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Angiogenic Role of Endogenous Basic Fibroblast Growth Factor Released by Rat Aorta after Injury

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The autocrine role of basic fibroblast growth factor (bFGF) in angiogenesis was studied in the rat aortic ring-collagen gel model using serum-free culture conditions. Immunohistochemical staining of the rat aorta showed bFGF in the cytoplasm of endothelial and smooth muscle cells. Aortic rings mechanically injured during the dissection procedure released bFGF, which was demonstrated in the conditioned medium by slot and Western blot analysis. bFGF-containing aorta-conditioned medium and purified bFGF increased both the number and length of microvessels sprouting from the explants. This effect was particularly evident during the second week of culture, when the release of endogenous bFGF was minimal. Neutralizing anti-bFGF antibodies induced a 40% reduction of angiogenesis. Regression of microvessels, which regularly occurred toward the end of the second week, was prevented by purified bFGF. These data support the idea that bFGF released by vascular cells plays an important role in the autoregulation of angiogenesis after injury. (Am J Pathol 1993, 143:181–190)

Angiogenesis, the process by which endothelial cells form new microvessels, plays a fundamental role in embryonal development, wound healing, and pathological conditions such as tumor growth, diabetic retinopathy, and complicated atherosclerosis. New microvessels form as a result of complex interactions between endothelial cells, soluble cytokines, extracellular matrix molecules, and matrix-degrading proteases.[1–5]

Advances during the past decade have resulted in the isolation, purification, sequencing, and cloning of several angiogenic cytokines.[1,6] Among these, basic fibroblast growth factor (bFGF) has been shown to play a major role in experimental angiogenesis; bFGF promotes angiogenesis *in vivo*[7] and *in vitro*[8] by stimulating the migration,[9] proliferation,[10] and proteolytic activity[4,5] of endothelial cells. However, whereas several effects of exogenous bFGF on endothelial cells have been described, it has remained unclear how endogenous bFGF produced by vascular endothelial and smooth muscle cells regulates angiogenesis. In fact, bFGF, despite its potent angiogenic activity, can be found in the wall of quiescent blood vessels.[11] In addition, the mechanisms of secretion of bFGF are poorly understood, because this cytokine lacks the signal peptide sequence necessary for the entrance of proteins into the rough endoplasmic reticulum and subsequent secretion through conventional exocytotic pathways.[12] It has recently been shown that endothelial injury, either transient or permanent, induces release of bFGF through cell membrane disruptions.[13–15] Thus, bFGF released after injury may act as a wound hormone, stimulating endothelial cells through autocrine and paracrine mechanisms.[15,16] This idea has been studied *in vitro* with monolayer cultures of endothelial cells[9] but has not been tested in models of angiogenesis. One reason for this gap has been the lack of bioassays that could support an angiogenic response in the absence of exogenous growth factors.

We describe here the role of endogenous bFGF in the rat aorta model of angiogenesis. This model exploits the ability of the rat aorta to generate microvascular networks *in vitro*.[17] Because angiogenesis occurs in the absence of serum or other growth supplements,[18] we have hypothesized that injured aortic explants provide the chemical signals required for the formation of microvessels. Data presented

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here demonstrate that bFGF is present in rat aortic endothelial and smooth muscle cells and is released by wounded aortic explants during angiogenesis *in vitro*. Our findings support the hypothesis that endogenous bFGF plays a role in the autocrine regulation of angiogenesis after injury.

Materials and Methods

Living Cultures

Thoracic aortas excised from 2- to 3-month-old male Fischer 344 rats were rinsed in serum-free Molecular and Cellular Developmental Biology (MCDB) 131 growth medium (Clonetics, San Diego, CA) containing 50 μg/ml gentamicin, cleaned of periadventitial fibroadipose tissue, and cross-sectioned at ~1-mm intervals. The resulting rings were extensively rinsed in serum-free MCDB 131 medium and embedded in collagen gels as previously described.[18] Collagen gel cultures were transferred to 35-mm dishes and grown at 35.5 C in serum-free MCDB 131, which was changed three times a week. In a separate set of experiments, the cultures were fed with MCDB 131 containing 50% rat aorta-conditioned medium. Conditioned medium was prepared from a thoracic aorta, which was minced in 5 ml MCDB 131 and incubated for 24 hours at 35.5 C. In an additional group of experiments, purified bovine bFGF (R&D Systems, Minneapolis, MN) was added to the collagen gel or to the growth medium. Rat aorta cultures were also treated with neutralizing rabbit anti-bFGF antibody (R&D Systems) or equal amounts of nonimmune rabbit IgG (Sigma Chemical Co., St. Louis, MO).

Cultures were also treated with 50 μg/ml protamine sulfate, a substance believed to block bFGF activity by binding to the bFGF cell receptor.[19] To evaluate the reversibility of the protamine sulfate effect, collagen gel cultures treated for 1 week with protamine sulfate (Sigma) were rinsed several times with fresh medium and further maintained in the absence of protamine sulfate. Protamine sulfate was also added to 7-day-old cultures to evaluate its effect on developing microvessels.

To investigate whether microvessels could grow under serum-free conditions in the absence of an exogenous extracellular matrix, aortic rings were cultured in 35-mm dishes without a collagen gel. The explants were laid vertically on the bottom of 35-mm dishes and kept in a small drop of MCDB 131 for 30 minutes at 35.5 C to ensure adhesion to the plastic before addition of medium.

Quantitation of Angiogenesis

Angiogenesis in collagen gel cultures of rat aorta was quantitated by counting the number of newly formed microvessels according to published criteria.[18] The length of the microvessels was measured by digitizing morphometry with a Bioquant IV image analysis system, including a personal computer, a digitizing pad, a Leitz Laborlux microscope, a Dage MTI videocamera, and a high resolution Auditron monitor.

Statistical Analysis

Data were analyzed with a personal computer using SPSS/PC+ statistical software. The Student's *t*-test was used to determine if there were significant differences between experimental groups. Probability values < 0.05 were required for statistical significance.

Immunohistochemical Studies

For the immunolocalization of bFGF, aortic rings were snap-frozen in melting isopentane and sectioned with a Tissue Tek II cryostat. Frozen sections were collected on 1% gelatin-coated slides and immunostained with monoclonal mouse (1:10; UBI, Lake Placid, NY) or polyclonal rabbit (1:50; Collaborative Research, Bedford, MA) anti-bFGF antibodies. In negative controls, the primary antibody was substituted with nonimmune rabbit serum, bovine serum albumin (BSA), or an irrelevant antibody. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide for 10 minutes, followed by rinsing in phosphate-buffered saline (PBS). The sections were then reacted with primary antibody diluted in PBS containing 0.1% BSA (PBS-BSA) for 2 hours at room temperature. After washing in PBS-BSA, the sections were incubated for 30 minutes in biotinylated secondary antibody (1:250; Vector Laboratories, Burlingame, CA), followed by rinsing in PBS-BSA and 45 minutes incubation in avidin-biotin peroxidase complex (Vector). Peroxidase was localized by incubation for 5 minutes with 50 mg % diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in PBS. Finally, sections were rinsed with distilled H_2O and mounted in Permount.

Outgrowths from aortic explants cultured without collagen were fixed for 10 minutes in 95% alcohol and immunostained with a monoclonal mouse anti-human α-smooth muscle actin antibody (1:2000; Sigma) or with a polyclonal rabbit anti-human factor

VIII-related antigen (FVIII-RAg, 1:200; Dako Carpinteria, CA). The avidin-biotin peroxidase complex method as described above was used as detection system of the immunoperoxidase reaction. The same procedure was used for the immunostaining of formalin-fixed collagen gel cultures.

Ultrastructural Study of Cell Injury in Aortic Explants

Cell damage induced during cutting and manipulation of rat aortic explants was evaluated by testing the permeability of cell membranes to horseradish peroxidase (HRP). After cleaning the adventitial tissue, the aortic tubes were cut in small segments, rinsed several times with a syringe containing serum-free MCDB 131, immersed in MCDB 131 containing 30 mg/ml HRP, and cross-sectioned to obtain rings. The aortic rings were incubated in the HRP solution for 30 minutes at 35.5 C. They were then rinsed in fresh MCDB 131, kept for 30 minutes in HRP-free medium, rinsed in cold PBS, and fixed in 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer, pH 7.4, at 0 C. The peroxidase incorporated by the explants was then developed for 10 minutes in PBS containing 50 mg % diaminobenzidine tetrahydrochloride, 0.03% H_2O_2, and 0.03% cobalt chloride.[20] The tissue was postfixed in 1% OsO_4 in s-collidine buffer, processed for EPON embedding, thin-sectioned, and examined with a Zeiss 10A transmission electron microscope.

Slot Blot and Western Blot Analysis

For the biochemical study of bFGF released after injury, thoracic aortas cleaned of periadventitial fibroadipose tissue were perfused extensively with serum-free MCDB 131 growth medium. They were then cross-sectioned directly into 18-mm cultures wells (NUNC, Naperville, IL) and kept at 35.5 C. Each well contained 10 aortic rings in 0.5 ml serum-free MCDB 131. Conditioned medium collected daily was blotted onto nitrocellulose paper with a Bio-Rad slot blotting apparatus. During the first day, conditioned medium was also collected 5, 15, and 120 minutes after the initial cut. The nitrocellulose paper was then air-dried, rehydrated in 20 mmol/L Tris, 500 mmol/L NaCl buffer pH 7.8 (TBS), blocked for two hours with 3% dry milk (Carnation) in TBS, and incubated for 2 hours in affinity-purified rabbit anti-bFGF antibody (Collaborative Research, Bedford, MA) diluted 1/1000 in blocking buffer (TTBS: 1% milk and 0.05% TWEEN 20 in TBS). It was then washed with TTBS and reacted with goat anti-rabbit immunoglobulin antibody diluted 1:1000 in TTBS. After washing in TTBS, the paper was incubated for 1 hour in alkaline phosphatase-conjugated avidin diluted 1:1000 in blocking buffer, washed in TTBS, and developed with the bromochloroindolylphosphate-nitroblue tetrazolium substrate.

For Western blot analysis, the rat thoracic aorta was finely minced in serum-free MCDB 131 medium containing 1 mmol/L phenyl-methylsulfonyl fluoride, 5 mmol/L ethylenediaminetetraacetic acid, and 150 µg/ml heparin. After 2 hours incubation at 35.5 C, the conditioned medium was centrifuged at 15,000 rpm in a Beckman Microfuge E. The supernatant was lyophilized, reconstituted in 50 µl distilled water, diluted 1:1 in sodium dodecyl sulfate sample buffer, boiled for 5 minutes and applied to a 4% to 20% gradient sodium dodecyl sulfate polyacrylamide gel. The gel was then blotted onto nitrocellulose paper and immunostained for bFGF as described above for the slot blot.

Results

Angiogenic Response of Wounded Rat Aortic Explants in Collagen Gel Cultures

Aortic explants cultured in collagen gel under serum-free conditions generated microvascular outgrowths. Microvessels originated for the most part from the two wounded edges of the aorta with only occasional microvessels growing from the adventitial margins (Figure 1). Newly formed microvessels elongated, branched, anastomosed, and, during the end of the second week, underwent a process of remodeling that led to the regression of the smaller branches. The microvessels were positive for FVIII-RAg. Nonendothelial cells positive for α-smooth muscle actin were observed in periendothelial location and in interstitial spaces between microvessels.

Endothelial Growth from Rat Aorta Explants in the Absence of Collagen Gel

Aortic rings cultured on a plastic surface in the absence of collagen gel generated planar outgrowths of tightly cohesive cells surrounded by spindly or stellate single cells. The cohesive outgrowth organized at its periphery in cordlike structures, which resembled microvessels. Immunoperoxidase stain showed that this component of the outgrowth was endothelial on the basis of a positive reaction for

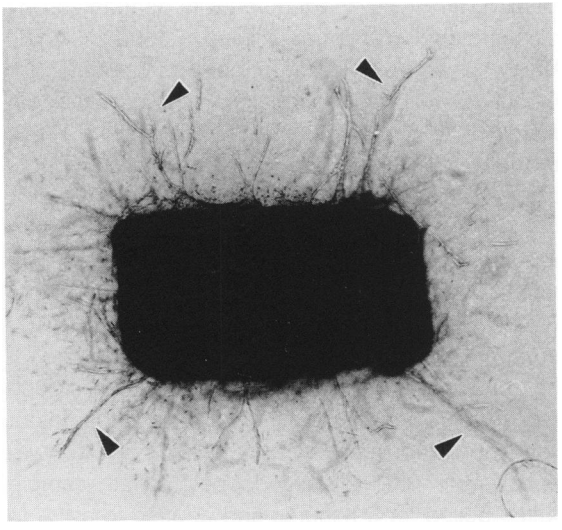

Figure 1. *Serum-free collagen gel culture of rat aorta. The aortic ring, viewed from the adventitial side, appears as a rectangular silhouette. Newly formed microvessels (arrowheads) have developed primarily from the edges of resection (top and bottom sides of the aortic silouette, ×25).*

FVIII-RAg (Figure 2) and negative α-smooth muscle actin. Conversely, the surrounding single cells were negative for FVIII-RAg and positive for α-smooth muscle actin. Cultures were discontinued at day 8 when endothelial cells showed signs of degeneration and were overgrown by smooth muscle cells.

Immunolocalization of bFGF in Rat Aorta

The rat aorta was immunostained for bFGF using monoclonal and polyclonal antibodies. Both antibodies demonstrated a similar pattern of staining, although a stronger reaction was seen with the polyclonal antibody. Strong expression of bFGF was found in the cytoplasm of intimal endothelial and medial smooth muscle cells (Figure 3A). Smooth muscle cells of the inner layers of the tunica media were more consistently and strongly stained than those lying in the deeper portions, which contained unreactive cells. The possibility that bFGF was present also in the extracellular matrix could not be ruled out due to the thinness of the basement membranes, which could not be resolved by light microscopy. However, elastic laminae, adventitial collagen, and fibroblasts were negative. Negative controls had a very low nonspecific background staining (Figure 3B).

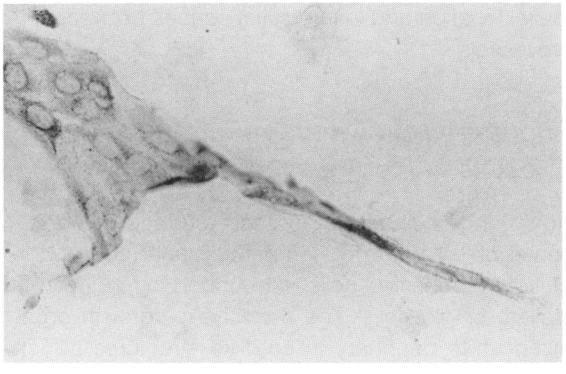

Figure 2. *Endothelial outgrowth of 5-day-old rat aortic culture maintained without serum and collagen and immunostained for FVIII-RAg. Note: a sheet of cohesive cells positive for FVIII-RAg has formed a cordlike structure (×210).*

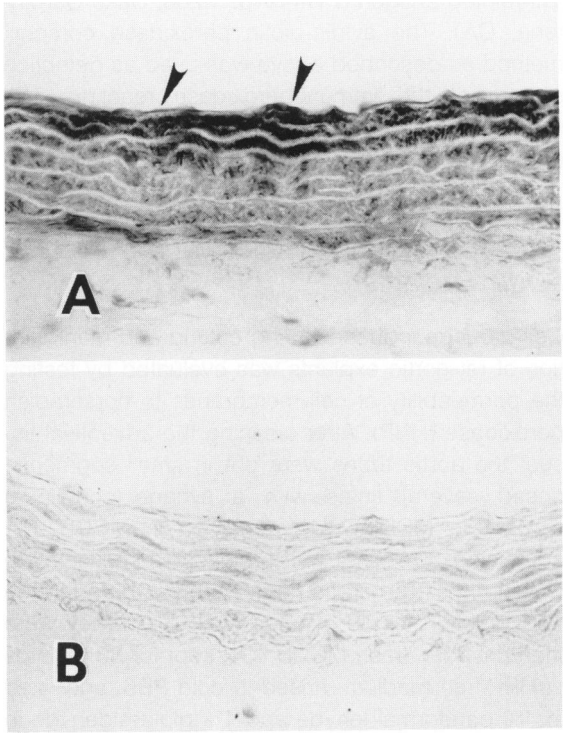

Figure 3. **A:** *Rat aorta immunostained for bFGF. Endothelial cells (arrowheads) and smooth muscle cells of the inner layers of the tunica media are strongly stained. Patches of unreactive smooth muscle cells are present in the central and outer portions of the tunica media. Adventitial fibroblasts are negative.* **B:** *A negative control section shows low background staining (×210).*

Ultrastructural Study of Cell Damage in Aortic Explants

HRP is a 40-kd protein that is normally prevented from entering the cell cytoplasm of viable cells and is taken up by the endocytotic pathway.[21] However, HRP diffuses into the cytosolic compartment through membrane permeabilization following cell injury.[14] Resealing of the plasma membrane traps the protein in the cell cytoplasm. Thus, peroxidase, visualized as an electron dense stain by the diaminobenzidine tetrahydrochloride technique, acts as a

cell damage tracer.[14,15,22] Electron microscopy showed HRP-positive endothelial cells mainly at the cut edges of the aortic explants. These cells characteristically displayed long and thin cytoplasmic processes, suggesting activation. The HRP tracer permeated the cytosol, imparting it with a dark staining but not the undamaged organelles that retained a normal electron density (Figure 4A). Some permanently damaged endothelial cells were unstained because the HRP leached out of their irreversibly permeabilized cell membrane. Uninjured endothelial cells showed pinocytotic uptake of the HRP, whereas their cytoplasmic matrix was unstained (Figure 4B). Subendothelial myointimal cells (Figure 4B) and scattered fibroblasts of the adventitia were heavily permeated by HRP.

Release of bFGF by Wounded Rat Aorta

Western blot analysis demonstrated the presence of bFGF in medium conditioned by rings of rat aorta (Figure 5). Immunostaining of aorta-conditioned medium blotted onto nitrocellulose paper showed that the release of bFGF was maximum at day 1 and gradually decreased over time (Figure 6). Strong staining for bFGF was seen as early as 5 minutes after injury (data not shown). Reinjury on day 9, caused by cross-sectioning the explants a second time, induced a net increase of bFGF levels in the following 24 hours (Figure 6). The pattern of bFGF release into the medium correlated with the angiogenic response of the aortic explants in collagen gel culture so that maximum release of bFGF was followed by vascular proliferation, whereas the low release of bFGF during the second week coincided with arrest of growth and regression of microvessels (Figures 6 and 7).

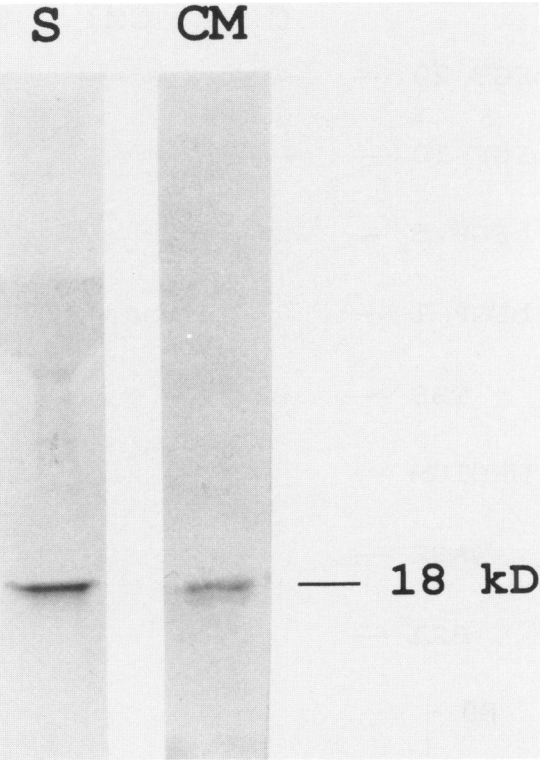

Figure 5. *Western blot of rat aorta-conditioned medium (CM) and purified bFGF standard (S) immunostained for bFGF.*

Effect of Rat Aorta-Conditioned Medium and Purified bFGF on Rat Aortic Angiogenesis

Addition of medium conditioned by aortic explants to collagen gel cultures of rat aorta significantly increased the number of microvessels (Figure 8). In addition, there was a tendency of the most forward segments of the sprouts to detach from the parent microvessels and migrate as isolated break-away capillaries at the advancing front of the outgrowth

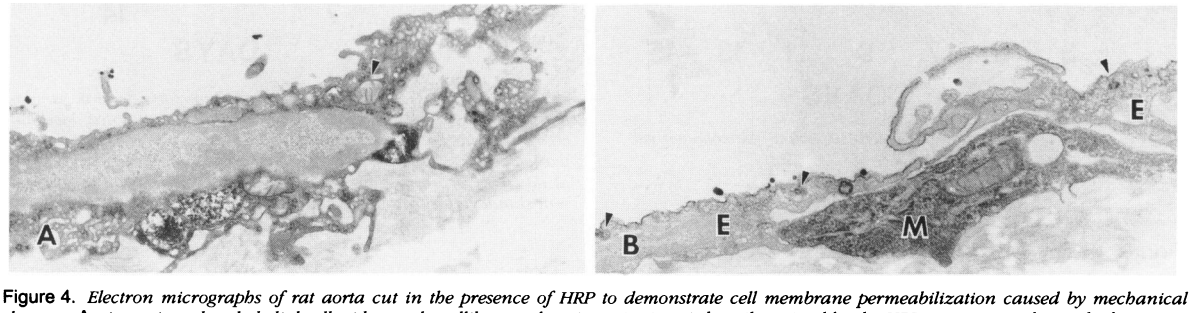

Figure 4. *Electron micrographs of rat aorta cut in the presence of HRP to demonstrate cell membrane permeabilization caused by mechanical damage.* **A:** *An activated endothelial cell with pseudopodlike cytoplasmic projections is heavily stained by the HRP reaction product, which appears electron dense. Note: the dark staining is restricted to the cytosol, whereas the mitochondrial matrix (arrowhead) and the nucleus have a normal density (×22,000).* **B:** *Undamaged endothelial cells (E) show unstained cytoplasm and HRP-decorated pinocytotic vesicles (arrowheads), whereas a damaged subendothelial myointimal cell (M) is heavily stained (×27,500). All magnifications are original.*

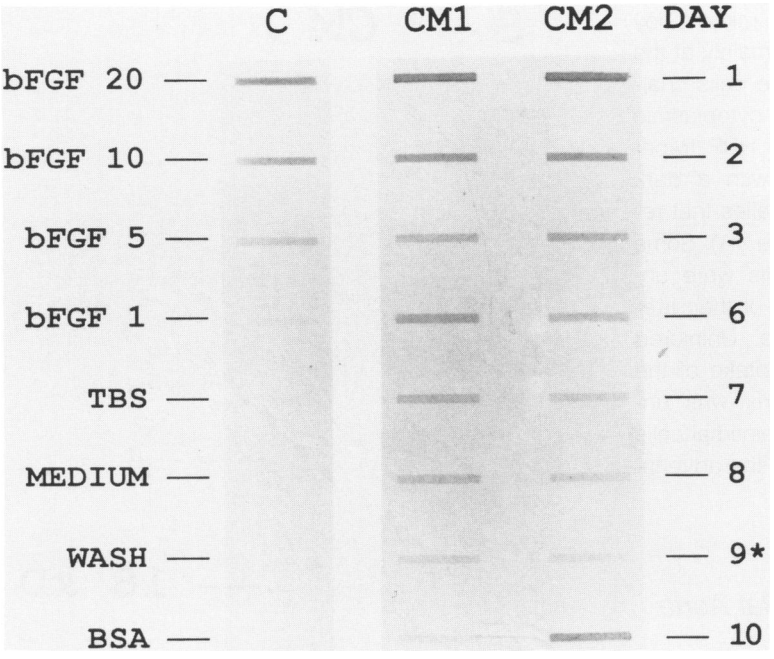

Figure 6. *bFGF-immunostained slot blot of rat aorta-conditioned medium collected at daily intervals after cross-sectioning the aorta. The medium was collected from two sets of aortic rings: CM1 and CM2. Set labeled CM2 was re-injured at day 9 (*) by cross-sectioning the aortic rings a second time. bFGF release by injured aortic explants was maximum at day 1 and decreased over time. Reinjury (CM2) induced a new release of bFGF, which was demonstrated at day 10.* Lane C *shows bFGF standards at various concentrations (ng/ml) and negative controls including buffer solution (TBS), MCDB 131 growth medium, last wash of the aorta before cutting (wash), and BSA.*

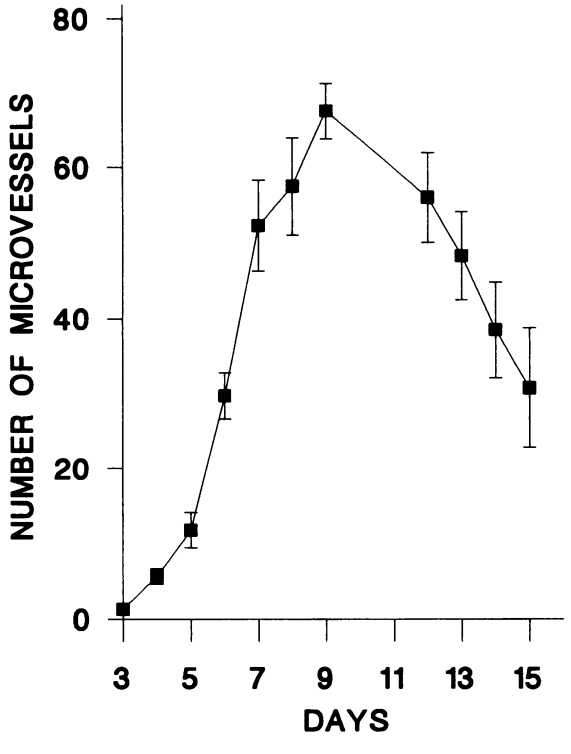

Figure 7. *Curve of microvascular growth in serum-free collagen gel culture of rat aorta.*

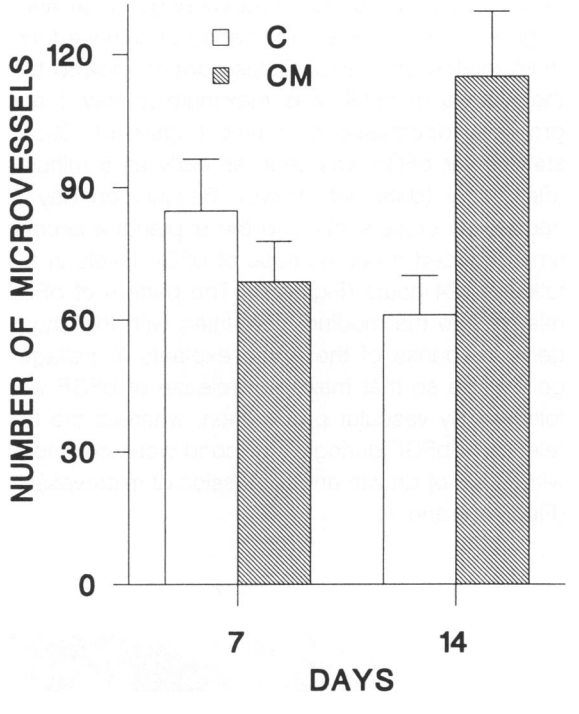

Figure 8. *Effect of aorta-conditioned medium on rat aortic angiogenesis in collagen gel culture. Addition of conditioned medium (CM) stimulated the formation of microvessels. Note that the effect was evident at day 14 ($P < 0.007$), whereas there was no difference at day 7. (C: control cultures)* $n = 8$ *(C), 7 (CM). Error bars represent standard error of the mean (SEM).*

(Figure 9). This effect was particularly evident in the second week of culture (Figure 10). Rat aorta-conditioned medium induced also an 80% increase in microvascular length. Microvessels treated with rat aorta-conditioned medium were thinner than mature microvessels of control cultures and resembled immature capillaries even in later stages of angiogenesis.

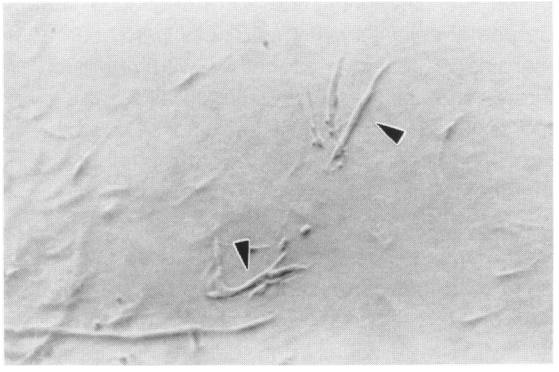

Figure 9. *Break-away capillaries (arrowheads) formed in collagen gel culture of rat aorta stimulated with exogenous bFGF. Capillaries with similar morphology were observed in cultures treated with aorta-conditioned medium.*

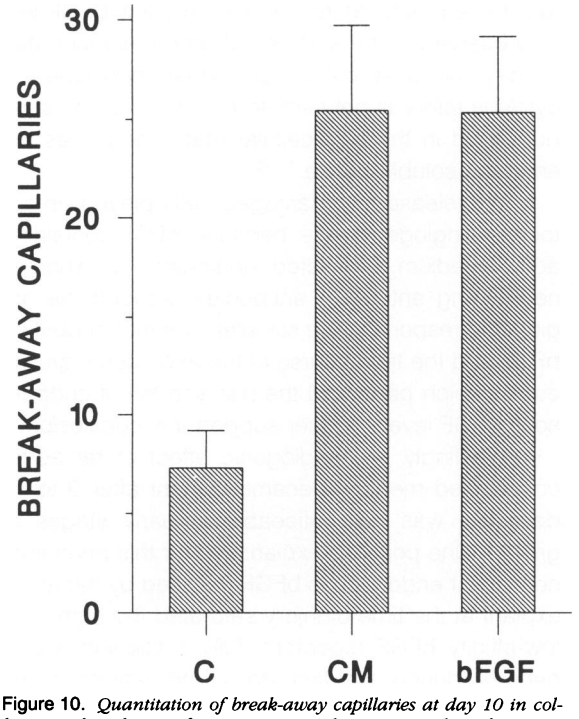

Figure 10. *Quantitation of break-away capillaries at day 10 in collagen gel culture of rat aorta. Cultures treated with aorta-conditioned medium (CM) or purified bFGF had a significantly higher number of break-away capillaries as compared to control (C) (P < 0.001)* n = *14 (C), 7 (CM), 6 (bFGF). Error bars represent SEM.*

Purified bFGF added to the collagen gel induced a dose-dependent increase in the number of microvessels (Figure 11). Purified bFGF also induced a 110% increase in the length of the microvessels and promoted the formation of break-away capillaries as seen with the rat aorta conditioned medium (Figures 9 and 10).

Addition of exogenous bFGF to the growth medium after the growth phase (day 14) prevented the regression of the microvessels, which was characteristically seen when cultures were kept more than

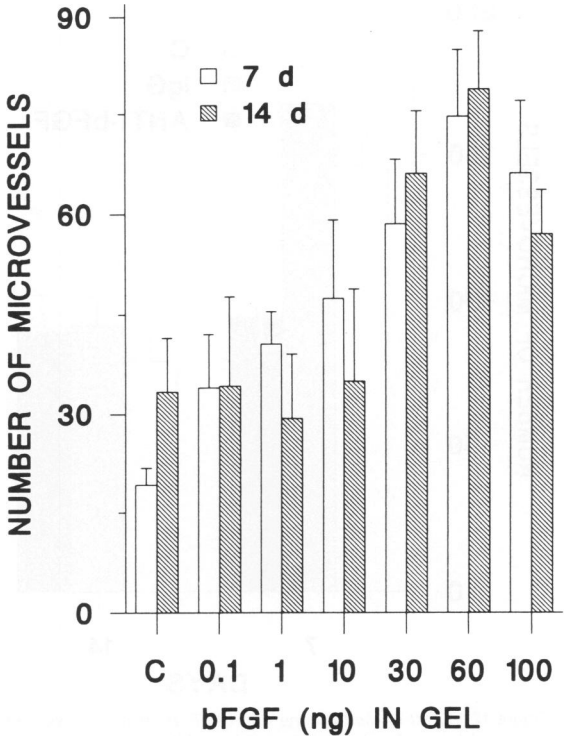

Figure 11. *Effect of purified bFGF added at increasing concentrations to the collagen gel of rat aorta cultures. bFGF induced a dose-dependent increase in the number of microvessels. The increase at day 7 was significant at all doses (0.1 ng/ml,* P < *0.05; 1 and 10 ng/ml,* P < *0.005; other concentrations,* P < *0.0001), whereas at day 14, it was significant only at higher doses (30 ng/ml,* P < *0.02; 60 ng/ml,* P < *0.003; 100 ng/ml < 0.02),* n = *9 (C, 100 ng/ml bFGF), 3 (0.1 ng/ml, 1 ng/ml, 10 ng/ml), 6 (30 ng/ml, 60 ng/ml). Error bars represent SEM.*

2 weeks under serum-free conditions. The number of microvessels decreased by 60% between days 14 and 21 in control cultures, but only by 9% in parallel cultures treated with bFGF. Furthermore, the length of the microvessels increased 127% from day 14 to day 21 in cultures treated with bFGF, whereas it remained unchanged in control cultures.

Effect of Neutralization of Endogenous bFGF Activity on Rat Aortic Angiogenesis

Addition of neutralizing anti-bFGF antibodies to serum-free collagen gel culture of rat aorta caused a 40% reduction of angiogenesis (Figure 12). This effect was evident during the early stages of growth, whereas no significant effect was observed at day 14. Protamine sulfate had a marked anti-angiogenic effect that was partially reversed by removing the drug from the culture medium (data not shown). In addition, protamine sulfate added to cultures that had been allowed to grow in its absence

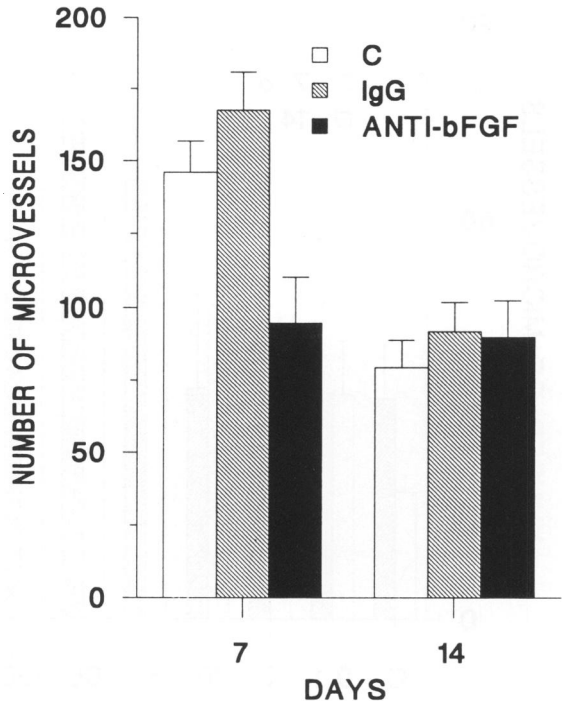

Figure 12. *Effect of neutralizing anti-bFGF antibody on angiogenesis in collagen gel culture of rat aorta. Addition of 50 μg/ml anti-bFGF to the culture medium and to the gel induced a decrease in microvascular growth at day 7 ($P < 0.05$, compared to untreated control; $P < 0.005$, compared to nonimmune IgG) but not at day 14; n = 7 (C, anti-bFGF), 6 (IgG). Error bars represent SEM.*

altered the morphology of developing microvessels, which became blunt and regressed.

Discussion

Our data demonstrate that injured rat aortic explants release bFGF, which plays an important autocrine role during angiogenesis *in vitro*. The previous observation that angiogenesis in collagen gel culture of rat aorta occurred in the absence of exogenous growth factors, suggested modulation of the angiogenic response by endogenous cytokines.[18] The present study supports this hypothesis and shows that aortic explants do not require an exogenous extracellular matrix to initiate angiogenesis, as they can form microvessellike structures also on plastic surfaces without the support of a collagen gel. The rapid appearance of the angiogenic cytokine bFGF in the conditioned medium of freshly cut aortic rings indicates that mechanical damage triggered the release of bFGF from injured aortic cells. This finding confirms and expands in a model of angiogenesis previous reports demonstrating that bFGF, which lacks a signal peptide sequence for secretion, is released by mechanically wounded cells through permeabilization of the plasma membrane.[13,15] Sectioning the aorta caused a variable degree of cell injury. Necrotic cells were seen at the margins of resection, together with reversibly injured endothelial and myointimal cells that showed morphological features of activation. Reversibly injured cells may play an important role in sustaining the angiogenic response of the aorta because it has been shown that transient permeabilization of cell membranes favors the selective release of bFGF as compared to the irreversible permeabilization associated with cell death.[15] Because signs of cellular activation were usually not seen in uninjured aortic cells, it is possible that coupling of injury and bFGF release was required for autocrine cell activation.[14] The decrease over time of bFGF release by the aorta may be due to the gradual healing of reversibly injured cells. Moreover, the very low bFGF levels observed in the aorta-conditioned medium after 10 days suggest that under normal conditions this cytokine either is not secreted or, if secreted, is sequestered in the extracellular matrix and does not enter the soluble phase.[13,22–25]

bFGF released by damaged cells played an autocrine angiogenic role because bFGF-containing aortic medium stimulated angiogenesis, whereas neutralizing anti-bFGF antibodies reduced the angiogenic response. The stimulatory effect of purified bFGF and the time course of the angiogenic growth curve, which paralleled the rise and fall of endogenous bFGF levels, further support this conclusion.

Interestingly, the angiogenic effect of rat aorta-conditioned medium became evident after 9 to 10 days but was not noticeable in early stages of growth. One possible explanation for this phenomenon is that endogenous bFGF released by the aortic explant at the time of injury saturated the high- and low-affinity bFGF receptors, fully sustaining angiogenesis during the first days and rendering the growth factor added with exogenous conditioned medium relatively ineffective. This interpretation is supported by the finding that the anti-angiogenic effect of anti-bFGF antibodies was significant only in young cultures when there was maximum release of endogenous bFGF. However, the window of time during which the culture is sensitive to exogenous bFGF may vary with the experimental conditions, because in some cultures we obtained early stimulation of angiogenesis by adding purified bFGF to the collagen gel and not to the growth medium.

bFGF-containing rat aorta-conditioned medium and purified bFGF stimulated formation of capillaries, which broke away from their roots, migrating actively toward the periphery of the collagen gel. These findings confirm in a 3-dimensional model of

angiogenesis previous observations showing a stimulatory effect of bFGF on endothelial cell motility in 2-dimensional cultures.[9] The fact that the migratory effect was particularly evident in the most forward segments of the microvessels supports the idea that angiogenesis is the result of a coordinated interaction of endothelial migration and proliferation, with the former occurring mainly at the tip and the latter at the trailing end of the capillary.[26]

bFGF added after the growth phase, when the vascular outgrowths were regressing, stabilized the microvessels and promoted their elongation, but failed to further stimulate vascular proliferation. This is in agreement with recent observations showing that intravascularly injected bFGF stimulates growth of injured endothelial cells but not of quiescent endothelial cells.[27] The inability of bFGF to stimulate new sprouting in late aortic cultures suggests that additional factors regulate the angiogenic response of the aortic explants. One possibility is that quiescent endothelial cells down-regulate their bFGF receptors. Alternatively, mature microvessels may produce or activate cytokines that modulate bFGF or its receptors, promoting differentiation rather than proliferation. For example, it has recently been shown that pericytes inhibit growth of endothelial cells and that this phenomenon is mediated by transforming growth factor β-1, which is activated when the two cells are in physical contact.[28] This mechanism is supported by the observation that pericytes in aortic cultures increase in number during the maturation of the microvessels.[29] The observation that the rat aorta produces, in addition to bFGF, other cytokines capable of influencing endothelial behavior raises the possibility that rat aortic angiogenesis is regulated by a redundant system of growth factors.[30] The inability of anti-bFGF antibodies to completely suppress angiogenesis is consistent with this hypothesis.

The reversible inhibition of microvascular sprouting by protamine sulfate further supports the angiogenic role of endogenous bFGF. In fact, it has been shown that protamine sulfate, which has anti-angiogenic properties *in vivo*, inhibits bFGF-mediated proliferation of baby hamster cells by preventing the binding of bFGF to its cell-surface receptor.[19,31] In our experiments, protamine sulfate inhibited the formation of new microvessels and arrested the growth of developing microvessels. The effect was reversible and angiogenesis was restored after withdrawal of protamine sulfate from the medium.

Of additional interest in this study was the distribution in the rat aorta of bFGF, which appeared particularly abundant not only in endothelial cells but also in the smooth muscle cells of the innermost layers of the tunica media. This suggests that the rat aorta contains a subpopulation of smooth muscle cells that, by constitutively expressing bFGF, may play an important role in the response of the aortic wall to injury. In fact, in early phases of experimental atherosclerosis, dividing smooth muscle cells are preferentially located in subintimal layers of the tunica media underneath the developing plaques.[32,33] Thus, it is possible that bFGF released by aortic cells after injury promotes, through autocrine and paracrine mechanisms, not only angiogenesis but also smooth muscle cell proliferation.[27,34–37]

In conclusion, our experiments support the idea that endothelial and smooth muscle cells release bFGF after injury and indicate that endogenous bFGF plays an important role in the autoregulation of angiogenesis during vascular wound healing.

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