Production of a Heparin-binding Angiogenesis Factor by the Embryonic Kidney

Werner Risau and Peter Ekblom
Max-Planck-Institut für Entwicklungsbiologie and Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 35-39, D-7400 Tübingen, Federal Republic of Germany

Abstract. Embryonic mouse kidneys induce angiogenesis when transplanted on the quail chorioallantoic membrane (Ekblom, P., H. Sariola, M. Karkinen, and L. Saxén, 1982, Cell Differ., 11:35-39). In these experiments all blood vessels were derived from the quail host, suggesting that kidney endothelium is derived from outside blood vessels. We have now analyzed whether kidney angiogenesis is regulated by kidney-derived soluble factors that stimulate the growth of new blood vessels. In the rabbit cornea, ll-d embryonic kidneys induced angiogenesis, whereas uninduced 11-d kidney mesenchymes did not. To characterize and purify this activity from an embryonic organ, we dissected between 600 and 1,000 14-17-d-old embryonic mouse kidneys for each purification experiment. Growth factor activity for capillary endothelial cells was found to bind to heparin-Sepharose and eluted at 0.9-1.1 M sodium chloride. Gel filtration revealed a molecular weight of 16,000-20,000 of this factor. A major 18,000-mol-wt band was seen after gel electrophoresis and silver staining of partially purified growth factor material. The chromatographed factor is mitogenic for endothelial cells but not for smooth muscle cells and stimulates angiogenesis in vivo in the rabbit cornea. Adult kidneys contained two heparin-binding endothelial cell growth factors. The differentiation-dependent production of an angiogenesis factor by the embryonic kidney suggests an important role of angiogenesis in organogenesis.

Angiogenesis is a central phenomenon in embryogenesis, tissue repair, and tumor formation (2, 3, 16, 17). Angiogenesis-stimulating growth factors have been studied for a number of years, but until recently (9, 15, 34, 35) they have not been well characterized. These factors are usually isolated from adult brain and some tumor cells, but until now efforts to isolate these factors during normal morphogenesis have been very few.

Embryonic kidneys strongly stimulate angiogenesis (1, 11, 12, 31-33). The kidney model system is particularly well suited for analysis of angiogenesis since the formation of capillaries is developmentally regulated. Kidney differentiation is driven by interactions between cells of a different developmental history. The epithelial ureter bud induces the differentiation of the mesenchyme (20) and this in turn leads to a stimulation of blood vessels (12, 31). The mesenchyme can be stimulated to differentiate in vitro, and our previous biological assays suggest that these induced mesenchymes stimulate neovascularization on the chorioallantoic membrane (CAM) (12, 31). Quail CAMs were selected since the quail nucleoli can be used to distinguish quail cells from mouse cells (25). In the CAM assay, quail blood vessels could be seen invading the developing kidney in an organotypic fashion. Moreover, the quail vessels organized themselves histiotypically and deposited a quail type basement membrane (32).

Previous studies thus show that the blood vessels of the kidney are of outside origin. Hence, we thought that the differentiating kidney may produce angiogenesis factors. Since the differentiating mouse kidney can stimulate blood vessels from a different species, the secreted factor apparently has a broad specificity for many endothelial cells. In the present study, we have characterized a soluble polypeptide growth factor from the embryonic kidney. This embryonic kidney-derived angiogenesis factor (EKdAF) specifically stimulates the proliferation of capillary endothelial cells and induces angiogenesis in vivo.

Materials and Methods

Tissues

Embryonic kidneys were dissected from hybrid mouse embryos (NMRI x C57Bl). Day of plug appearance was day 0. Kidneys were stored at -20°C in small amounts of phosphate-buffered saline (PBS), pH 7.1.

Smooth Muscle Cells

To study the cell specificity of the growth factor, we also tested the mitogenic activity for smooth muscle cells. Bovine smooth muscle cells (passage 3, isolated from the aortic media as described in reference 30) were plated par-
allel to the capillary endothelial cells in 24-well plates at a density of 10,000 cells per well in medium containing 10% fetal calf serum. 15 h later, the medium was aspirated and new medium containing 0.5% fetal calf serum and the growth factor was added. 3 d later the cell number was determined as described for the capillary endothelial cells.

**In Vivo Assays of Angiogenesis Activity**

The rabbit cornea assay was performed as described by Gimbrone et al. (18). This assay was used to test whether the embryonic kidney tissues secrete soluble angiogenic activity, and it was also used to verify that the heparin-binding growth factor obtained from the embryonic kidney was angiogenic.

Growth factor activity obtained after heparin-Sepharose affinity chromatography was incorporated into Elvax pellets (an ethylene-vinyl-acetate polymer, a gift from Dr. P. D'Amore at Harvard Medical School, Boston, MA; see reference 23). Rabbit serum albumin was added to the growth factor to achieve a 20% loading of the polymer. Control pellets contained material from heparin-Sepharose fractions that did not show mitogenic activity. Albumin was purified from the serum of rabbits using ammonium-sulfate precipitation and gel filtration on Sephacryl S-300 columns (Pharmacia, Bromma, Sweden). Elvax pellets were prepared as described (23).

**Column Chromatography**

The frozen embryonic kidneys were thawed, and homogenized on ice in 1 ml PBS per 100 kidneys in an all glass homogenizer (loose fitting) in the presence of 1 nM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). After 30 min on ice, the homogenate was centrifuged at 3,000 g for 10 min and the supernatant re centrifuged at 12,000 g for 15 min. The supernatant was directly applied to heparin-Sepharose (10 ml column bed volume; Pharmacia) and Sephacryl S-200 (1 × 90 cm), respectively. The bound material on the heparin-Sepharose column was eluted using a 0.1-2.0 M linear NaCl gradient containing 10 mM Tris, pH 7.0. The absorbance at 280 nm and the conductivity were recorded simultaneously using flow-through cuvettes. Dialysis was performed in spectrapor tubing (6,000-8,000-mol-wt cutoff).

**Growth Factor Activity of Fractions**

Column fractions were analyzed for growth factor activity on 3T3 cells as described (22). Briefly, BALB/c-3T3 cells (clone A31, gift of Dr. P. D'Amore) were plated in 96-well tissue culture plates (Nunc, Roskilde, Denmark) and used when they had formed a quiescent confluent monolayer. Growth factor activity was determined by measuring the incorporation of [3H]thymidine into the DNA after 2-3 d of incubation in the presence of the growth factor. Growth factor activity for capillary endothelial cells was analyzed from the same samples by measuring the increase in number of cells as described by Klagsbrun and Shing (22). Briefly, bovine capillary endothelial cells (passages 6-12; gift of Dr. J. Folkman, Harvard Medical School, Boston, MA) were plated on 24-well plates (Costar, Cambridge, MA) at 10,000 cells per well. 15 h later, new medium was added to the wells together with the fractions to be tested in duplicate. 3 d later the cells were trypsinized and the cell number was determined with a Coulter counter.

**PAGE**

An extract from ~1,000 embryonic kidneys (14-17-d-old embryos) was separated on heparin-Sepharose. Pooled fractions containing the growth factor activity that bound heparin were recycled on heparin-Sepharose. The material of the fractions containing growth factor activity was dialyzed, lyophilized, and separated on a 13-17% SDS polyacrylamide gel (24). Proteins were visualized by silver staining (28). Molecular weight standards were from Bethesda Research Laboratories, (Gaithersburg, MD).

**Results**

**Differentiation-dependent Production of Angiogenic Activity**

On the chorioallantoic membrane (CAM), only kidneys induced to differentiate will become vascularized (31). In the CAM, both insoluble and soluble factors can stimulate angiogenesis. Since the cornea assay is a more reliable assay for soluble factors inducing angiogenesis, we implanted 11-d embryonic mouse kidneys as well as uninduced nephrogenic mesenchyme of the same age into the rabbit corneal pocket (18). The 11-d whole kidney is composed of the nephrogenic mesenchyme and the ureter bud. When the 11-d whole kidney is transplanted, differentiation of the mesenchyme occurs in the cornea as a result of an induction by the ureter bud. In contrast, no such induction of differentiation occurs when the nephrogenic mesenchyme is transplanted without the ureter bud (20, 21, 31). Thus, by transplanting either mesenchyme or mesenchyme together with the ureter bud, we could test whether the inductive interaction between the ureter and the mesenchyme leads to secretion of angiogenic activity. The results demonstrate that the mesenchyme without the ureter does not stimulate angiogenesis (Fig. 1, A and B) whereas the whole kidney with both the ureter and the mesenchyme stimulates angiogenesis in vivo (Fig. 1, C and D).

**Characterization and Purification of EKdAF**

To avoid the dissection of large amounts of the small 11-d embryonic mouse kidneys, we tested extracts of kidneys at different ages of development in the capillary endothelial cell proliferation assay. All extracts were active showing maximal activity from the 15th embryonic day to birth (data not shown). Implantation of 14-d-old kidneys into corneal pockets verified that the larger kidneys were angiogenic. The 14-15-d kidneys are ~20-fold larger than the 11-d old kidneys and they are thus a more convenient source of the factor than the small 11-d-old kidneys. Between 600 and 1,000 kidneys from 14-17-d-old embryos were therefore dissected, collected, and used for each purification experiment. Heparin-Sepharose was used as a crucial step for the purification. The affinity of endothelial cell growth factors for heparin has first been demonstrated by Shing et al. (34). A growth factor for endothelial cells isolated from embryonic kidneys also bound to heparin-Sepharose and eluted at 0.9-1.1 M salt as a single peak (Fig. 2 A). Separation of the crude extract on a Sephacryl S200 gel filtration column showed that a fraction with a molecular weight between 16,000 and 20,000 was responsible for the stimulation of capillary endothelial cells (Fig. 2 B). We then used two cycles of heparin-Sepharose affinity chromatography to further purify this growth factor. This material was subjected to SDS PAGE (24) and silver staining (28). A major band with a molecular weight of ~18,000 was obtained (Fig. 2, C). The partially purified factor stimulated the proliferation of capillary endothelial cells but not smooth muscle cells (Fig. 3).

**EKdAF Is Angiogenic In Vivo**

Heparin-Sepharose-purified growth factor was incorporated into Elvax pellets and implanted in the rabbit cornea. As a control, heparin-Sepharose fractions which did not exhibit mitogenic activity for capillary endothelial cells were simultaneously prepared and implanted. Only the growth factor material showed angiogenesis activity in the cornea (Fig. 4).

**Adult Kidneys Produce Endothelial Cell Growth Factors**

Since endothelial cell growth factors have been demonstrated in a number of adult tissues, we wanted to determine whether adult kidneys also contain endothelial cell growth factors. We
Figure 1. In vivo assay of the production of an angiogenesis factor derived from embryonic kidney. Kidneys were implanted into rabbit corneal pockets as described by Gimbrone et al. (18). Previous experiments by Grobstein and Parker (21) have already shown that uninduced mesenchymes do not differentiate when implanted to the cornea, and these could therefore be used as good controls. (A) From three 11-d-old embryonic kidneys, the ureter buds were dissected out, and the remaining mesenchymes were implanted to the right eye. (B) Drawing traced from Fig. 1 A of the limbus blood vessels underneath the area of implantation. (C) Three 11-d-old embryonic kidneys, which contain both the mesenchymes and the inducer tissue (the ureter buds), were implanted to the left eye. (D) Drawing traced from Fig. 1 C showing the blood vessels penetrating the cornea in the direction of the implanted kidneys. The vessels penetrating the avascular cornea were first observed after 4 d in C, but not in A. Both pictures were taken 9 d after implantation. Bars, 400 μm.

Therefore analyzed extracts of adult mouse kidneys after separation on heparin-Sepharose for growth factor activity on 3T3 cells and capillary endothelial cells. We found two capillary endothelial cell growth factor activities that bound to heparin-Sepharose and eluted at 0.8–1.0 and 1.2–1.4 M NaCl, respectively (Fig. 5).

Discussion

Our data presented here show that the differentiating kidney secretes a heparin-binding growth factor that stimulates the proliferation of endothelial cells. The factor is apparently soluble, since only soluble factors reach the limbal blood vessels in the corneal pocket assay used here. In the developing kidney, the secretion of angiogenic activity is thought to be differentiation dependent (31). The kidney is known to develop as a result of an inductive interaction between the nephrogenic mesenchyme and an inducer tissue (20). The induced cells then in turn start to attract a third cell lineage, the endothelium (11, 12, 31–33). The results of the cornea assays suggest that an induction of the nephrogenic mesenchyme leads a secretion of angiogenic activity. When the nephrogenic mesenchyme is implanted into the cornea together with its inducer, angiogenic activity can be demonstrated, but no such activity is seen when the nephrogenic mesenchyme is implanted without the inducer. The angiogenic activity may be due to a secretion of the heparin-binding growth factor that we have characterized from the older embryonic kidneys. However, direct evidence that induction of differentiation of the mesenchyme would lead to a secretion of the factor is not available. A differentiation-dependent secretion of angiogenesis factors has previously been reported for adipocyte cells by Castelliot et al. (7). However, the factor secreted by the differentiating adipocytes is apparently a prostaglandin (10) and shows no similarity with other angiogenesis factors found in tumors and in the central nervous system (4–6, 8, 9, 15, 19, 22, 26, 27, 34, 37).

It was found that older differentiating kidneys also secreted
Figure 2. Characterization and purification of EKdAF. (A) An extract from 600 embryonic mouse kidneys was applied to a heparin-Sepharose column. The bound material was eluted using a 0.1-2.0 M linear NaCl gradient containing 10 mM Tris, pH 7.0. (B) An extract from 120 15-d-old kidneys was separated on a 1 x 90-cm Sephacryl S200 column in 0.5 M NaCl, 10 mM Tris, pH 7.0. Fractions were analyzed for growth factor activity on 3T3 cells and capillary endothelial (CE) cells. (C) PAGE of growth factor material from 1,000 embryonic kidneys that had been purified by two cycles of heparin-Sepharose chromatography (see Materials and Methods). Indicated molecular weight standards were as follows: ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300; insulin, 6,200.

This factor. This is not surprising since the differentiation of the kidney involves a continuous growth with a very strong ingrowth of blood vessels throughout embryogenesis. The older, far bigger embryonic kidneys were used to partially purify this factor. It is not known whether this factor is the only angiogenesis factor produced by the embryonic kidney.

It has previously proved difficult to characterize angiogenesis factors, and until recently no clearly defined factors were known. However, it has now been found that angiogenesis factors bind to heparin and they can be purified by a single step purification by affinity chromatography on heparin-Sepharose columns (34). Acidic and basic endothelial cell growth factors have been isolated from adult brain tissue, and different names had been given to them. Most present evidence suggest that the heparin-binding growth factors are different forms of fibroblast growth factor (FGF). Interestingly, a truncated form of FGF has now been found in the adult kidney (4). The EKdAF is similar to the anionic hepa-
Figure 3. Cell specificity of EKdAF. (A) Heparin-Sepharose-purified EKdAF was analyzed for growth factor activity on capillary endothelial (CE) cells. Bar 1, control medium alone; bar 2, EKdAF; bar 3, an extract from bovine retinas (prepared as described in reference 9) was used at maximal activity as a positive control. (B) Bovine smooth muscle cells (SMC) were plated parallel to the capillary endothelial cells, and growth factor activity was determined as described in Materials and Methods. Bar 1, control medium alone; bar 2, same amount of EKdAF as in A; bar 3, 20% fetal calf serum was added as a positive control. In contrast to the purified EKdAF, the crude extract stimulated the proliferation of smooth muscle cells to a small extent.

Figure 4. In vivo assay of purified EKdAF. Heparin-Sepharose-purified EKdAF (from 20 embryonic kidneys) in Elvax pellets and control pellets were implanted into rabbit corneas. Vessels penetrated the cornea of the EKdAF-containing eye 4 d after implantation. Pictures were taken after 15 d. (A) Control. (B) EKdAF. Bars, 1 mm.
tissues. Their functions in normal adult tissues such as brain or the kidney are as yet not clear. A factor derived from adult kidneys has recently been characterized, and the amino acid sequence is identical to the basic pituitary FGF (4).

However, adult kidneys are not angiogenic, and there is normally no active vascularization process in the adult kidney. In contrast to adult tissues, vascular beds in embryonic tissues are constantly expanded and remodeled, and this process is instrumental for organogenesis. We have here shown that the embryonic kidney contains an angiogenic factor, EKdAF. It is difficult to understand why embryonic but not adult kidneys are angiogenic, although they both contain endothelial cell mitogens. It is possible that adult tissues contain inhibitors of angiogenesis.

Like other endothelial growth factors, the embryonic angiogenesis factors that we have analyzed (29; this report) share the affinity for heparin, supporting the hypothesis of Klagsbrun and Shing (22) that the various endothelial cell growth factors may have in common a structural domain that is involved in binding to heparin. Compared with the angiogenesis factor from the embryonic brain (29), EKdAF elutes from heparin-Sepharose at a lower salt concentration, suggesting that these two embryonic angiogenesis factors are different.

The detection of an angiogenesis factor in embryonic tissues known to stimulate vessel ingrowth at a well-defined developmental stage suggests that angiogenesis factors play a role in embryonic development. It is noteworthy that it could be detected from the small embryonic tissues, since much more material is usually used to isolate angiogenesis factors from tumors or adult tissues. Hence, embryonic tissues could be potent producers of angiogenesis factors. Because soluble mitogens transported by the blood are required for growth of the embryonic organs (13, 36), it is essential for the growing organs to develop a vascular bed at early stages. The secretion of embryonic angiogenesis factors may be of fundamental importance in the ordered development of the vasculature. Similar suggestions have recently been made for another factor, named angiogenin, which is also thought to be angiogenic (35). However, this factor has no similarity with EKdAF, and no studies on embryonic tissues were performed.

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