Journal of Cellular Biochemistry 29:275–287 (1985)
Perspectives in Inflammation, Neoplasia, and Vascular Cell Biology 63–75

Angiogenesis Is Stimulated by a Tumor-Derived Endothelial Cell Growth Factor

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A growth factor mitogenic for BALB/C 3T3 cells and capillary endothelial cells was isolated from a rat chondrosarcoma and purified to homogeneity. Purification was accomplished by a combination of BioRex 70 cation exchange chromatography and heparin affinity chromatography. The pure chondrosarcoma-derived growth factor (ChDGF) had a molecular weight of about 18,000. The angiogenesis activity of pure ChDGF was tested by measuring its ability to vascularize the chorioallantoic membrane (CAM) and yolk sac membrane of the developing chick. The ability of ChDGF to induce the growth of limbal vessels in the rat cornea was also measured. To quantitate the angiogenesis response, a unit system based on the growth factor activity of ChDGF for 3T3 cells was adopted. ChDGF was found to have a specific activity of about 5 units/ng when applied to 3T3 cells. About 300–600 units of ChDGF in the two types of developing chick membrane and 30–50 units of ChDGF in the rat cornea were found to stimulate noninflammatory angiogenesis.

Key words: endothelial cell, angiogenesis, growth factor, chondrosarcoma, heparin

Angiogenesis is a complex process in which capillary blood vessels grow in an ordered sequence of events [1,2]. When a new capillary sprout grows from the side of a venule, endothelial cells degrade basement membrane, migrate toward an angiogenic source, proliferate, form a lumen, join the tips of two sprouts to generate a capillary loop, and manufacture new basement membrane. Our laboratory has used in vitro methods to study two key aspects of angiogenesis: capillary endothelial cell migration and proliferation. In particular, we have focused our efforts on the purification of polypeptides that stimulate capillary endothelial cell migration and proliferation in vitro. Initially, we described the partial purification from a transplantable rat chondrosarcoma of a 16,000–20,000 molecular weight polypeptide that stimulated capillary endothelial cell migration [3]. More recently, we used heparin-affinity

Received February 14, 1985; revised and accepted June 17, 1985.

chromatography to purify to homogeneity an 18,000 molecular weight chondrosarcoma-derived capillary endothelial cell mitogen [4,5]. Biochemical analysis suggested that the migration and proliferation stimulating factors were the same. The next question was whether the chondrosarcoma-derived growth factor (ChDGF), which stimulated capillary endothelial cell proliferation and migration in vitro was also an angiogenic factor in vivo. Several techniques have been developed to measure angiogenesis. These include analysis of the neovascularization of chorioallantoic [6] and yolk sac [7] membranes in the developing chick embryo and neovascularization of the rabbit cornea [8]. In this report we demonstrate that pure ChDGF is a noninflammatory angiogenic factor.

MATERIALS AND METHODS

Chondrosarcoma-Derived Growth Factor

Chondrosarcoma-derived growth factor was purified as described previously by a combination of BioRex 70 and heparin-Sepharose chromatography [4,5]. A unit of ChDGF was defined as the amount of growth factor in 250 μl of medium necessary to stimulate half-maximal DNA synthesis in BALB/C 3T3 cells. Pure ChDGF was estimated to have a specific activity of 5 units/ng. In angiogenesis experiments, the amount of ChDGF used was quantitated in terms of 3T3 growth factor activity units.

Chick Chorioallantoic Membrane Assay

The use of the chick chorioallantoic membrane (CAM) as a model of angiogenesis has been previously described [6,9]. In the angiogenesis assay, ChDGF or controls were incorporated along with 10 μg of bovine serum albumin and 2 units of heparin into 0.5% methylcellulose, 10 μl of which were implanted onto the CAM of a 9.5-day-old chick embryo through a window previously made in the egg shell on day 4 [9]. Angiogenesis was analyzed after a 24-hr incubation period. The CAM was photographed and fixed in formalin for histology.

Yolk Sac Membrane Assay

Fertilized chick embryos were removed from their shell on day 3, placed in a petri dish, and incubated as previously described [7,10]. Ten microliters of 0.5% methylcellulose-containing samples were implanted on the center of the anterior or posterior vitelline vein of the yolk sac. After 24 hr the membranes were analyzed for angiogenesis, photographed, and fixed in formalin for histology.

Rat Cornea Assay

This assay was adapted from the rabbit cornea assay first described by Gimbrone et al [8]. The rat cornea assay has the advantages of (1) being a syngeneic system for testing the in vivo activity of the rat-derived growth factor and (2) being a smaller scale system than the rabbit cornea thereby using less growth factor. ChDGF was incorporated into ethylene-vinyl acetate copolymer pellets using methods previously described [11,12]. The pellets (0.1 mm^3, 0.1 mg) were implanted into the corneas of anesthetized 200-g male Sprague-Dawley rats using the method described by Fournier et al [13]. Briefly, a 0.3-mm transverse incision was made through one-half the thickness of the cornea at its center with a 15-gauge scalpel. A pocket (1.5 $\times$ 3 mm) was created in the stroma of the cornea by using an ophthalmological spatula. A pellet

was introduced into the pocket. After 6 days the rats were anesthetized and the cornea was analyzed for angiogenesis after injection of ink. To introduce the ink, a 5-cm vertical incision was made over the lateral neck on the side of the implanted cornea and the carotid artery was exposed. A tiny arteriotomy was created and the beveled tip of a piece of polyethylene PE-10 tubing attached to a 30-gauge needle was inserted and secured. The needle was connected to a syringe containing ink, which was then injected. The cornea was removed and fixed for analysis of angiogenesis, photography, and histology. In a positive angiogenic response, vessels were found to extend directionally into the cornea from the cornea-scleral limbus toward the pellet containing ChDGF.

Histology

CAM, yolk sac membranes, and rat corneas were examined by routine histological techniques. Tissues were fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned with a microtome. Afterward the tissues were deparaffinized and stained with hematoxylin and eosin. The rat corneas were cut in cross section and the CAM and yolk sac membranes were cut en face as described previously [14].

RESULTS

Purification of Chondrosarcoma-Derived Growth Factor

An 18,000 molecular weight growth factor that was mitogenic for capillary endothelial (CE) and 3T3 cells was purified from a transplantable rat chondrosarcoma as described previously [4,5]. The chondrosarcoma-derived growth factor (ChDGF) was purified by a combination of BioRex 70 cation exchange and heparin-Sepharose affinity chromatography. In this two-step procedure ChDGF was purified approximately 500,000-fold and the recovery was about 10% (Table I). It was convenient to measure the biological activity of ChDGF in terms of 3T3 units, where one unit was defined as the amount of growth factor in 250 μl required to stimulate half-maximal DNA synthesis in 3T3 cells. The specific activity of ChDGF was found to be about 5 units/ng (Table I).

Angiogenesis in Developing Chick Embryo Membranes

Figure 1 shows the profile of growth factor activity after chromatography of ChDGF on heparin-Sepharose. Pure ChDGF that was mitogenic for both capillary

TABLE I. Purification of Chondrosarcoma-Derived Growth Factor

Purification step[a]	Protein (mg)	Total activity (units)[b]	Specific activity (units/μg)	Recovery (%)	Purification (fold)
Extracellular matrix	10,000	100,000	0.01	100	—
BioRex 70	30	45,000	1.5	45	150
Heparin-Sepharose	0.002	10,000	5,000	10	500,000

[a]Chondrosarcoma (20 tumors, 12 grams/tumor) was digested with 2 mg/ml collagenase and separated into cellular and extracellular matrix fractions [5]. ChDGF was purified from the extracellular matrix by consecutive steps of BioRex 70 cation exchange chromatography and heparin-Sepharose affinity chromatography.

[b]A unit of growth factor activity was defined as the amount of growth factor in 250 μl of medium required to stimulate half-maximal DNA synthesis in BALB/C 3T3 cells.

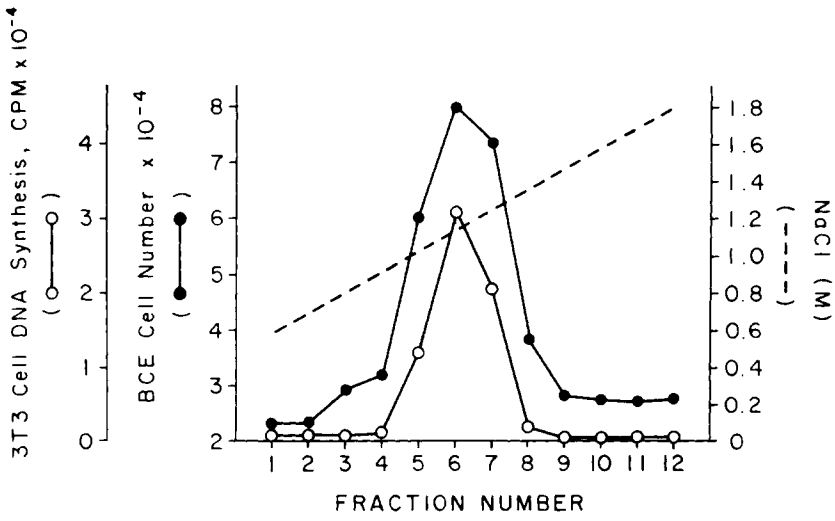

Fig. 1. Heparin-Sepharose affinity chromatography. ChDGF (3,000 units) partially purified by BioRex 70 cation exchange chromatography was further purified on a column (1 × 15 cm) of heparin-Sepharose (Pharmacia, Piscataway, NJ). ChDGF was eluted with a 300-ml gradient of 0.6 M to 2 M NaCl in 10 mM Tris, pH 7, at a flow rate of 30 ml/hour. Fractions were collected and assayed for the ability to stimulate proliferation of capillary endothelial cells (●) and DNA synthesis in BALB/C 3T3 cells (○).

endothelial and 3T3 cells was tested for the ability to induce angiogenesis in the developing CAM and developing chick yolk sac membrane. About 15 eggs were used in this experiment. The 3T3 unit system was used for quantitation. About 600 units of pure ChDGF was required to induce a qualitatively strong angiogenic response in the CAM in a 24-hr period (Fig. 2, right). The angiogenic response was characterized by the directional converging of blood vessels toward ChDGF. Many newly formed loops were seen. The induction of angiogenesis was dose dependent. Only a weak reaction was found with 200 units (results not shown) and no angiogenesis occurred in the absence of ChDGF (Fig. 2, left). The CAM was sectioned en face and analyzed histologically. Figure 3 shows an en face histological section of the CAM at about × 45. Two events were noticeable. First, blood vessels could be seen converging toward the ChDGF implantation site from all directions. Second, there was a marked effect on the morphology of the mesenchymal cells present at the site of ChDGF implantation. These two occurrences were examined at a higher magnification of about × 450. Figure 4 shows an apparently newly formed capillary loop with at least four sprouts extending in the same direction. Although it is impossible to ascertain that these sprouts were induced specifically by ChDGF, the common directionality of the sprouts suggests a directional stimulus. Figure 5 shows the ChDGF implantation site. It appears that the mesenchymal cells in this area are elongated and have acquired a morphology reminiscent of 3T3 fibroblasts in culture that had been stimulated to migrate and divide by growth factors [15]. The mesenchymal cells at the site of ChDGF implantation are surrounded by a zone of blood vessels, which can be seen in cross section emerging from the plane of the CAM. The effects of ChDGF on capillaries and mesenchymal cells in vivo are consistent with the actions of this

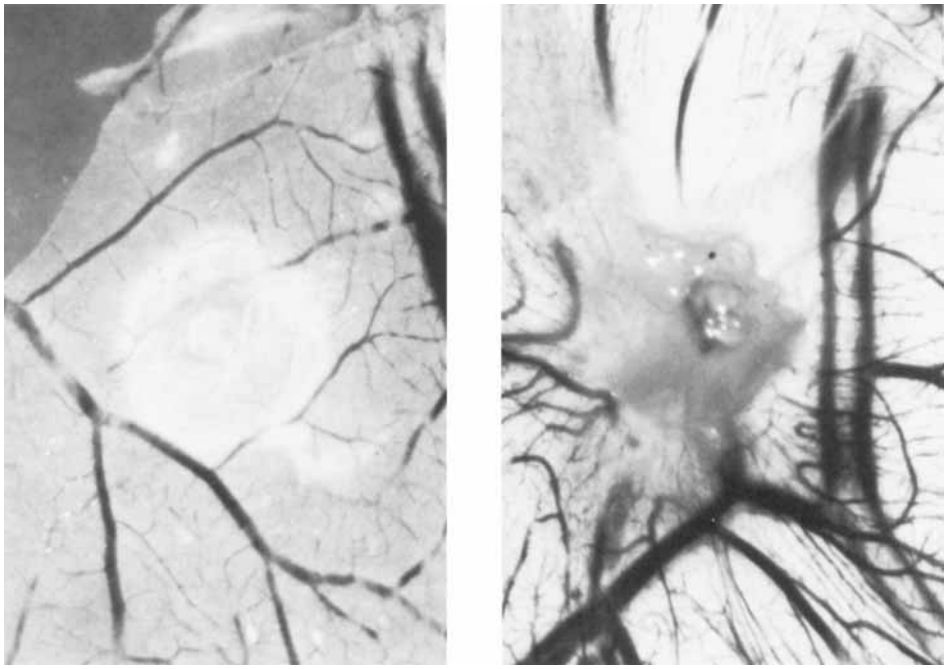

Fig. 2. Stimulation of angiogenesis on the chorioallantoic membrane (CAM). ChDGF was incorporated into methylcellulose as described in Materials and Methods and applied to the CAM of a 9.5-day-old developing chick membrane. Fifteen eggs were assayed. After approximately 24 hr the CAMs were excised, fixed, and photographed (magnified × 6). Left: Control. Right: ChDGF (600 units).

mitogen in vitro. ChDGF is a stimulator of both capillary endothelial cell and 3T3 fibroblast proliferation (Fig. 1).

Histological analysis at high magnification (Fig. 4,5) also indicated a virtual absence of inflammatory cells in the vicinity of the newly forming vessels, suggesting that ChDGF was inducing angiogenesis rather than inflammation.

Angiogenesis was also measured on the developing chick yolk sac membrane. In these experiments a quantitative analysis using over 70 eggs was carried out. ChDGF was purified by heparin affinity chromatography as shown in Figure 1. The fractions collected were pooled into three groups (Table II) and tested for the ability to stimulate angiogenesis activity in a 24-hr period. Group B contained the fractions eluting in the growth factor activity peak (fractions 4–8), while groups A (fractions 1–3) and C (fractions 9–12) contained the fractions eluting before and after the growth factor activity peak, respectively. About 46% of the group B yolk sac membranes were positive for angiogenesis activity compared to 0% for group A and 4% for group C. Some of the negative responses in group B were attributed to technical problems such as detachment of the methylcellulose polymer from the yolk sac membrane. Representative yolk sac membranes tested with individual fractions 2, 6, and 10 before pooling are shown in Figure 6. Only fraction 6, which is in the center of the growth factor activity peak shown in Figure 1, was positive. About 400 3T3 growth factor activity units of fraction 6 were required to induce angiogenesis. Histological analysis showed strong angiogenic responses in the absence of inflam-

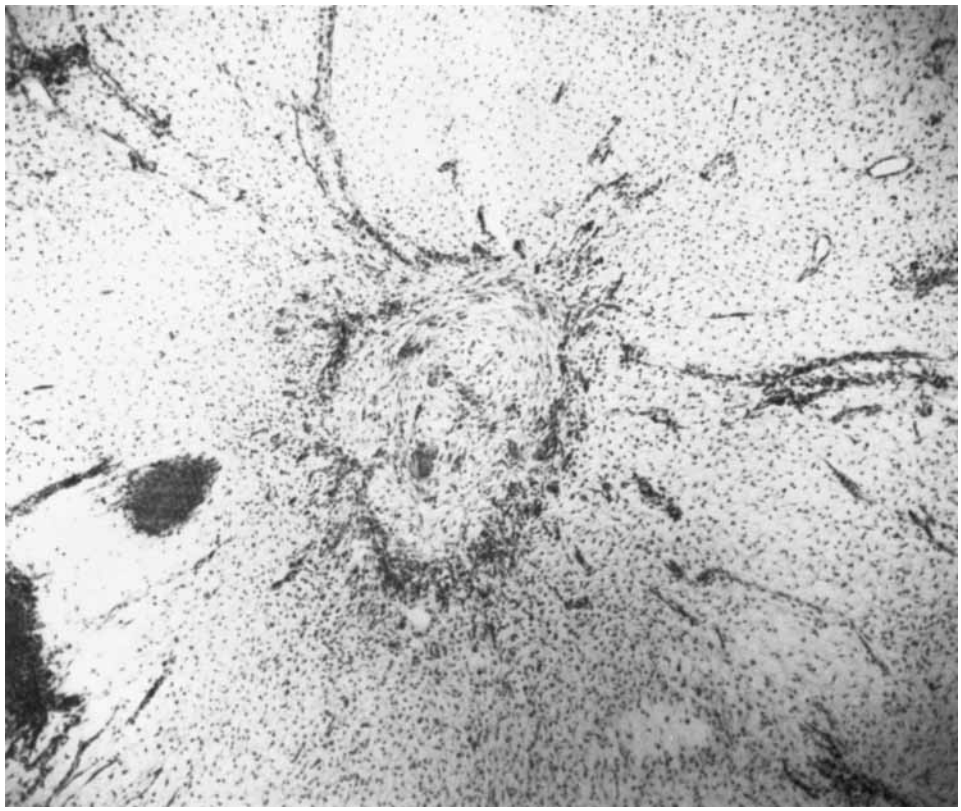

Fig. 3. En face histology of angiogenesis on the CAM. The CAM shown in Figure 2 was fixed, sectioned en face, and stained with hematoxylin and eosin. The area in the center is where ChDGF was implanted. It is surrounded by a thick zone of blood vessels, which are seen in cross section emerging from the plane of the CAM. Also note the many blood vessels converging from all directions toward the implantation site. × 45.

mation in fraction 6 and no angiogenesis in fraction 2 or 10. However, these results are not shown because the presence of yolk makes it very difficult to prepare adequate photomicrographs.

Angiogenesis in the Rat Cornea

The induction of angiogenesis by ChDGF in the rat cornea was also tested. Pure ChDGF was prepared. Half of the ChDGF was boiled for 15 min, resulting in about a 98% inactivation of growth factor activity. The active and inactive samples were each incorporated into slow release ethylene-vinyl acetate copolymers. A portion of each polymer weighing approximately 0.1 mg and containing 30–50 units was implanted into each rat corneal pocket. Six days after implantation the corneas were injected with ink and removed for blood vessel analysis, photography, and histology. Table III shows that of the six corneas implanted with 30–50 units of active ChDGF, five were positive in that blood vessels were found to extend directionally from the cornea-scleral limbus toward the pocket containing the polymer with active ChDGF. The average length of a new blood vessel was about 1 mm. Of the five corneas

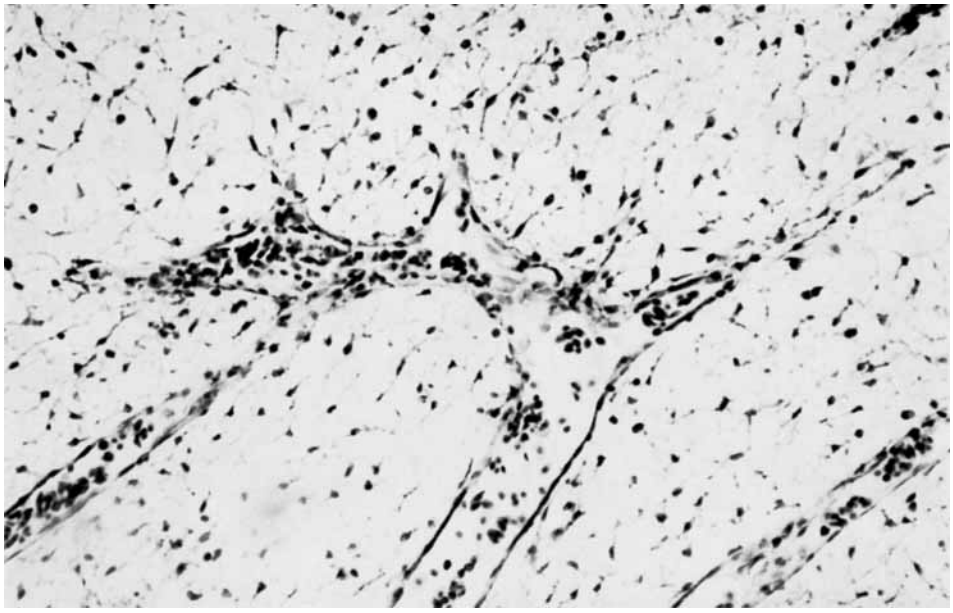

Fig. 4. En face histology of angiogenesis on the CAM. A detail of Figure 3 at a higher magnification (× 450), a vascular loop is seen directed toward the upper right hand corner. Note that there are at least four sprouts that can be seen emerging from the loop and that they are all pointed in the same direction, toward the implantation site of ChDGF.

TABLE II. Angiogenesis Induced by ChDGF on the Yolk Sac Membrane

		Positive angiogenesis responses	
Group[a]	Fractions	Positive eggs/total eggs	% Positive eggs
A	1–3	0/16	0
B	4–8	15/33	46
C	9–12	1/23	4

[a]ChDGF was purified by chromatography on a column of heparin-Sepharose (Fig. 1). Fractions were collected and pooled into three groups: A, B, and C. These fractions were dialyzed against doubly distilled H_2O. A calculation was made as to the volume of group B that contained 400 units of 3T3 growth factor activity. This volume and an equivalent volume of group A and C were lyophilized, incorporated into methylcellulose, and applied to yolk sac membranes.

implanted with inactive ChDGF, none were positive. A photomicrograph of a representative cornea implanted with inactive ChDGF is shown in Figure 7 (top) and one with active ChDGF is shown in Figure 7 (bottom). Figure 8 shows a histological analysis of a ChDGF-induced angiogenic response in the rat cornea. At a magnification of × 70, one can see the site of the ChDGF-containing polymer implant at the upper right. Ink-stained blood vessels can be seen in the limbus at the lower left and in the vicinity of the polymer at the upper right. Figure 9 shows a higher magnification (× 450) of the ink-stained blood vessels in the vicinity of the ChDGF-containing polymer. The epithelial cell layer is intact. Virtually no inflammatory cells can be found, suggesting that angiogenesis rather than inflammation is occurring.

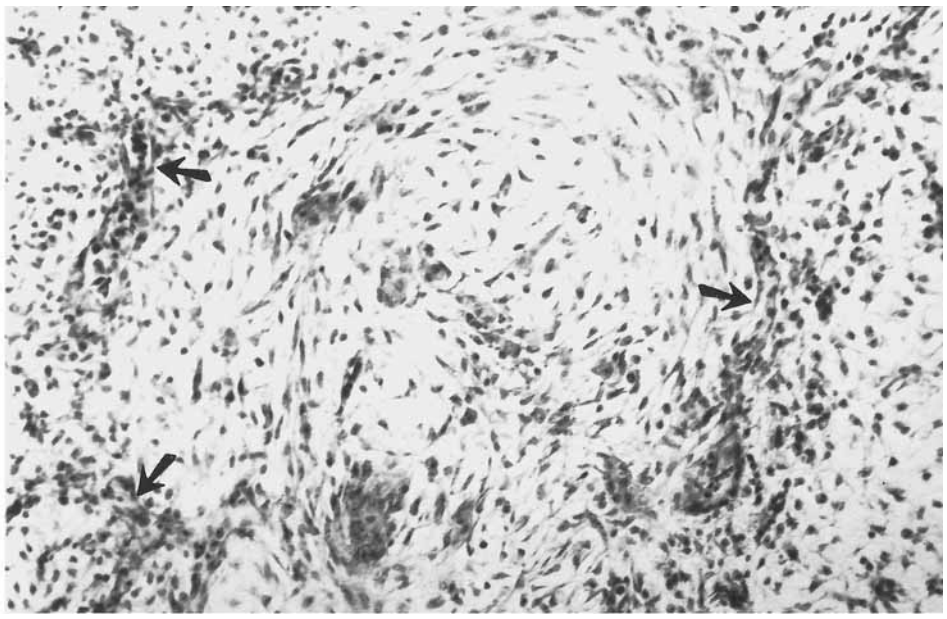

Fig. 5. En face histology of angiogenesis on the CAM. The implantation site of ChDGF in Figure 3 is shown at higher magnification (× 450). Note the elongated morphology of the mesenchymal cells in the center. Surrounding this area are numerous blood vessels (arrows), which are seen in cross section emerging from the plane of the CAM.

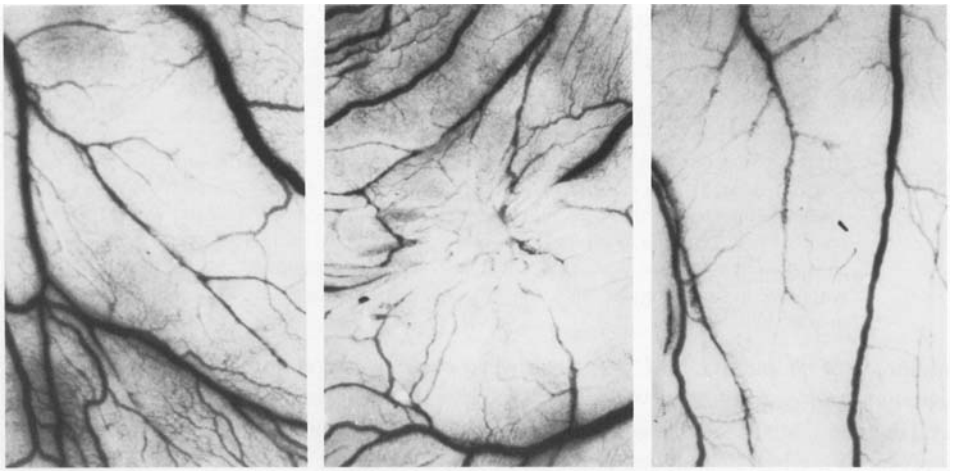

Fig. 6. Stimulation of angiogenesis on the yolk sac membrane. Pure ChDGF was prepared by heparin-Sepharose affinity chromatography. The column is shown in Figure 1. Fractions 2 (left) and 10 (right), which elute before and after the ChDGF peak, respectively, and fraction 6 (center) in the center of the growth factor peak (400 units of ChDGF) were tested on the 4-day-old yolk sac membrane for angiogenesis stimulation. ×6.

TABLE III. Angiogenesis Induced by ChDGF in the Rat Cornea

Sample	Positive angiogenesis responses[a] (Positive cornea/total cornea)
Active ChDGF	5/6
Inactive ChDGF[b]	0/5

[a]A positive response is defined as mean blood vessel length of 1 mm. In a negative response no blood vessels migrate toward the polymer.
[b]ChDGF was inactivated by boiling for 15 min. Both active (30–50 units) and inactive ChDGF were incorporated into ethylene-vinyl acetate polymer pellets, which were subsequently implanted into the rat cornea.

DISCUSSION

Chondrosarcoma-derived growth factor, an 18,000 molecular weight polypeptide that stimulates the migration [3] and proliferation [4,5] of capillary endothelial cells in vitro is also angiogenic in vivo. The CAM and yolk sac membrane of the developing chick embryo and the avascular rat cornea were used as model systems for testing angiogenesis activity. The results were scored both visually and histologically. In the developing chick embryo membranes, pure ChDGF rapidly stimulated angiogenesis within 24 hr of application. The rapid stimulation of angiogenesis in vivo by ChDGF is probably due to an increase in the rate of both capillary endothelial cell migration and proliferation. The early blood vessels of the chick embryo arise by aggregation of mesenchymal cells to form a closed network of tubules lined by endothelial cells. Additional capillaries then develop by a process of sprouting, which involves both proliferation and migration of endothelial cells from the existing capillaries. All larger vessels are formed by subsequent remodeling of this primitive capillary network [16]. ChDGF, by inducing the migration and proliferation of capillary endothelial cells, probably accelerates sprouting and the remodeling of capillaries, processes that are already ongoing in the rapidly developing chick embryo chorioallantoic and yolk sac membranes. Thus, the angiogenesis response to ChDGF is quite rapid. In the avascular rat cornea, where no capillaries are present, blood vessels are seen to migrate from the limbus to the corneal pocket containing ChDGF in about 6 days after growth factor implantation. In both the chick embryo and rat cornea assays, histological examination shows the virtual absence of inflammatory cells. Several controls were used to affirm the validity of the results: (1) ChDGF was purified by heparin-Sepharose affinity chromatography [4,5], and fractions with and without growth factor activity were tested. Angiogenesis in vivo was stimulated only by fractions that had growth factor activity but not by fractions that contained other proteins. (2) ChDGF growth factor activity was inactivated by boiling for 15 min. The inactivated samples were not angiogenic in vivo.

An attempt was made to quantitate the angiogenic response using a growth factor unit system. ChDGF is a potent polypeptide that is active in stimulating 3T3 cells at about 5 units/ng. In the developing chick membrane model systems, a strong positive angiogenic response was induced by about 300–600 units of ChDGF, an amount equal to about 60–120 ng of pure protein. In the rat cornea about 30–50 units (6–10 ng) were required. It is impossible to ascertain what portion of the ChDGF was actually delivered to the target. Losses undoubtedly occurred because of diffusion, incomplete release from sustained release polymers, and inactivation.

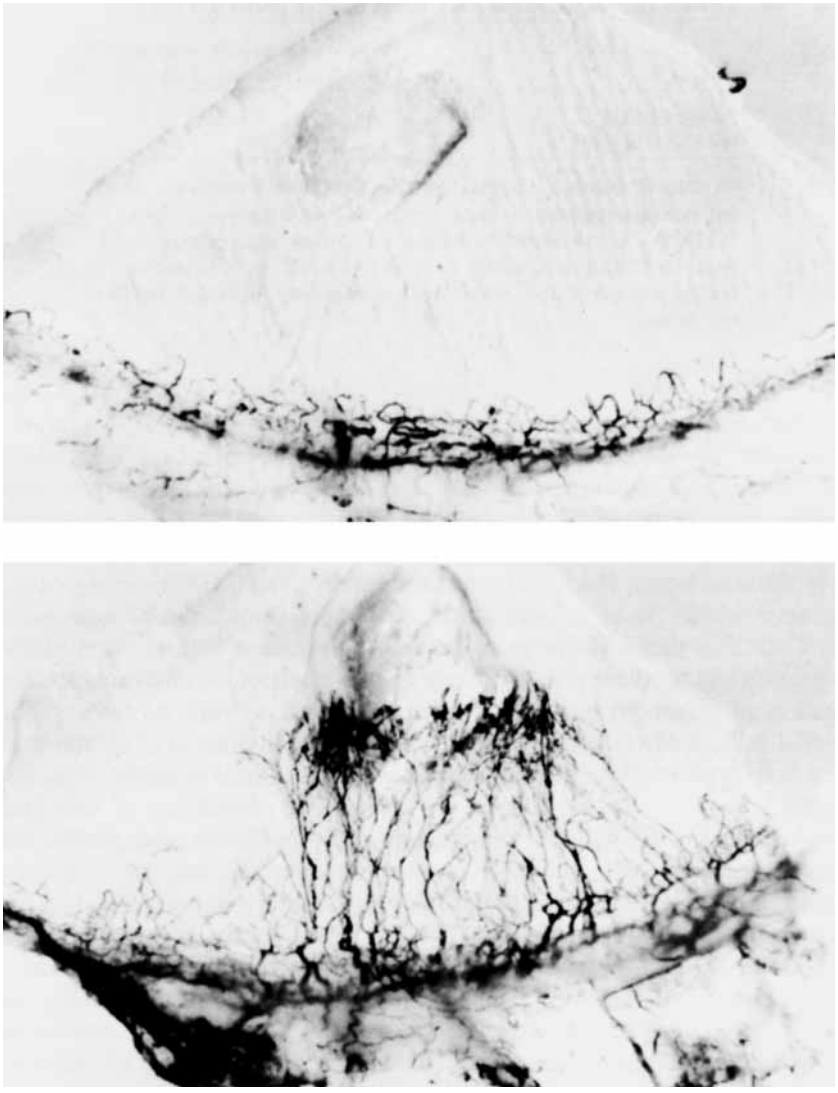

Fig. 7. Stimulation of angiogenesis in the rat cornea. Active ChDGF and ChDGF inactivated by boiling for 15 min were incorporated into ethylene-vinyl acetate polymers, which were implanted into the rat cornea. After 6 days the rat eyes were injected with ink and the corneas were removed and photographed (× 12). Top: Inactive ChDGF. Bottom: Active ChDGF (30–50 units). Note the ink-stained blood vessels originating from the limbus (bottom) and converging on the polymer (top).

Histological analysis of a chick CAM after exposure to ChDGF suggested that the growth factor affected other cells besides capillary endothelial cells. The mesenchymal cells at the site of ChDGF implantation were found to be markedly elongated. They appeared to have a morphology that was similar to that of 3T3 fibroblasts that had been stimulated to migrate and divide by growth factors in vitro [15]. The possibility that ChDGF has an effect on both mesenchymal cells and blood vessels is consistent with some recent results obtained with cartilage-derived growth factor (CDGF), a 19,000 molecular weight polypeptide with strong structural similarity to

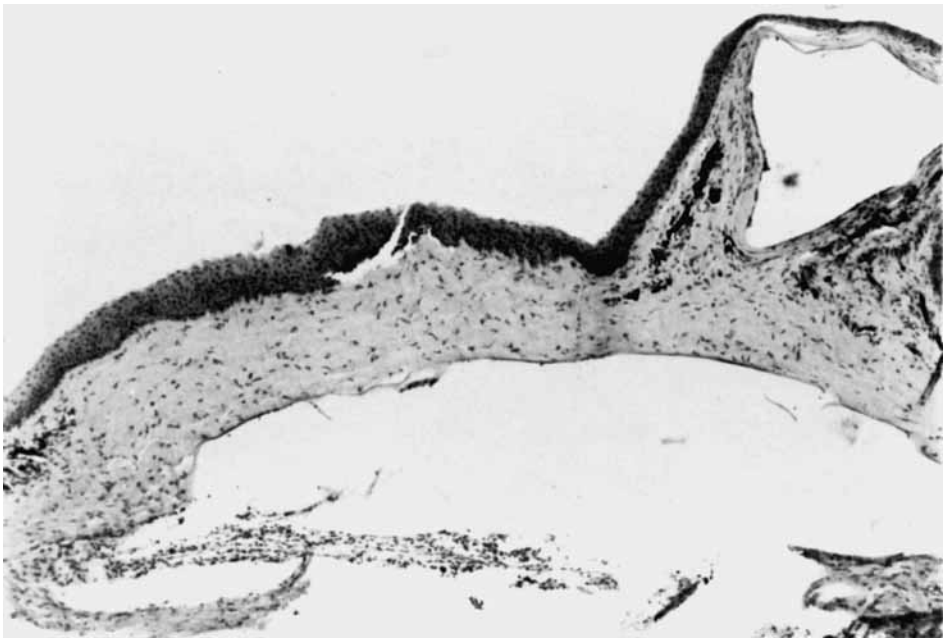

Fig. 8. Histology of angiogenesis in the rat cornea. After injection of the rat eye with ink (6 days after implantation), the rat cornea was excised, fixed, sectioned, and stained with hematoxylin and eosin. Note the ink-stained blood vessels in the area of the polymer implant at the upper right and in the limbus at the lower left. The external epithelial layer (top) and the internal Descemet's membrane (bottom) are intact. × 70.

ChDGF [17]. In these studies polyvinyl sponges were implanted into a rat, and after a 6-day incubation period, the sponges were injected with CDGF [18]. It was found that compared to control sponges, CDGF-treated sponges were much more heavily infiltrated with granulation tissue and that the granulation tissue was highly vascular. Thus, it appears that CDGF in the rat sponge model and ChDGF in the developing chick membrane model are able to mobilize both blood vessels and fibroblasts. These biological activities in vivo reflect the ability of ChDGF and CDGF to stimulate both 3T3 fibroblast and endothelial cell proliferation in vitro. We speculate that angiogenesis sometimes may be accompanied by fibroblast migration and proliferation.

The purification of ChDGF [4,5] has been greatly facilitated by the use of heparin-Sepharose affinity chromatography. Recently, we and other laboratories have purified from normal tissue several endothelial cell growth factors that have a strong affinity for heparin. These growth factors include pituitary and brain fibroblast growth factors [19–21], endothelial cell growth factor [20–22], and retina-derived growth factor [23]. Both fibroblast growth factor [24] and retina-derived growth factor [25] have been shown to be angiogenic in the rabbit corneal model. Thus, it appears that heparin-binding angiogenesis factors are found in both normal tissues and tumors. Perhaps in normal tissue, angiogenesis factors are expressed according to a cellular program, while in neoplasia, these factors are expressed continually. The major difference between normal and tumor angiogenesis may be a matter of temporal control.

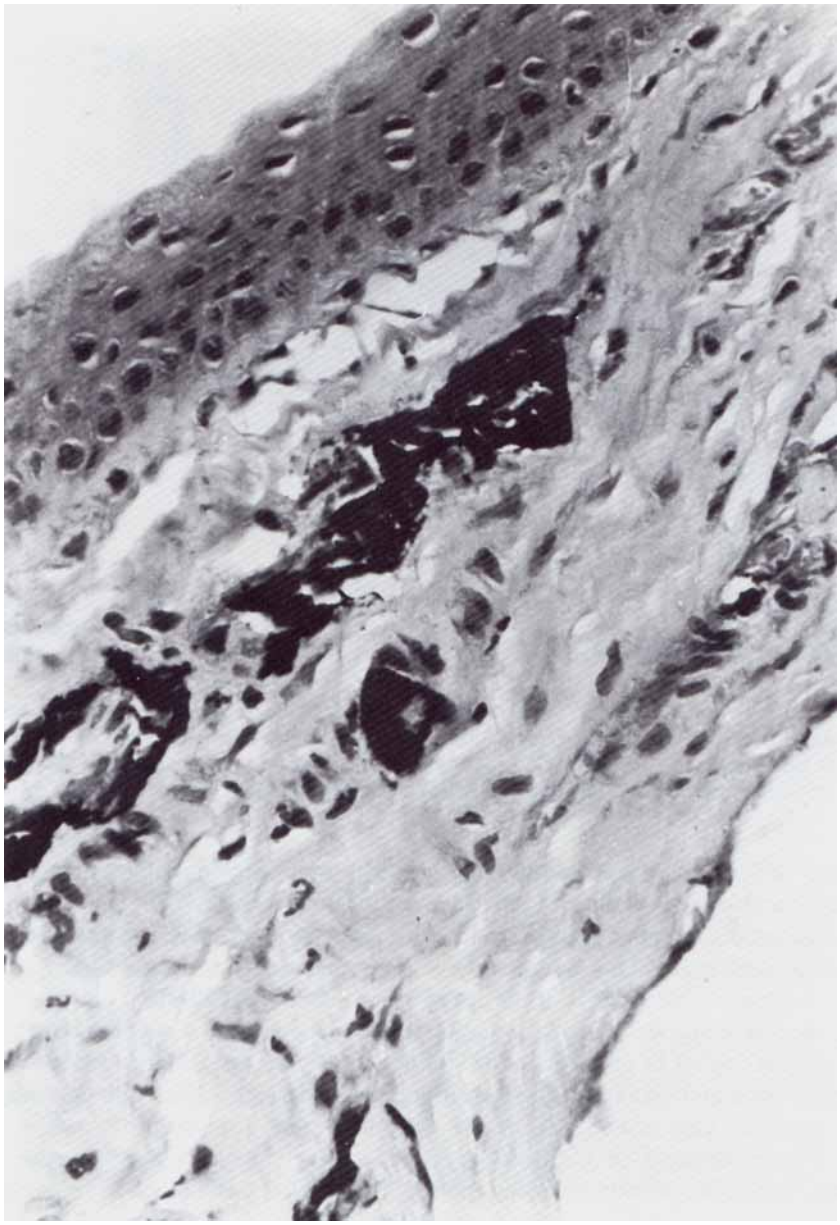

Fig. 9. Histology of angiogenesis in the cornea. An area of ink-stained blood vessels (6 days after implantation) in the vicinity of the ChDGF-containing polymer shown in Figure 8 is now shown at a higher magnification of × 450. Virtually no inflammatory cells (less than four in this entire section) are present.

ACKNOWLEDGMENTS

This work was supported by National Cancer Institute grants CA 21763 to M.K. and CA 14019 to J.F., the Claude E. Welch research fellowship from the Department

of Surgery, Massachusetts General Hospital to D.L., and a grant to Harvard University from the Monsanto Company.

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