Stimulation of renal microvascular development under organotypic culture conditions

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ABSTRACT **The development of the renal vascular system requires the coordinated action of soluble morphogenic factors and specific extracellular matrix components. Despite intensive research it remains unknown whether the humoral or the environmental component is more important in the development of renal microvessels. The prolonged serum-free culture of embryonic kidney cortex explants was achieved by means of a newly developed perfusion culture system. This system made the investigation of renal vascular development under defined organotypic conditions possible. Thus, growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hormones (aldosterone, vitamin D_3) could be applied without the interference with serum components. Medium supplementation with VEGF or aldosterone in combination with vitamin D_3 resulted in the coordinated proliferation of endothelial cells in the explant. A well-developed collecting duct epithelium and numerous tubular structures were always observed. In contrast, only a uniform cell layer was found between fibrous organ capsule and the collecting duct epithelium after bFGF application, but neither tubular structures nor endothelial cells. Thus, the experiments indicate that bFGF alone has no stimulating effect on the growth of the renal microvasculature under perfusion culture conditions.—Kloth, S., Ebenbeck, C., Kubitza, M., Schmidbauer, A., Röckl, W., Minuth, W. W. Stimulation of renal microvascular development under organotypic culture conditions.** ***FASEB J.*** **9, 963–967**

Key words: kidney • vascular development • perfusion cell culture • endothelium • growth factors

IN THE HEALTHY ORGANISM THE ENDOTHELIAL CELLS of the blood vessels are known for their extremely low proliferation rate. However, under pathological conditions such as wounding (1) or tumor development (2), endothelial cell growth is strongly stimulated. Vascular endothelial growth factor (VEGF)[2] (3) and basic fibroblast growth factor (bFGF) (4) have been reported to play a key role in inducing the proliferation of this otherwise contact inhibited cell type. Furthermore, both growth factors induce migration and tube formation in embryonic endothelial cells, which are prerequisites for angiogenesis (5).

The development of renal vasculature has been investigated by different techniques: light and electron microscopic methods (6–8), the culture of kidney rudiments (9), transplantation experiments (10), and transfilter- and coculture techniques (11, 12) have all been used. In addition to soluble growth factors such as VEGF and bFGF, components of the extracellular matrix (10, 13) and their cellular receptors (14) are involved in the highly coordinated process of vascularization. To this day, however, the dominant trigger mechanism essential for renal vascular development remains unknown.

In the present study a new cell culture model for the embryonic kidney and two newly generated endothelium detecting antibodies were chosen to investigate endothelial development under controlled culture conditions. Renal tissue explants were prepared by a microsurgical method (15) and cultured for 13 days without any serum supplements. Excellent tissue preservation under serum-free culture conditions was achieved by continuous medium exchange (16). Purified VEGF and bFGF were applied as endothelium stimulating factors triggering the development of the renal microcirculature within an organotypical environment.

MATERIALS AND METHODS

Tissue culture

Tissue explants were prepared from neonatal rabbit kidneys. One- to 3-day-old rabbits were anesthetized with diethylether (Merck, Darmstadt, Germany) and killed by cervical dislocation. Both kidneys were prepared under sterile conditions and washed in phosphate buffered saline (PBS, pH 7.2), after which the adipose tissue was removed. The fibrous organ capsule was stripped off with a pair of sharp, fine forceps (Dumont No. 5, Laborcenter, Nürnberg, Germany) (17). A thin tissue layer consisting of mesenchyme, collecting duct *ampullae*, endothelial cells, and developing nephrons remained attached to the fibrous organ capsule (**Fig. 1*B***). These cortex explants were mounted like a drumhead

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[2]Abbreviations: BSA, bovine serum albumin; bFGF, basic fibroblast growth factor; FITC, fluoresceine isothiocyanate; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate buffered saline; VEGF, vascular endothelial growth factor.

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in a set of sterile holding rings. The set was put into a perfusion chamber (Fig. 1A, Minucells and Minutissue, Bad Abbach, Germany), which allowed the continuous exchange of medium at a flow rate of 1 ml/h over the whole culture period of 13 days. The explants were cultured in serum-free medium (IMDM, 25 mM Hepes, Gibco-BRL Life Technologies, Germany). Different medium supplements were applied: VEGF (5 ng/ml), bFGF (1 ng/ml, Biomol, Hamburg, Germany) with 0.1% bovine serum albumin (Sigma, Deisenhofen, Germany), 1×10^{-7} M aldosterone (Aldocorten, Ciba-Geigy, Basel, Switzerland), and 1×10^{-9} M 1,25 dihydroxyvitamin D_3 (Biomol). Control explants were cultured in basal medium (IMDM, 25 mM Hepes) and in medium supplemented with 0.1% BSA. After 13 days of culture the tissue explants were washed in PBS and frozen in liquid nitrogen.

Immunohistological detection of endothelial cells

Immunoreactive material was detected in cryostat sections (HM 500, Microm, Heidelberg, Germany) following two different incubation protocols. Water-insoluble chromogens (diaminobenzidine, Sigma) and fluoresceine isothiocyanate (FITC) were applied for the visualization of bound primary antibodies. The methods using horseradish peroxidase conjugated streptavidine (16) and FITC-conjugated antibodies (8) have been described elsewhere in great detail. Biotin-SP conjugated donkey anti-mouse immunoglobulin (Dianova, Hamburg, Germany) was applied in conjunction with a streptavidin-horseradish peroxidase detection complex according to the manufacturer's instructions (Vectastain, Vector, Burlingame, Calif.). Donkey anti-mouse immunglobulin-FITC conjugates were supplied by Dianova (Hamburg).

Primary antibodies

Monoclonal antibodies (EnPo 1, EC1, both IgG_1) detecting endothelial cells at different developmental stages were raised according to the method described by Köhler and Milstein (18). The immunohistological and biochemical characterization has been described elsewhere (16, 19). Both antibodies were applied as undiluted hybridoma culture supernatants.

Controls

The specificity of the antibody reaction has been verified by different controls that were routinely included in the immune incubation experiments. One part of the control sections was incubated with mouse preimmune serum. Other kidney sections were incubated without primary antibody or with irrelevant primary antibodies. None of these controls revealed a positive reaction with endothelial structures.

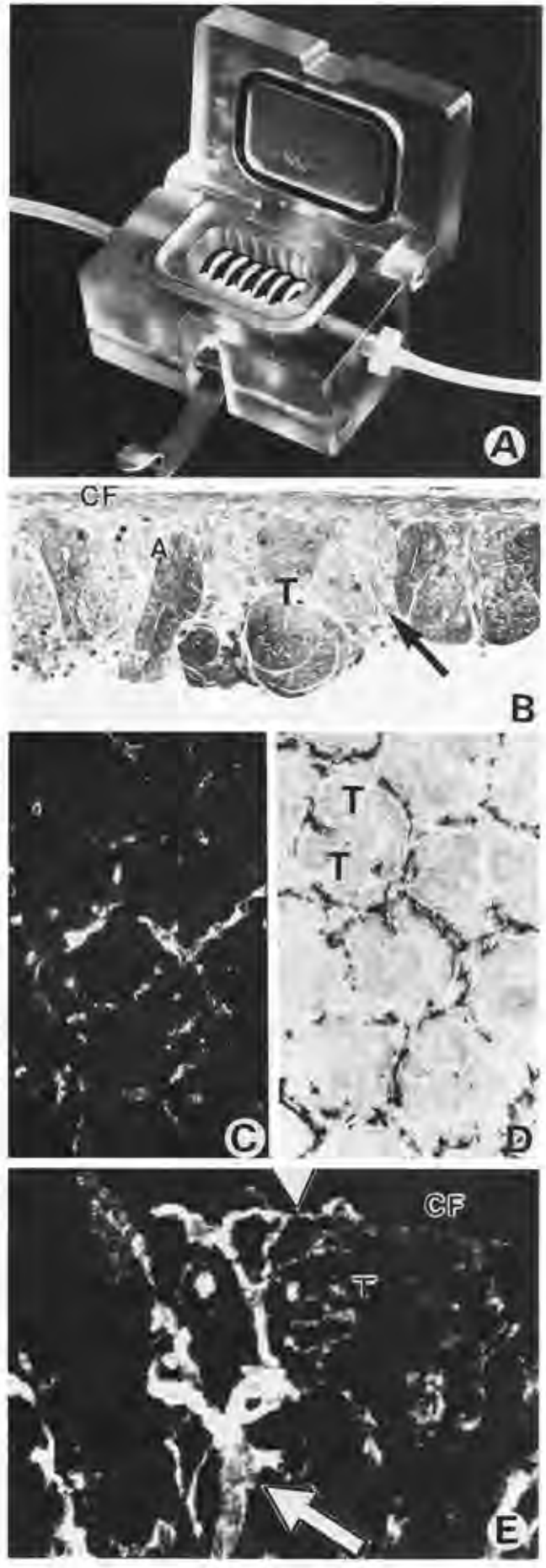


Figure 1. *A)* The renal cortex explants were mounted in a set of holding rings, inserted in the perfusion culture chamber, and perfused at a constant flow rate of 1 ml/h for 13 days. IMDM with different supplements but without fetal calf serum served as culture medium. Magnification: 1:1. *B)* Semithin longitudinal section of a freshly prepared cortex explant. Freshly prepared cortex explants were fixed, dehydrated, and embedded in epoxy resin as described previously (15). The immature tissue region consisted of mesenchymal cells, collecting duct *ampullae* (A), developing nephrons (T), and endothelial cells (arrow). CF, fibrous organ capsule. Magnification: 1:250. *C, D)* Tangential (cross) sections through the cortex corticis of the neonatal rabbit kidney showed a filigree network of cells labeled by both endothelium detecting antibodies EC1 and EnPo 1. *C)* Endothelial cells labeled by EnPo 1. An anti-mouse immunglobulin-FITC conjugate served as the detecting antibody a. *D)* Tangential section incubated with EC1 and an anti-mouse immunglobulin horseradish peroxidase conjugate. Each labeled endothelial mesh enclosed a collecting duct *ampulla* and two developing nephrons (T). Magnification: 1:250. *E)* In longitudinal sections of the renal cortex, the connection between tangentially located endothelial meshs and the functional vascular system of the kidney could be demonstrated. Arrowhead, mesh; arrow, branch of the interlobular artery; T, developing nephron; CF, fibrous organ capsule labeled by EnPo 1. Magnification: 1:500.

RESULTS

The structure of the microvascular network within the outer cortex of the neonatal rabbit kidney was investigated using two newly developed monoclonal antibodies (EC1, EnPo 1 [8]). In tangential sections of this cortex region, a filigree network of endothelial cells with a surprisingly high degree of spatial organization was detected (Fig. 1*C, D*). Extending beneath the fibrous organ capsule, the embryonic endothelial cells had formed a honeycomb-like network. As revealed in longitudinal tissue sections this filigree network is connected to vessel-like structures in contact with branches of the *arteria interlobularis* of the midcortical region (8). Within each mesh of the endothelial network a collecting duct *ampulla* and one developing nephron were present (Fig. 1*E*). The development of the nephrons, the maturation of the collecting duct, and the spreading of the renal vasculature happen in close spatial vicinity (8).

The developing cortex region of the neonatal rabbit kidney can be isolated easily. The explants consist of the fibrous organ capsule with adherent mesenchyme, collecting duct *ampullae*, spreading endothelial cells, and developing nephrons (Fig. 1*B*). Such explants were cultured under serum-free conditions for 13 days under permanent medium perfusion. Immunohistology revealed excellent tissue preservation. One side of the tissue is covered by capsule material and on the other side a monolayer of collecting duct epithelium has developed (**Fig. 2**; 17). Between the capsule and the collecting duct epithelium several cross-sectioned tubules can be observed. Large clusters of EnPo1- and EC1-labeled cells can be detected within tissue explants cultured without any medium supplements (Fig. 2*F*). However, the high degree of spatial organization in the vascular network cannot be obtained under culture by perfusion with basal medium alone.

Supplementation of the culture medium with VEGF resulted in the expression of a vascular network very similar to the situation found in vivo (Fig. 2*C*). In longitudinal sections of the explants, branches of endothelial cells were found arborizing from labeled cell streaks. Even better results were obtained when tissue explants were cultured in the presence of aldosterone and vitamin D_3 (Fig. 2*B*). Excellent tissue preservation and vascular network development were observed. Honeycomb-like endothelial structures similar to the endothelial network observed in tangential sections of the kidney were detectable in cultured explants (Fig. 2*B*). In comparison to the developing vasculature of the kidney (Fig. 2*A*), the endothelial branches detected in cortex explants are considerably broader and more elongated (Fig. 2*B*).

It is surprising that similar results cannot be obtained after medium supplementation with bFGF (Fig. 2*D*). Only very few EC1-labeled cells were detectable. Continuous endothelial cell streaks forming networks were not found. Moreover, the tissue explant did not include tubular cross sections, which were found frequently in all the other cultured explant sections. The combination of bFGF with aldosterone and vitamin D_3 resulted in the expression of small vessel-like structures labeled by EC1 (Fig. 2*E*). However, the broadening and elongation of these vessel-like structures that was evident after VEGF or aldosterone/vitamin D_3 stimulus (Fig. 2*B*) were not found.

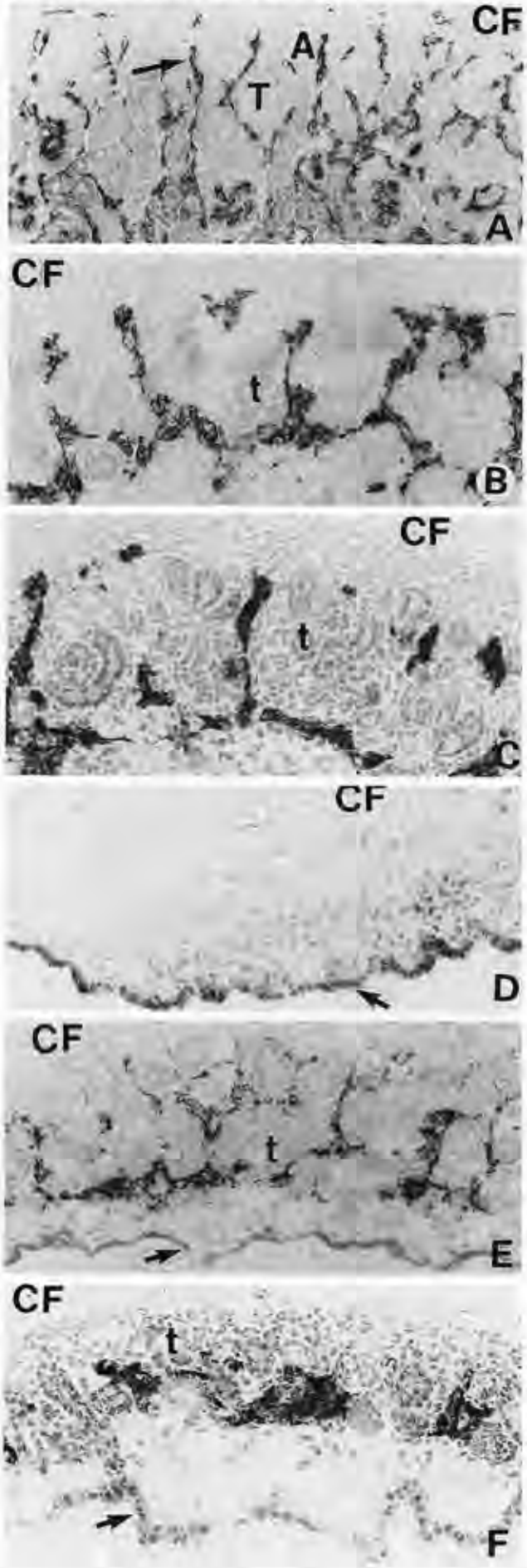
DISCUSSION

A culture system has been established that allows the investigation of renal microvascular development under the controlled application of different growth factors such as VEGF and bFGF. Cortical explants from neonatal rabbit kidneys were prepared by microsurgical methods. Unlike other preparation protocols that result in single-cell suspensions, our method omits the application of proteases. Thus, the integrity of the tissue is preserved, which allowed the further development of embryonic tissue components within their natural environment.

The cortical explants consisted of the immature tissue of the kidney attached to the fibrous organ capsule. Note that parts of this developing cortex region were not supplied by functional vessels (7, 19). Soluble growth and differentiation stimulators have to reach the embryonic cells by diffusion. The application of growth factors via permanent perfusion of the culture medium thus resembled the situation in vivo.

By means of this organotypic culture system, the developing endothelial network of the renal cortex could be maintained and propagated in vitro for the first time. Under perfusion culture the addition of VEGF or a combination of aldosterone and vitamin D_3 resulted in the proliferation of the developing endothelial network. In contrast, no EC1/EnPo 1-positive endothelial cells were detectable within cultured explants after stimulation with bFGF. Whether this is due to a down-regulation of endothelium-specific antigens or to the reduction and absence of endothelial cells remains to be investigated. However, bFGF-treated explants were characterized by a homogeneous tissue composition. Tubular structures were never observed, but were always found within unstimulated explants as well as in VEGF or aldosterone/vitamin D_3-treated tissue (Fig. 2) (16). This speaks in favor of a different tissue composition under stimulus with bFGF alone. Even control explants showed more typical renal tissue components than bFGF-incubated material. The combination of bFGF with aldosterone and vitamin D_3 resulted in a vascular network that resembled the endothelial structures found in longitudinal sections of the neonatal kidney. Vessel-like structures remained a filigree network and did not show the broadening and elongation that was a typical feature of tissue treated with VEGF or aldosterone/vitamin D_3 alone.

Both endothelial stimulating factors VEGF and bFGF are different with respect to origin and to receptor expression pattern. VEGF is the only one of the applied factors with exclusive specificity for endothelial cells (20). Different VEGF receptors have been described in the past, but distribution of the recognition molecules was restricted to the endothelium (21, 22). However, VEGF is produced by a wide variety of different cell types such as pituitary follicular cells (20), podocytes (3), mesangial cells and monocytes (23), glomerular endothelial cells (24), and tumor cells (21, 25). Recently VEGF was detected within the collecting duct



epithelium of the mouse kidney (26). The stimulation of endothelial cells by VEGF is mediated by auto- and paracrine mechanisms.

In contrast, bFGF induces proliferation and differentiation in many different cell types (4, 27, 28). The growth factor binds to different receptors with distinct ligand affinities (29–31). Like VEGF, it can be released from stores within the extracellular matrix (32). Until now inhibitory effects on endothelial proliferation have not been observed for bFGF in the concentration range used. On the contrary, mitogenic activity of bFGF on endothelial cells has been reported for in vitro as well as in vivo applications (5, 33). We only can speculate about the reasons for the negative effect of bFGF in our renal tissue culture system. The results cannot be explained by a nonactive bFGF preparation. The lot has been tested for biological activity and has proved to be highly active (W. Röckl, personal communication).

Many studies using bFGF have been performed in the presence of fetal calf serum and in culture systems consisting of one single cell type. Probably an unknown serum cofactor is required for proper receptor binding or signal transduction. On the other hand, bFGF-perfused cortex explants showed profound changes in tissue composition compared to control explants. Neither EC1 labeling nor tubular structures were observed after bFGF application. Thus, the explanation might be the broad range of cells stimulated by bFGF. It is not known whether any of these cell types produce inhibitory molecules in response to bFGF stimulus that down-regulate the proliferation of other tissue components.

For the development of complex organs the coordinated action of inhibitory and stimulatory elements is required. The

Figure 2. Comparison of the cortex corticis of neonatal rabbit kidney with renal explants perfusion cultured in the presence of different stimulating growth factors and hormones. All panels show tissue sections at the same magnification (1:250). *A)* The microvascular network of the renal cortex was labeled by the monoclonal antibody EC1. The developing tissue zone was crossed by numerous parallel branches of small vessels (arrow). CF, fibrous organ capsule; arrowhead, glomerulus; A, collecting duct *ampulla*. *B)* A cortex explant cultured for 13 days with serum-free IMDM medium supplemented with 1×10^{-7} M aldosterone and 1×10^{-9} M 1.25-dihydroxyvitamin D_3. Compared with the capillaries in panel A, EC1-labeled cell streaks have become considerably broader and more elongated. However, the characteristic pattern of the developing vascular network has been preserved. CF, capsula fibrosa; t, tubule. *C)* After application of VEGF (5 ng/ml) broad bands of EC1 positive cells could be detected in cultured explants. CF, capsula fibrosa; t, tubule. *D)* Medium supplementation with bFGF (1 ng/ml) resulted in a homogeneous tissue organization of the explant. No EC1-labeled cell streaks and tubular structures were observed. A uniform cell layer has developed between the fibrous organ capsule (CF) and the collecting duct epithelium (arrow). *E)* The negative bFGF stimulus could be overcome by the application of aldosterone and vitamin D3 in conjunction with bFGF. A network of small EC1-labeled cell streaks was detected between the fibrous organ capsule (CF) and the well-developed epithelium (arrow). Tubular cross sections (t) were found frequently. *F)* Control: The explant was perfusion cultured without any supplements for 13 days. A collecting duct epithelium (arrow) has grown out of the collecting duct *ampulla*. Areas consisting of EC1-positive cells and cross-sectioned tubules (t) were observed. CF, capsula fibrosa.

results obtained after application of bFGF in combination with aldosterone and vitamin D_3 may be discussed under this aspect. Aldosterone combined with vitamin D_3 alone gave rise to endothelial proliferation and an enlargement of the honeycomb-like endothelial network. In the presence of both hormones and bFGF, thin endothelial cell streaks and a small network were observed that resembled even more the in vivo situation. It is not known whether the hormones directly stimulated the cells inhibited by bFGF or whether there are different target cells.

VEGF and bFGF are well-known angiogenic factors (3, 4, 5, 20). This is not the case for aldosterone and vitamin D_3. Receptor distribution for aldosterone and vitamin D_3 within the kidney has been reported for the nephron and the collecting duct, but not for endothelial cells (34, 35). All cells of the collecting duct *ampulla* expressed receptors for both hormones, whereas the mature collecting duct epithelium showed a heterogeneous distribution of the recognition molecules. Receptor expression for vitamin D_3 or aldo-sterone on the vascular endothelium of the kidney has not been reported yet. It is assumed that the stimulatory effect on endothelial cells of both hormones is mediated by indirect mechanisms. The development of the collecting duct system, the nephrons, and the renal microvasculature take place in very close proximity (8). It is conceivable that binding of the hormones to the collecting duct epithelium results in the production of factor (or factors) stimulating endothelial development. VEGF produced by the collecting duct epithelium (26) is a likely candidate. However, the nature of the induced endothelial stimulators as well as the producing cells in the perfused explants remain to be investigated. FJ

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