (20 ng/ml). The dishes were then washed three times with phosphate-buffered saline, and the cells were overlaid with a casein/agar/plasminogen mixture as described (15). In control experiments, plasminogen was omitted from the assay mixture. The cultures were incubated at 37°C, and photographs were taken 5 hr later under dark-field illumination.

Zymographic Assay for PAs. Confluent cultures of endothelial cells were prepared in 35-mm plastic dishes and incubated for 24 hr in 2 ml of serum-free medium in the absence or presence of FGF (3 ng/ml) or PMA (20 ng/ml). At the end of the incubation, the culture medium was collected.

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Abbreviations: FGF, fibroblast growth factor; PA, plasminogen activator; t-PA and u-PA, tissue-type and urokinase-type PAs; PMA, 4β-phorbol 12-myristate 13-acetate.

The cells were washed twice with phosphate-buffered saline and harvested by scraping into 0.5 ml of 0.2% Triton X-100 in 0.1 M Tris·HCl. The culture media and cell lysates were centrifuged at 1000 × *g* for 10 min, and the supernatants were collected. Aliquots (15 μl) of both the culture media and the cell lysates were subjected to $NaDodSO_4$/PAGE and zymography as described (16). Photographs were taken under dark-field illumination after 4 hr of incubation at 37°C. Immunoadsorptions with anti-urokinase-type and anti-tissue-type PAs were performed as described (16).

RESULTS

Cells grown to confluence on the surface of three-dimensional collagen gels formed a monolayer of closely apposed cells (Fig. 1*a*). Within 24 hr after the addition of FGF (3 ng/ml), numerous endothelial cells could be distinguished by their irregular or dendritic morphology, and their plane of focus, which was slightly beneath that of the original monolayer (Fig. 1*b*). After 2–3 days of incubation with FGF, these cells organized into short branching cords that formed a discontinuous network under the surface monolayer. Longer incubations with FGF for up to 5 days did not result in further changes in the organization of the cultures. Similar effects were obtained with higher doses of FGF (up to 30 ng/ml). In contrast, concentrations of FGF <3 ng/ml produced a weaker effect. Only a few scattered endothelial cell cords could be observed in cultures treated with FGF at 300 pg/ml even though this concentration of FGF is reported to maximally stimulate cell growth (8, 10). A progressively weaker response to effective concentrations of FGF was also observed in late-passage cultures, and morphological changes were barely detectable beyond the 22nd or 23rd passage.

Semi-thin sections cut perpendicular to the culture plane showed that the endothelial cell cords seen in phase-contrast microscopy were tubular structures containing small lumina (Fig. 2 *a* and *b*). The tubules were located inside the collagen matrix in close proximity to the surface monolayer, but some tubules were occasionally seen to penetrate deeper into the matrix. In thin sections, the tubules consisted usually of either a single endothelial cell folded on itself (Fig. 2*c*) or two endothelial cells joined by intercellular junctions (not shown). Intracellular lumina were also occasionally observed.

Low-density cultures of the endothelial cells were tested for plasminogen-dependent proteolytic activity by a substrate overlay procedure that allows detection of catalytic activity around individual cells. Whereas untreated cells did not express lytic activity, numerous zones of substrate lysis were seen to develop progressively in FGF-treated or PMA-treated cultures (Fig. 3). Phase-contrast microscopy confirmed that the lytic areas were localized around individual cells or small groups of cells. The proportion of catalytically active individual cells was determined after 5 hr of incubation by scoring 100 cells in each condition in three separate experiments. Large lytic zones surrounded 92–100% of the cells in the PMA-treated cultures, while small lytic zones were observed around 39–73% of the FGF-treated cells and 0–6% of the cells in control cultures. Because lysis of the substrate did not occur when plasminogen was omitted from the assay medium, the lytic areas are directly correlated with the production of PAs by the cells.

The PAs present in the endothelial cell cultures were characterized by zymographic analysis and by immunoadsorption with specific anti-human urokinase-type PA (u-PA) and anti-human tissue-type (t-PA) IgG. In samples obtained from PMA-treated cultures, three enzymes, with apparent M_rs of 48,000, 72,000, and 105,000, were resolved (Fig. 4, lanes c). The M_r 48,000 enzyme was determined to be u-PA, whereas the M_r 72,000 and 105,000 activities were

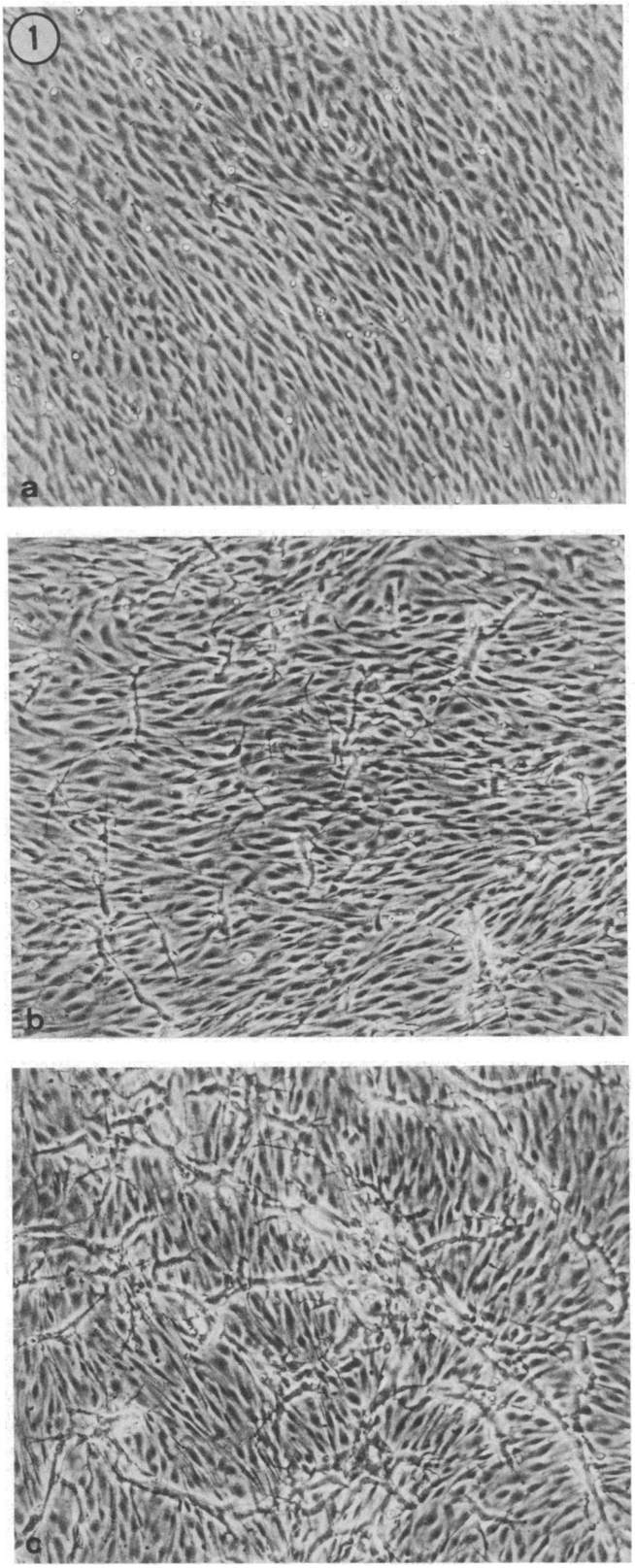

FIG. 1. Effect of FGF on capillary endothelial cells grown on collagen gels (phase-contrast microscopy). (*a*) Control cells form a monolayer of closely apposed cells. (*b*) FGF-treated cells after 24 hr of treatment with FGF (3 ng/ml). Numerous endothelial cells differ in shape and orientation from those forming the confluent monolayer. Fine focusing showed that these cells were located immediately beneath the monolayer. (*c*) FGF-treated cells after 72 hr of treatment. An incomplete network of branching cell cords has formed underneath the confluent monolayer. (×85.)

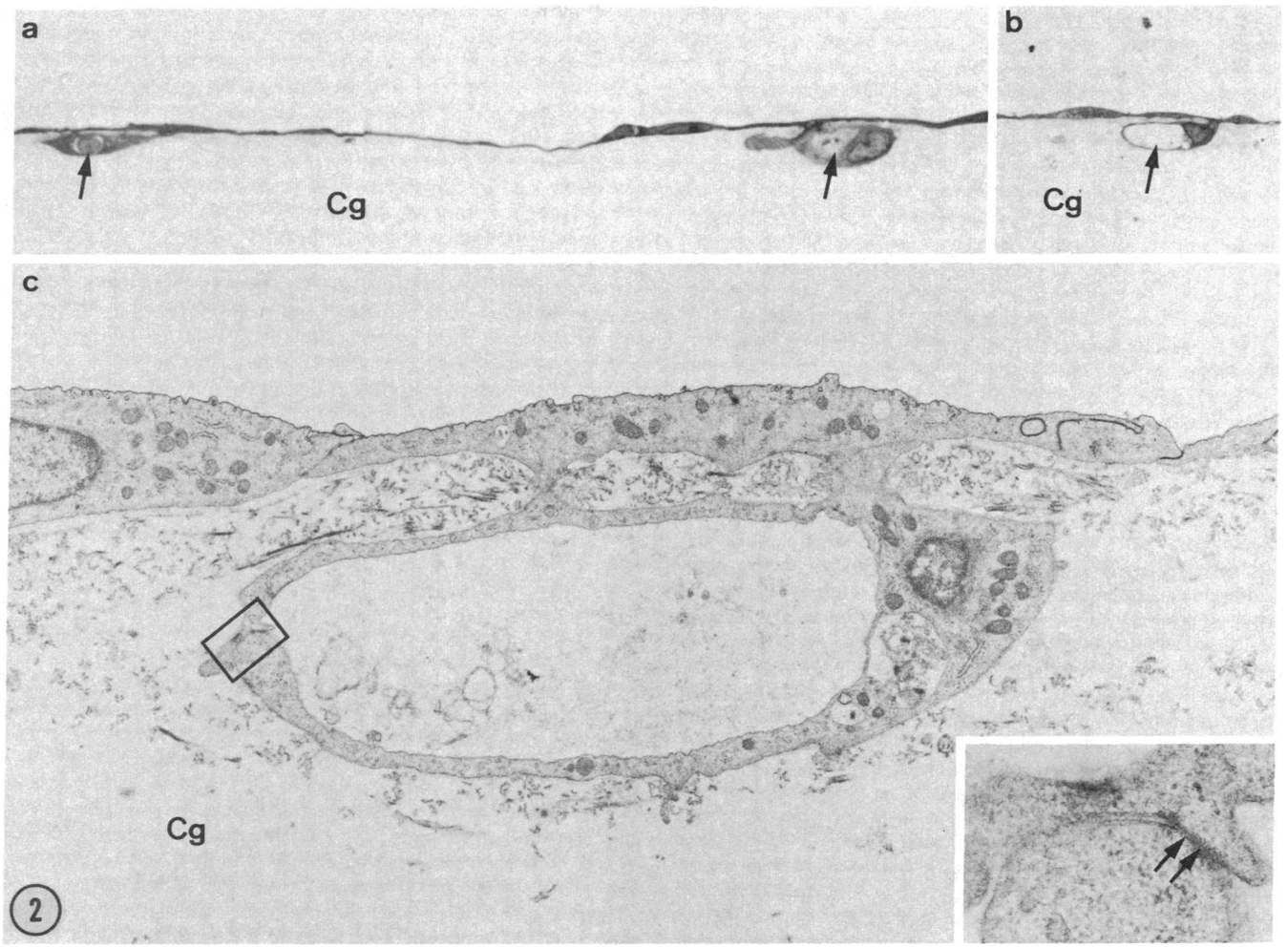

FIG. 2. Semi-thin (*a* and *b*) and thin (*c*) sections perpendicular to the culture plane of FGF-treated capillary endothelial cells. Tubular structures enclosing narrow lumina (arrows) have formed inside the collagen gel (Cg) in close proximity to the surface monolayer. The tubule in *c* consists of a single endothelial cell folded on itself. (*Inset*) Higher magnification of the area outlined in black, showing the junction between the cytoplasmic extensions of the endothelial cell. (*a* and *b*, ×750; *c*, ×6700; *Inset* ×37,000.)

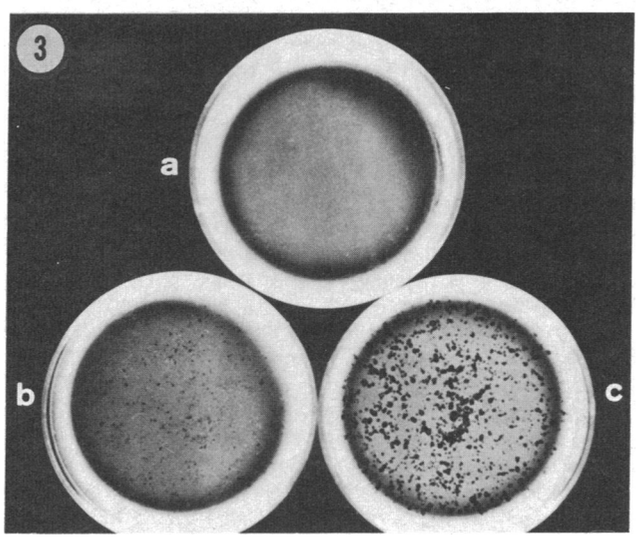

FIG. 3. PA plaque assay. Control (*a*), FGF-treated (*b*), and PMA-treated (*c*) cultures were overlaid with a mixture of casein, agar, and plasminogen. Zones of lysis of the substrate appear as dark plaques on a clear background and indicate the production of enzyme by the cultured cells.

related to t-PA on the basis of their respective absence in samples that had been specifically immunodepleted prior to zymography. Very little activity was detected in the culture medium or in cell lysates obtained from untreated cultures (lanes a), although longer incubations of the zymogram revealed the presence of M_r 48,000 u-PA in these samples. Samples obtained from FGF-treated cultures (lanes b) contained increased levels of u-PA as compared to untreated cultures.

Analysis of the serially diluted samples provided a semi-quantitative evaluation of the amount of u-PA in the different cultures. As compared to control cultures, the enzyme activity in the cell lysates and the culture medium was increased 5- to 10-fold in FGF-treated cultures and more than 30-fold in PMA-treated cultures.

DISCUSSION

Although angiogenesis has been mostly studied *in vivo*, as, for example, in the rabbit cornea (17) or the chorioallantoic membrane of the chicken embryo (18), the development of methods for the isolation and culture of capillary endothelial cells (19) has provided an opportunity to study *in vitro* the properties of endothelial cells that are relevant to neovascularization. Recent studies have shown that partially purified

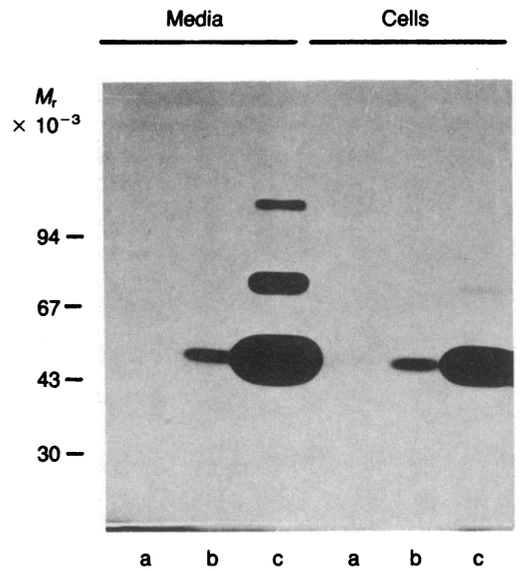

FIG. 4. Zymographic assay for PAs. Samples of the conditioned culture media (Media) and of the cell lysates (Cells) were processed from control (lanes a), FGF-treated (lanes b), and PMA-treated (lanes c) cultures and were subjected to $NaDodSO_4$/PAGE and zymography as described in *Materials and Methods*.

preparations of angiogenic substances can stimulate three distinct processes at the cellular level: increased rate of multiplication (20–24), migration and chemotaxis (24–29), and production of various proteases including PA and collagenase (24, 30). Unfortunately, the culture of cells on a plastic substratum of tissue culture dishes still has a major limitation—i.e., the loss of three-dimensional cell–matrix interactions. To obviate this shortcoming and to approximate as closely as possible the *in vivo* situation, we applied the strategy of growing capillary endothelial cells on the surface of reconstituted collagen fibrils (3). By using a three-dimensional matrix, we have shown that basic FGF, an endothelial cell mitogen with potent angiogenic activity *in vivo* (8–12), induces cultured capillary endothelial cells to invade the underlying collagen matrix and to organize themselves as distinct tubules resembling blood capillaries.

The effect of FGF was not as pronounced as that observed with phorbol esters (3). The endothelial cell tubules formed in response to FGF were shorter and less numerous, contained smaller lumina, and did not penetrate as deeply into the collagen matrix as those induced by PMA. These differences in the biological effects of PMA and FGF may be the result of their activation of a key enzyme in signal transduction, protein kinase C (7, 31, 32). Endogenous mediators, like FGF, act through a transient release of diacylglycerol (7), while PMA produces a persistent activation of protein kinase C (7). Because phorbol esters represent a more efficient and/or persistent stimulus for endothelial cells than physiological angiogenesis factors, they should induce a quantitatively greater but qualitatively identical effect on the endothelial cell cultures. Indeed, in spite of their different magnitude, the phenomena triggered by FGF and PMA appear qualitatively similar and they both mimic the crucial events that take place during angiogenesis *in vivo*—i.e., the invasion of collagen matrices and the formation of capillary sprouts.

The synthesis and secretion of proteases, including PAs and collagenase, by endothelial cells is thought to be related to the invasive properties of these cells during angiogenesis. Both purified preparations of angiogenic activity and PMA have been shown to increase production of these enzymes (24, 30, 33). In a previous study by Gross *et al.* (30), crude preparations of basic FGF had been unable to increase the production of PA or collagenase by capillary endothelial cells. It was thus important to reevaluate this point using our highly purified and "angiogenically active" preparation of basic FGF. The results presented here demonstrate that the production of u-PA is markedly stimulated in FGF-treated cultures. Differences in the preparations of FGF and/or in the procedures used to assay for PAs may account for the discrepancy between our results and those reported previously (30). Interestingly, and perhaps for the reasons cited earlier, the stimulation of u-PA production by FGF was significantly less than that obtained with PMA. The relative effectiveness of these two agents in inducing tubule formation and protease production is thus similar. Although there is at present no evidence for a role of PAs in the invasion of the collagen matrix in our culture system (3), the correlation between the angiogenic behavior of endothelial cells in response to FGF, the known angiogenic activity of FGF *in vivo* (8–12), and the increase in u-PA production in the presence of FGF, supports the proposed role for this enzyme (34) and FGF in the neovascular response *in vivo*.

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