Kidney International, Vol. 53 (1998), pp. 970–978

Basic fibroblast growth factor is a morphogenic modulator in kidney vessel development

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Basic fibroblast growth factor is a morphogenic modulator in kidney vessel development. During kidney organogenesis the development of renal vessels must be synchronized with the maturation of nephrons and the collecting duct system. Several reports showed that hormones and mitogenic peptides as basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) are involved in this regulatory process. It is a known fact that bFGF receptors are expressed by differentiating tubular epithelium and mesenchyme, but little information is available about the function of bFGF in kidney organogenesis. The role of bFGF during kidney development was investigated using an organotypic culture system and immunohistological techniques. Renal cortex explants were prepared from the kidneys of neonatal rabbits with a microsurgical method, retaining the natural tissue composition. The explants were cultured serum free under continuous medium perfusion. Our results indicate a new and unexpected role of bFGF during the differentiation process. When bFGF alone was applied, vessels could no longer be detected. The inhibitory influence of bFGF could be overcome by addition of VEGF or hormones such as retinoic acid and aldosterone/vitamin D_3. The combination of these factors with bFGF resulted in the expression of small vessel-like structures. We conclude that bFGF has a morphogenic rather than a mitogenic function during kidney vessel development.

The development of vessels may depend on a number of very different mechanisms. Whether new capillaries are formed by sprouting from vessels, by the conversion from mesenchymal to endothelial cells or by the insertion of new walls in existing vessels depends on the organ and its developmental stage as well as on a large number of regulatory molecules [1–3]. The requirements for the different mechanisms of vessel formation are basically very similar; however, all of them depend on a partial remodeling of the extracellular matrix, endothelial cell proliferation, cell migration and cell differentiation. In every case, the sequence of these steps has to be initiated, coordinated and finally terminated.

Numerous soluble factors have been described that stimulate the proteolytic degradation of the extracellular matrix [4] or the synthesis of new matrix material [5–8]. These factors may also induce endothelial proliferation and migration [9–12]. Mitogens and chemoattractants stimulating endothelial cells are synthesized by a number of different cells [1, 13, 14]. Many of these substances are small peptides like vascular endothelial growth factor (VEGF), which acts on endothelial cells with high specificity [15, 16], or as basic fibroblast growth factor (bFGF), which possess a low endothelial specificity [14].

Hormones such as retinoic acid or vitamin D_3 have been known for many years for their ability to induce differentiation [17, 18]. First indications of the vital role of retinoic acid in kidney organogenesis came from feeding experiments with pregnant animals [19]. These results were confirmed recently when kidney agenesis or malformations were observed in retinoic acid receptor deficient transgenic mice [20]. However, retinoic acid receptors could not yet be detected within the embryonic kidney. In contrast, aldosterone and vitamin D_3 receptors are expressed in large amounts by the cells of the developing collecting duct system [21, 22]. Furthermore, the vitamin D receptor was found in organotypically cultured mouse metanephroi [23]. Correspondingly, a stimulating activity of aldosterone on the differentiation of collecting duct epithelium was observed [24]. Otherwise, the physiological function of vitamin D_3 in the collecting duct epithelium at this stage of kidney development is still unknown.

In this study a tissue culture system was used to investigate whether hormones and peptide growth factors act directly on renal cells during kidney vessel development. The nephrogenic zone of the kidney of neonatal rabbits was microsurgically prepared [25] and cultured for extended periods. Proteases were not applied. With this method, both the complex cellular composition of the tissue and its extracellular matrix remained unchanged. The tissue pieces were cultured under serum free conditions with continuous medium perfusion. The culture medium was supplemented with defined concentrations of bFGF, VEGF and different hormones to analyze the modulating effect of these molecules on the development of the renal vascular network.

METHODS

Tissue culture

Tissue explants were prepared from kidneys of one- to three-day-old rabbits. The animals were killed by cervical dislocation, and the organs were removed immediately. The *capsula fibrosa* was stripped off under sterile conditions [25]. The nephrogenic zone, composed of collecting duct ampullae, mesenchymal cells, endothelium and nephrons in different developmental stages, remained attached to the fibrous organ capsule.

Key words:: growth factor, tissue culture, vascular differentiation, kidney, fibroblasts.

Received for publication August 5, 1997
and in revised form November 13, 1997
Accepted for publication November 14, 1997

Table 1. Stimulation of vessel development in renal explants

Medium supplement	Length of supplement application	Length of culture	Single endothelial cells	Endothelium arranged in clusters	Vessel-like structures
Without supplements	13d	13d	+	+	−
BSA, 0.1 mg/ml	13d	13d	+	+	−
bFGF, $5.5 \times 10^{-12} - 5.5 \times 10^{-10}$ M	13d	13d	−	−	−
bFGF, $5.5 \times 10^{-12} - 5.5 \times 10^{-10}$ M	1d	14d	+	+	−
Aldosterone, 10^{-7} M	13d	13d	+	−	(+)
Vitamin D_3, 10^{-9} M	13d	13d	+	−	(+)
Aldosterone, 10^{-7} M +Vitamin D_3, 10^{-9} M[a]	13d	13d	+	−	++
9-cis Retinoic acid, 3.3×10^{-7} M	3h	13d	+	−	++
VEGF, 1.25×10^{-10} M[a]	13d	13d	+	−	++
bFGF, 5.5×10^{-11} M +Aldosterone + Vitamin D_3[a]	13d	13d	+	−	++
9-cis Retinoic acid +bFGF, 5.5×10^{-11} M	3h 13d	13d	+	−	++
bFGF 5.5×10^{-11} M +VEGF, 1.25×10^{-10} M	13d	13d	+	−	(+)

Symbols are: − not detectable, + detectable, (+) network partially preserved, ++ network completely preserved. Abbreviations are: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; VEGF, vascular endothelial growth factor. Renal explants were cultured for 1 to 14 days under continuous medium perfusion. The serum-free culture medium was supplemented with various hormones or growth factors.

[a] Data published previously [28].

The explants were mounted in tissue carriers and inserted into perfusion containers ([24]; Minucell and Minutissue, Bad Abbach, Germany) that were placed on a warming plate (37°C; Medax, Kiel, Germany). The tissue was cultured for up to 13 days under continuous medium flow. Fresh culture medium was pumped through the chamber at a flow rate of 1 ml/hr. Experiments were conducted with IMDM culture medium (Gibco BRL-Life Technologies, Eggenstein, Germany), supplemented with 0.1 mg/ml bovine serum albumin (BSA; Sigma, Deisenhofen, Germany), and hormones/growth factors as specified below. The medium was stored at 4°C and prewarmed to 37°C immediately before reaching the perfusion culture container.

Hormone and growth factor experiments

The culture medium was supplemented with basic fibroblast growth factor (PBH, Hannover, Germany) at concentrations of 5.5×10^{-12} M, 5.5×10^{-11} M and 5.5×10^{-10} M (equivalent to 0.1, 1 and 10 ng/ml; Table 1). Purified vascular endothelial growth factor ($VEGF_{164}$), a generous gift of Prof. Dr. H. Weich, GBF Braunschweig, Germany, was added in a concentration of 1.25×10^{-10} M (equivalent to 5 ng/ml). Hormones were applied alone or in combination at the following concentrations: 1×10^{-7} M aldosterone (Ciba Geigy, Basel, Switzerland), 1×10^{-9} M 1,25-dihydroxyvitamin D_3 (Biomol, Hamburg, Germany), 3.3×10^{-7} M 9-cis retinoic acid (Sigma). Furthermore, basic fibroblast growth factor (bFGF) was applied in combination with 1×10^{-7} M aldosterone and 10^{-9} M 1,25 dihydroxyvitamin D_3. In another set of experiments freshly prepared tissue was stationary preincubated for three hours with 3.3×10^{-7} M 9-cis retinoic acid prior to perfusion culture with 5.5×10^{-11} M bFGF (equivalent to 1 ng/ml). All culture experiments were performed at least in triplicate.

Kinetic experiments

To analyze the time course of bFGF stimulation kinetic experiments were carried out. Basic fibroblast growth factor (bFGF) was applied for 1 to 13 days under perfusion culture conditions. Tissue pieces were sampled daily and stored in liquid nitrogen prior to further analysis.

In another set of experiments explants were stationary precultured with various concentrations of bFGF (0.1 to 10 ng/ml) for one day and further cultured with basic culture medium only for 13 days.

Immunohistological analysis

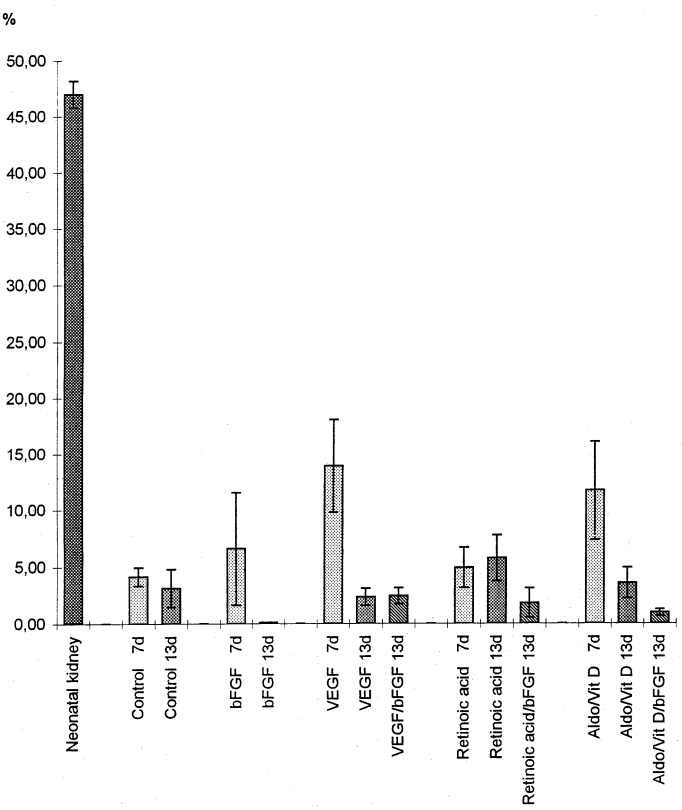
Following perfusion culture the explants were washed in basic medium, placed in a drop of TissueTek O.C.T. 4583 Compound (Miles, Elkhart, USA) and frozen in liquid nitrogen. Corticomedullary oriented sections of 8 μm thickness were cut with a cryomicrotome (Micron, Heidelberg, Germany).

The fixation and incubation procedure for the detection of antigens has been described in detail elsewhere [26]. In brief, a solution of 4.2% paraformaldehyde (Merck, Darmstadt, Germany), 16% picric acid (Fluka, Buchs, Switzerland), 0.002% cobalt chloride and 0.1% glutaraldehyde (Serva, Heidelberg, Germany) in phosphate buffered saline (PBS; pH 7.2) was applied for fixation. Then the sections were incubated in blocking buffer (0.1 M Tris-buffer, pH 7.4, 25% fetal calf serum, 1% NaCl, 1% Triton X-100) for 45 minutes.

The monoclonal antibody EC1 [26] was used to detect endothelial cells. EC1 is specific exclusively for rabbit endothelial cells. Proliferating cells were labeled using the monoclonal antibody Ki-67 [27]. Primary antibodies were applied as undiluted culture supernatants overnight.

Biotin-conjugated donkey anti-mouse Ig antiserum (Dianova, Hamburg, Germany) was used to detect bound primary antibody. Finally, avidin conjugated with horseradish peroxidase (Vectastain; Vector, Burlingame, CA, USA) was applied, and the enzyme reaction was started by the addition of the substrate solution (0.5 mg diaminobenzidine; Sigma; 0.1 M Tris, pH 7.4, 0.002% cobalt chloride, 0.04% nickel chloride, 0.012% H_2O_2). The sections were embedded in DePeX (Serva) and analyzed with a Zeiss Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

Table 2. Cell proliferation after growth factor and hormone application



Cell proliferation was detected by incubation of explant sections with the monoclonal antibody Ki-67. The Ki-67 antigen is expressed by all proliferating cells. To measure the decrease of cell proliferation, the percentage of Ki-67 labeled section area has been determined. At least 3 explants per experiment and 6 sections per explant have been analyzed. The standard deviation of the mean is indicated.

Controls: To determine the amount of proliferating cells within the nephrogenic zone, section of the neonatal kidney has been analyzed. Explants cultured for 13 days in the presence of 0.1% bovine serum albumin were used as control for tissue cultured without growth factor or hormone supplement.

Agfa Pan 25 film (Agfa, Leverkusen, Germany) was used for photographic documentation.

Specificity controls

In order to ensure the specificity of the antibody labeling different controls were included in the experiments. Pre-immune serum as well as irrelevant primary antibodies were applied. Furthermore, control sections were incubated with the detecting antibody and the enzyme complex alone. None of these control sections showed any positive antibody labeling of the vessels.

A commercially available endothelium-specific antibody (PAL-E; Progen, Heidelberg, Germany; [28]) was applied to verify the absence of endothelial cells in bFGF stimulated explants.

Evaluation of cell proliferation

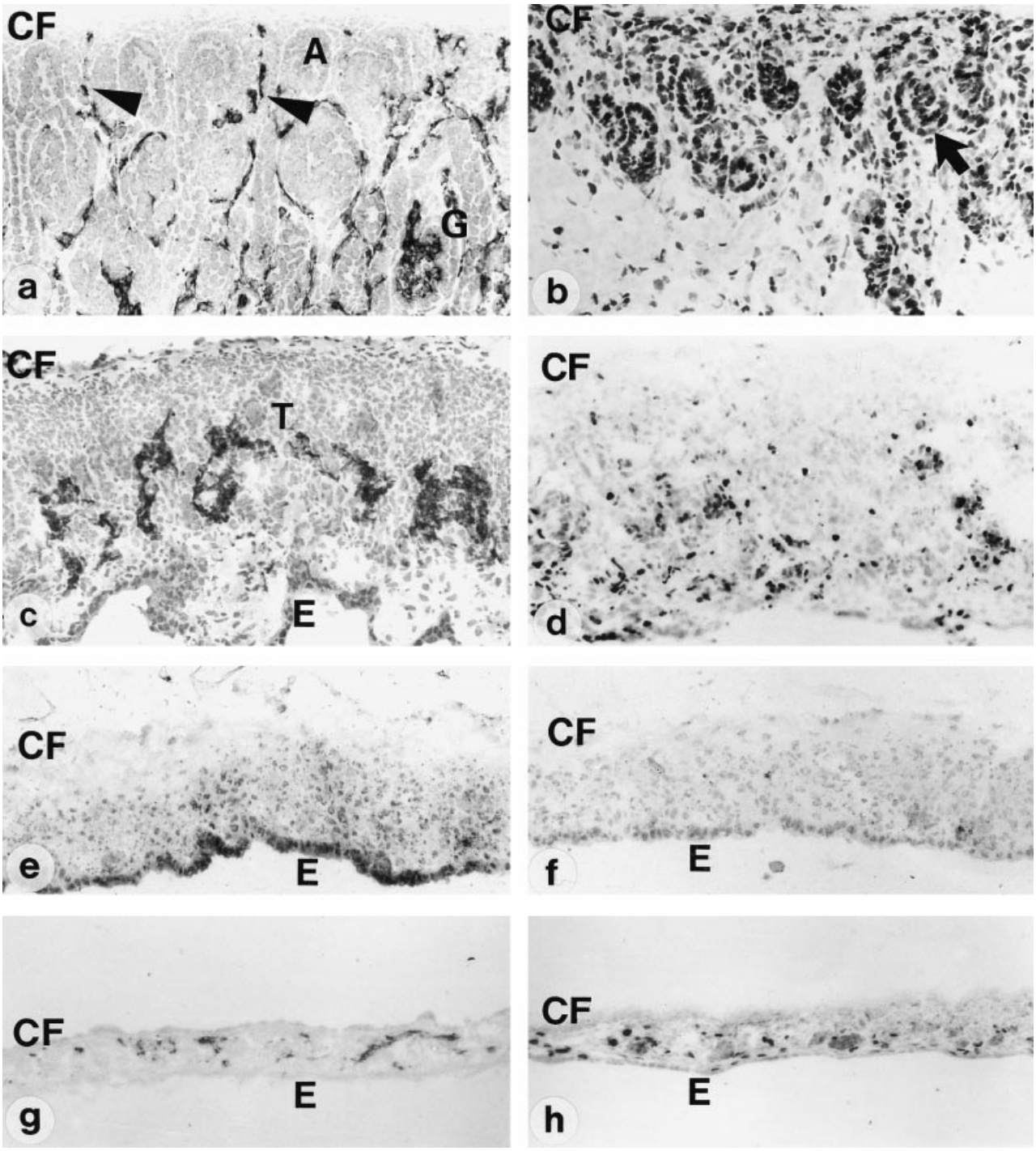
To determine the amount of cell proliferation, longitudinal sections of cortex explants were probed with the monoclonal antibody Ki-67, which recognizes a nuclear antigen specifically expressed by proliferating cells [27]. Proliferating cells were observed within all parts of the explants, but featured an inhomogeneous distribution. Therefore, we determined the labeled area of the section instead of counting single cells (Table 2). Area measurements were performed on digitized video images with the image-processing program SOM 462 (BioRad, Munich, Germany). Proliferation activity in the tissue explants is expressed by the percentages of the Ki-67 labeled areas. At least three explants were analyzed for each experiment, and six sections of each explant were evaluated. Percentages of labeled areas +/− SDS are given in Table 2.

RESULTS

Culture strategy

To investigate the cellular interactions during renal vessel development a tissue culture model was used. Tissue explants were prepared from neonatal rabbit kidneys by a microsurgical method [25]. Proteases were not applied, leaving the natural

Fig. 1. Endothelial cells and cell streaks as well as vessels within the nephrogenic zone of the neonatal kidney are shown, which have been labeled by the monoclonal antibody EC1 (*a*). The monoclonal antibody EC1 was used for the detection of vessels of different developmental stages. The developing vascular network showed a high degree of regular spatial organization. Endothelial cell streaks (arrowheads) running parallel to each other towards the organ capsule (CF) were frequently observed. Abbreviations are: A, collecting duct ampulla; G, glomerulus (magnification ×175). (*b*) Proliferating cells of the nephrogenic zone were detected by the monoclonal antibody Ki-67, which recognizes a nuclear antigen specifically expressed by proliferating cells. S-shaped bodies (arrow) and collecting duct ampullae were intensively labeled. Beyond the nephrogenic zone the number of proliferating cells decreased markedly (magnification ×175). (*c*) The nephrogenic zone was prepared and put in culture. The explants were cultured under continuous medium flow (1 ml/hr) for 13 days. Endothelial cell clusters labeled by EC1 were detected within explants which were cultured with basal medium alone (IMDM, 0.1% bovine serum albumin). While the endothelial antigen EC1 was continuously expressed in the absence of hormones and growth factors, the regular organization of the developing vascular network was not preserved. Abbreviations are: CF, fibrous organ capsule; E, collecting duct epithelium; T, cross section of tubule (magnification ×140). (*d*) A considerable number of proliferating cells were labeled by Ki-67 within explants cultured for 13 days with basal medium alone. Abbreviation is: CF, fibrous organ capsule (magnification ×140). (*e*) No endothelial cells were detectable within renal explants which were cultured for 13 days in the presence of bFGF. All endothelial structures observed within the nephrogenic zone (a) have disappeared. Tubular structures were absent as well. The tissue was of homogeneous composition (magnification ×140). (*f*) Proliferating cells could not be detected after 13 days of bFGF stimulation. The anti-proliferative effect of bFGF was underlined when the thickness of these explants was compared with other samples. bFGF-treated explants were considerably thinner (150 μm) than tissues cultured in the presence of BSA (c) or hormones (300 μm) (magnification ×140). (*g*) Kinetics of endothelial cell disappearance. At culture day 7 a few endothelial cells were detected in bFGF-stimulated tissue. A regular spatial organization of the endothelial cell streaks was not observed at this time point (magnification ×140). (*h*) Cell proliferation decreased continuously, but some proliferating cells labeled by the monoclonal antibody Ki-67 were detected within this explant. Other samples taken at culture day 7 already failed to show Ki-67 positive cells. Abbreviations are: CF, fibrous organ capsule; E, collecting duct epithelium (magnification ×140).



arrangement of the different tissue components and the organ-specific extracellular matrix unchanged.

The tissue was cultured under permanent medium exchange. Neither metabolites, nor paracrine factors can accumulate in the culture container as long as the tissue is continuously supplied with fresh medium. This perfusion culture method allows for the complete omission of serum supplement to the culture medium [29]. The influence of paracrine and endocrine growth and differentiation factors on the development of the renal vessel system could thus be analyzed without interference with unknown serum components.

Tissue explants cultured with IMDM medium alone or in the presence of 0.1% BSA showed no necrotic lesions even after 13 days (Fig. 1c and Table 1). In these explants the endothelial cells were arranged in large clusters but not in vessel-like structures. The typical three-dimensional structure of the developing renal vascular network (Fig. 1a) [30] could only be preserved in the presence of the growth and differentiation factors (Table 1 and Fig. 2).

Modulation of renal vessel development by basic fibroblast growth factor

From the reports of many authors we know that bFGF is an angiogenic factor [14, 31]. In order to analyze the influence of bFGF on renal vessel development, tissue explants were cultured serum free in the presence of bFGF. The application of culture medium supplemented with BSA and bFGF resulted in the disappearance of vessel-like structures (Fig. 1e). The endothelium-specific monoclonal antibody EC1 failed to label cell streaks like those observed within the nephrogenic zone of the kidney (Fig. 1a). Only few EC1-positive cells were detected within bFGF incubated explants. The incubation of these explants with a different endothelium-detecting antibody (PAL-E) confirmed this result (data not shown). PAL-E did not label any cells within these explants.

Another unexpected observation was that explants cultured with bFGF in a concentration range from 5×10^{-12} to 5×10^{-10} M were considerably thinner (less than 200 μm; Fig. 1 e, g) than those cultured with the hormone combination aldosterone/vitamin D_3 or with retinoic acid (Fig. 2, 300 to 500 μm). Furthermore, the bFGF-treated tissue was of a homogeneous composition. The typical collecting duct ampullae or renal tubules that were found in explants cultured with BSA (Fig. 1c) or hormones (Fig. 2) were not observed. However, a single collecting duct epithelium always completely covered the side of the explant which was not coated by the fibrous organ capsule.

Time course of endothelial cell disappearance

In another set of experiment the time course of endothelium disappearance was analyzed. Explants cultured for 1 to 13 days under the influence of bFGF were sectioned and incubated with EC1 and the proliferation marker Ki-67. After one day of stimulation with bFGF no morphological difference could be detected between the developing vascular network and freshly prepared explants. After seven days of culture in the presence of bFGF the vessels tended to disappear, and the endothelial cells formed small clusters (Fig. 1g). Extinction of endothelial cells was completed after 13 days (Fig. 1e). Not even EC 1-positive cell clusters, like those found in explants cultured with BSA (Fig. 1c) were detectable anymore.

To investigate whether the disappearance of endothelial cells was due to the permanent application of bFGF, some explants were preincubated for 24 hours with bFGF and then perfusion cultured with basic medium only for 13 days. These explants were approximately 300 μm thick and were characterized by endothelial cell clusters (Table 1). These samples could not be distinguished from explants cultured with BSA alone. Even after the application of higher concentrations of bFGF (5×10^{-10} M) no complete extinction of endothelial cells from the cultured tissue was observed. The spatial organization of the developing vascular network was disturbed in the same way as in control explants cultured with BSA alone. We have to conclude that the extinction of endothelial cells from the explant was coupled to the continuous presence of bFGF.

Stimulation of renal vessel development by retinoic acid

Retinoic acid is a known potent differentiation factor and an important factor for kidney development [18]. To investigate whether the hormone is directly involved in renal vessel formation, it was tested in our tissue culture system. Retinoic acid was applied for 1 to 24 hours. This short stimulus was sufficient to initiate a tremendous broadening of vessel-like structures in the explants (Fig. 2a and Table 1). Moreover, the regular spatial organization of the vascular network was preserved after 13 days of culture. Broad endothelial cell streaks showed an orderly spatial distribution. Retinoic acid stimulated explants were characterized by numerous tubular structures that were closely surrounded by endothelial cell streaks.

Development of the renal vascular network in the presence of growth and differentiation factors combined with basic fibroblast growth factor

Further experiments were performed to investigate whether the inhibitory effect of bFGF could be overcome by other growth and differentiation factors. The bFGF was applied in combination with retinoic acid, with aldosterone and vitamin D_3, or with VEGF (Table 1). These substances have been used successfully for the stimulation of renal vessel development under serum free perfusion culture conditions [26, 27].

All of these supplements were able to stimulate vessel development in the presence of bFGF. When bFGF was combined with retinoic acid or aldosterone/vitamin D_3, vessel-like structures in general were thinner than in the explants that were cultured without bFGF. This effect was prominent in retinoic-acid-stimulated tissue (Fig. 2c). Small vessel-like structures were detected in these explants, while retinoic acid alone led to a considerable broadening of the endothelial cell streaks. When both retinoic acid and bFGF were applied, smaller vessel-like structures were detected. The three-dimensional structure of the developing vessel network was also preserved.

Another interesting result of these experiments was the detection of another endothelial cell generation. Only in explants stimulated with bFGF and retinoic acid, a new generation of endothelial cell streaks was detected (Fig. 2c). These cells were located adjacent to the fibrous organ capsule. They were separated from the other vascular elements by a zone (30 to 50 μm) of unlabeled cells. These explants showed a large number of tubules and collecting duct ampullae. A single collecting duct epithelium covered one side of the explant, while the other side was covered by the fibrous organ capsule. A homogeneous composition of the explant as seen after bFGF stimulation alone was never observed.

Vascular endothelial growth factor (VEGF) is produced in the maturing kidney [1], and the developing renal vessels are stimulated by this angiogenic factor (Table 1) [29]. When VEGF and bFGF were applied in combination, the extinction of endothelial cells from the explant could be prevented. Endothelial cells arranged in clusters or streaks could be detected (Fig. 2e) after 13 days of culture with VEGF and bFGF. However, an orderly network arrangement of endothelial cells like that abundant after hormone treatment was not found in these explants.

Modulation of cell proliferation

It has been mentioned above that bFGF treated explants were considerably thinner than hormone or VEGF stimulated tissue. Freshly prepared explants were 100 to 150 μm thick. Following bFGF application no explants were obtained that were thicker than 200 μm (Fig. 1e), while hormone stimulated explants in general were thicker than 300 μm (Fig. 2). In order to investigate

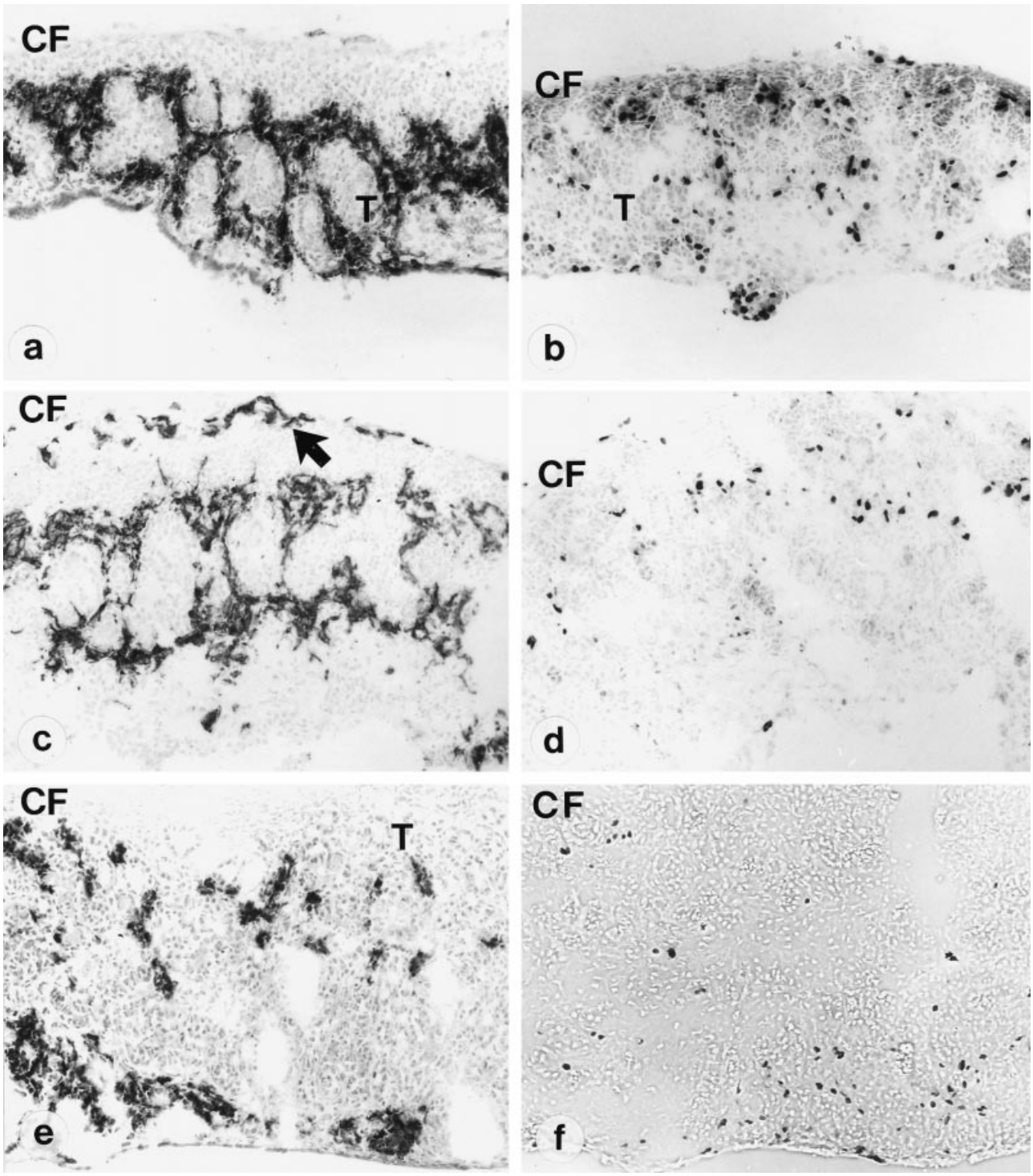


Fig. 2. Stimulation of vessel development by basic fibroblast growth factor (bFGF) combined with retinoic acid and vascular endothelial growth factor (VEGF). Explants cultured for three hours with 9-cis retinoic acid and subsequently with basic medium for 13 days were characterized by broad endothelial cell streaks (*a*), which enclose tubular structures (T). Proliferating cells (*b*) were detectable even after 13 days of culture. The combination of a preculture of the explants with retinoic acid and subsequent perfusion culture in the presence of bFGF (5.5×10^{-11} M) resulted in considerably smaller endothelial cell streaks (*c*). Beneath the fibrous organ capsule another generation of endothelial cells (arrow) could be detected. Few proliferating cells (*d*) could be observed after 13 days. When explants were treated with VEGF and bFGF the inhibitory effect of bFGF could be overcome as well (*e*). However, the endothelial cells were arranged in clusters, not in form of cell streaks (*f*). Some proliferating cells were detectable within these explants after 13 days of culture. Abbreviations are: CF, fibrous organ capsule; T, cross section of tubule. The magnification for all panels was the same: ×140.

whether the thickening of the explants correlates with the rate of cell proliferation, sections were incubated with the proliferation marker Ki-67 [27].

Within the nephrogenic zone of the cortex of the neonatal kidney nearly 50% of the section area was labeled by the antibody (Fig. 1b and Table 2). Proliferating cells within S-shaped bodies, collecting duct ampullae and mesenchyme were detected by Ki-67. Beyond the nephrogenic zone a remarkable decrease of cell proliferation was observed.

In cortex explants proliferating cells could be detected throughout the whole culture period (Fig. 1 d, f and Fig. 2 b, d, f), but the number of proliferating cells decreased considerably after the first seven days of culture (Fig. 1h and Table 2). The decrease of the cell proliferation clearly depended on the medium supplements.

A sharp decrease of proliferation was detected after seven days of culture with BSA. Less than 5% of the section area showed Ki-67 labeling (Table 2), but proliferating cell could be detected even after 13 days (3% of the section area; Fig. 1d).

Basic fibroblast growth factor-stimulated explants cultured for 7 and 13 days were analyzed for Ki-67 labeled cells. Cell proliferation decreased, but was still detectable in some of these explants on day 7 (Fig. 1h and Table 2). Then proliferation ceased completely and was not initiated again by day 13 of culture.

After application of retinoic acid cell proliferation decreased but could be detected during the whole culture period (Fig. 2b and Table 2). Retinoic acid-stimulated explants were approximately 400 μm thick. Compared with VEGF or aldosterone/vitamin D_3 stimulated tissue these explants showed the highest percentage of proliferating cells (5.7% labeled section area) after 13 days of culture. When VEGF or aldosterone and vitamin D were applied more than 10% labeled section area was measured at culture day 7, but then the proliferation rate decreased, and at culture day 13 less than 5% labeled area was found in tissue sections of these explants. The combined application of VEGF and bFGF did not result in a further reduction of the proliferation rate (Table 2).

When retinoic acid or aldosterone/vitamin D_3 were applied in combination with bFGF a significant reduction of cell proliferation took place (Table 2). The inhibitory effect of bFGF was most obvious when retinoic acid and bFGF were combined. While 5.7% of the section area was marked by Ki-67 when retinoic acid was applied alone, it was reduced to 1.8% when bFGF was added.

DISCUSSION

Known functions of basic fibroblast growth factor and its role in renal vessel development

The basic fibroblast growth factor (bFGF) fulfills a multitude of different functions in a variety of cells during organogenesis. bFGF has been described as a mitogen, a chemoattractant and a differentiation-promoting agent [10, 14, 31]. These various effects are mediated by different receptors and receptor isoforms [32] with tyrosine kinase activity. The receptor specificity of the different forms of FGFs is low. bFGF, for example, binds to three different FGF receptors with comparable affinity [32]. The expression of the FGF receptors is developmentally regulated [33, 34]. Remarkably, FGF receptor 1 is expressed mainly by the epithelia of the developing kidney, while FGF receptor 2 is restricted to the mesenchyme. In contrast, FGF receptors have not been detected on endothelial cells up to now [34], but are expressed by all renal epithelial cells during development [14]. bFGF has been discussed as an essential factor for epithelial-mesenchymal induction during the amphibian development [35]. However, it is not known whether this is the function of bFGF during nephron induction.

On the other hand, bFGF is a potent angiogenic factor stimulating the proliferation and migration of endothelial cells [31]. In view of these data the results of bFGF application in the renal tissue culture system were rather surprising. The application of biologically active bFGF in a complex tissue environment and under serum free culture conditions resulted in the obvious extinction of endothelial cells, visualized by the homogeneous tissue composition as well as the failure of different endothelial detecting antibodies to label cells in the tissue explant. As shown in the present investigation this process followed a set time course and depended on the permanent presence of bFGF (Table 1). The disappearance of endothelial cells could not be induced by a single dose of bFGF. The inhibitory effect of bFGF is further illustrated by the reduced thickness of bFGF-stimulated explants, and the complete cessation of cell proliferation after 13 days. bFGF was the only tested substance which completely abolished cell proliferation (Table 2). Even control explants treated with basic medium alone showed proliferating cells after 13 days. Consequently, explants stimulated with bFGF were considerably thinner than hormone stimulated tissues. In our serum-free tissue culture model, bFGF showed an anti-proliferative effect. Moreover, bFGF even caused the complete extinction of endothelial cells when permanently applied.

It has already been reported that the influence of a growth factor on the cellular reaction might depend on the test system used [31]. This may prove important especially in tissue pieces with a complex composition which were treated with bFGF under serum free culture conditions. We may assume that other cellular components than endothelial cells reacted to bFGF stimulation, eventually inducing the release of inhibitory substances. The mesenchyme itself or the epithelium of the collecting ducts are likely candidates for a direct bFGF stimulation, because these cells have been shown to express FGF receptors [33, 34]. Reports of a tight cooperation between mesenchymal and epithelial cells of the nephrogenic zone during renal organogenesis are numerous [12, 36]. Recently, the production of the angiogenic factor VEGF by the collecting duct epithelium was described [37]. However, for proper organ development not only mitogenic signals are required. On the contrary, cell proliferation has to be tightly regulated and terminated. This could be achieved by contact inhibition as well as by soluble inhibitors. Whether bFGF itself inhibits cell proliferation or whether it induces the production of inhibitory molecules has to be investigated.

The stimulating effect of endocrine and paracrine factors on developing renal vessels can be modulated by bFGF

As shown in the present study, bFGF alone does not stimulate proliferation of the renal endothelium or the preservation of its regular spatial organization. The combined application with VEGF promoted endothelial proliferation but was not sufficient to preserve the spatial organization of the developing vascular network completely (Table 1). It is concluded that other molecules in addition to bFGF and VEGF must be involved in renal vessel development.

Retinoids are potent stimulators of differentiation [18]. An expression of retinoid receptors in the kidney has not been

reported yet. However, transgenic mice expressing double null mutations of retinoid receptors showed severe kidney defects [20]. Retinoic-acid-deficient diets during pregnancy lead to malformations of the kidney [19]. In contrast, the application of high concentrations of retinoic acid during pregnancy caused urogenital defects [38]. Thus, a defined retinoid concentration seems to be required for proper development of the kidney.

Recently, it was reported that the incubation of kidney rudiments with retinoic acid derivatives leads to a considerable stimulation of organ growth by collecting duct branching [39]. These data are confirmed by the present experiments. The proliferation of endothelial cells in the nephrogenic zone is strongly stimulated by retinoic acid. The three-dimensional organization of the vascular network within this tissue region is preserved, while the vessel-like structures are considerably broadened. Thus, retinoic acid as well as the hormone combination aldosterone/vitamin D_3 [29] provide a sufficient stimulus for endothelial proliferation and for the preservation of the spatial organization of the developing vascular network.

The zone between the tips of the growing collecting ducts and the fibrous organ capsule is occupied by mesenchymal cells and vessel precursors [40]. Within this region new nephron generations and the dichotomous branching of the collecting duct ampullae are initiated by reciprocal induction of mesenchyme and embryonic collecting duct epithelium [36]. While VEGF and aldosterone in combination with vitamin D_3 mainly supported vessel broadening and elongation, a further generation of endothelial cell streaks was observed within the nephrogenic zone after stimulation of renal explants with retinoic acid and bFGF. Thus, these molecules did not only stimulate proliferation of the endothelial cells which were present in the explant at the time of excision from the organ, but they also supported the formation of new vessel generations. These vessels were found predominantly within mesenchymal tissue regions. Whether this finding indicates the formation of endothelial cells from mesenchyme [41] or a sprouting from existing vessels remains to be elucidated.

The application of retinoic acid alone induced an enlargement of the endothelial cell streaks. The combination of retinoic acid with bFGF resulted in considerably thinner vessels and a preservation of the spatial organization of the developing vessel network. It is still unknown whether these substances act directly on endothelial cells or whether they induce the production of regulating factors by other tissue components as epithelial cells or mesenchyme [42, 43]. With respect to bFGF, we assume a morphogenic rather than a mitogenic function during kidney development. We may speculate that this factor could induce the production of an antagonist for angiogenic factors, thus balancing mitogenic stimuli. Whether this is the physiological function of bFGF in the kidney will be the focus of further experiments.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft Kl 741/7-1.

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