Developing renal microvasculature can be maintained under perfusion culture conditions

Sabine Kloth[1)a], Anita Schmidbauer[a], Marion Kubitza[a], Herbert A. Weich[b], Will W. Minuth[a]

[a] Institute of Anatomy, University of Regensburg, Regensburg/Germany
[b] Institute for Molecular Cell Biology, University of Freiburg, Freiburg/Germany

The cortex corticis of the neonatal rabbit kidney consists of developing nephrons, vessels, collecting duct ampullae and the nephrogenic mesenchyme. Inductive interactions between embryonic mesenchyme and collecting duct ampullae lead to the coordinated development of the nephrons and the collecting duct system. The factors regulating nephrogenesis and vascular development within this tissue region are unknown. In order to analyze the hormonal regulation of vascular development an organotypic culture system was established. Cortex explants from neonatal rabbit kidneys were prepared, mounted in a set of holding rings and cultured under serum-free conditions for 14 days in conventional culture plates or under permanent medium perfusion in a newly developed culture container.

The detection of endothelial cells was carried out by means of two monoclonal antibodies. Within the renal cortex corticis EnPo 1 detected developing vasculature as well as podocytes and a subset of mesenchymal cells. EC1 displayed exclusive specificity for endothelial cells. The antibody did not discriminate between arteries and veins. Endothelial cells of different developmental stages were labeled with the same intensity. A combination of both antibodies allowed the discrimination between developing endothelial cells and podocytes.

Following 14 days of culture under permanent medium exchange, excellent tissue preservation as well as endothelial cell proliferation was observed in cortex explants. In contrast, tissue kept in stationary culture revealed a high degree of disintegration. Endothelial antigen expression was also severely disturbed. Tissue maintenance under stationary conditions was improved by the application of a hormone mixture consisting of aldosterone and 1,25-hydroxyvitamin D3. However, the high degree of spatial organization shown by developing endothelial cells in vivo was maintained exclusively in explants cultured in the presence of hormone under permanent perfusion.

Introduction

For a long time endothelial cells have been considered to be an inactive epithelial lining of the vessels. However, within the last few years it has become more and more clear that endothelial cells play an essential role in maintaining a multitude of body functions. The endothelium controls the exchange of soluble factors and cells between the blood and adjacent tissue [30, 37]. Furthermore, a variety of factors influencing, for instance, wound healing and immune response are produced by the endothelium itself [20, 31].

In order to investigate endothelial cell functions various cell culture systems were developed. It became evident that there are profound functional differences between the endothelium of the macro- and that of the microcirculation [27, 33]. Though endothelial cells derived from large vessels are easier to prepare and have been intensively used for in vitro investigations, different groups have now established preparation protocols for the microcirculation, too [5, 26, 27]. Besides the source, the culture technique used to propagate endothelial cells will also influence cellular behavior in vitro. For instance, the stimulating effect of transforming growth factor-β depends on the culture method used: proliferation of endothelial cells in adherent monolayers is inhibited, while tube formation is induced by this factor in cells cultured in gel matrices [12].

Our interests focus on renal microvasculature during the development of the neonatal rabbit kidney. The developing tissue zone can be easily prepared and has been used for investigations of the differentiation of the collecting duct epithelium [22, 24]. Recently, the close spatial relationship between renal vascular development and nephrogenesis in vivo was analyzed by means of the monoclonal antibody EnPo 1 [14]. The EnPo 1 antigen is expressed by endothelial cells of different developmental stages and by renal podocytes. Mesenchymal cells surrounding the tip of the ampullary collecting duct were found to be positive for EnPo 1 labeling, too. Further-

[1)] Dr. Sabine Kloth, Institut für Anatomie der Universität, Universitätsstr. 31, D-93053 Regensburg/Germany.

more, it could be demonstrated that these mesenchymal cell islets—representing one of the earliest stages of nephrogenesis on the cellular level [35]—were already in contact with differentiated parts of the renal vasculature. The connection was established by vessel-like structures. The term vessel-like structure was used for cell rows reacting specifically with EnPo 1 and detected within tissue zones which had previously been described as avascular [11]. The cell rows expressed endothelial antigens and were connected with the differentiated vasculature, but the existence of a lumen could not be proved up to now.

Renal vesicles and S-shaped bodies were surrounded by EnPo 1-positive cells mediating the coupling of the developing nephrons with the vascular system [15]. It is an open question whether the development of the renal microvascular system is influenced by ampullary induction, nephron development, or both events simultaneously. Thus, in a first approach, isolated renal embryonic tissue was brought in culture to investigate whether the complex composition of the developing microvasculature can be maintained under in vitro conditions.

Explants prepared by stripping off the capsula fibrosa of neonatal kidneys [22] consist of the embryonic ampullary collecting ducts, nephrons in different developmental stages, mesenchymal cells and fibrocytes of the capsula fibrosa. First, small capillaries accompanying the S-shaped bodies and vessel-like structures were found frequently. This tissue was used in order to establish an organotypic culture system for the investigation of renal endothelial development in vitro. In the present study, the influence of aldosterone, vitamin D3 and vascular endothelial growth factor (VEGF) on endothelial cells in organotypic renal cultures was investigated comparing conventional and perfusion cell culture techniques.

Materials and methods

Tissue preparation

Newborn New Zealand rabbits anesthetized with diethylether (Merck, Darmstadt/Germany), were killed by cervical dislocation, and the kidneys were removed immediately. Subsequently, tissue explants were prepared by stripping off the capsula fibrosa with a pair of sterile forceps from freshly sectioned kidney pieces. The explants were washed several times in sterile culture medium (IMDM, 25 mM Hepes, 100 IU/ml penicillin, 100 μg/ml streptomycin, Gibco-BRL Life Technologies, Eggenstein/Germany) before being mounted in a set of holding rings (Minucells and Minutissue, Bad Abbach/Germany, [23]). All mounted explants were cultured for 24 h under conventional stationary conditions (Heraeus incubator: 37°C, 95% air/5% CO_2 atmosphere) in 24-well culture plates (Becton Dickinson, Heidelberg/Germany) using the serum-free culture medium described above. Following this preculture, one part of the mounted explants was transferred to a perfusion container (Minucells and Minutissue) for prolonged culture under permanent perfusion (Fig. 1). Using conventional roller pump equipment (Ismatec, Werthein/Germany) the cultures were perfused with a constant speed of 1 ml/h. The containers were placed on a warming plate (Medax, Kiel/Germany) at 37°C. Supply medium and used medium were stored at 4°C. Throughout the culture period of 14 days only serum-free medium was
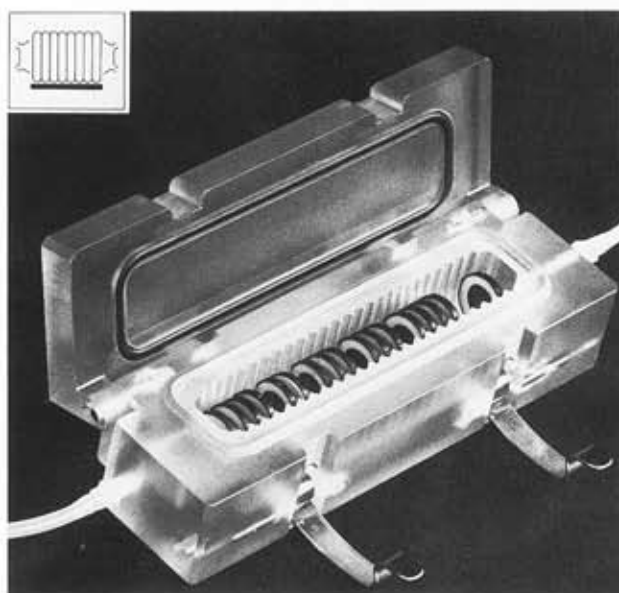
Fig. 1. Perfusion culture container. Following preparation, renal explants were mounted in a set of holding rings. The rings are transferred with a pair of sterile forceps to a perfusion container filled with culture medium. After one day of conventional stationary culture, explants were cultured for 13 days under permanent medium perfusion; flow rate: 1 ml/h.—The *inset* symbolically shows a perfusion culture container. In the following figures this symbol will indicate the usage of the perfusion culture. Tissue held under stationary conditions is marked by an *inset* showing a conventional culture dish.

used. Cultured explants were frozen and stored in liquid nitrogen prior to further analysis.

Medium supplements: Some experiments were run with medium supplements. Aldosterone (1×10^{-7} M, Aldocorten, Ciba-Geigy, Basel/Switzerland) was used in combination with 1,25 hydroxyvitamin D3 (1×10^{-9} M, Biomol, Hamburg/Germany). VEGF was applied in a concentration of 5 ng/ml.

Light microscopical techniques

Cryosections (8 μm) of frozen neonatal rabbit kidneys and renal explants were cut by means of a cryomicrotome (Microm, Heidelberg/Germany). For antigen detection by indirect immunofluorescence, the sections were fixed in 100% ice-cold ethanol (Merck) for 10 min, washed in phosphate-buffered saline (PBS, pH 7.2) and incubated with blocking buffer (PBS, pH 7.2, 1% bovine serum albumin, BSA; Sigma, Deisenhofen/Germany; 10% horse serum, Boehringer, Mannheim/Germany) for 30 min. After a 90 min incubation with undiluted culture supernatants containing primary antibodies, the sections were washed and incubated for another 45 min with detecting antisera (donkey anti-mouse Ig-fluorescein isothiocyanate (FITC), 1:400, Dianova, Hamburg/Germany) diluted in PBS, pH 7.2, 1% BSA. Finally, the sections were washed and embedded in FITC-Guard (Testoc, Chicago, IL/USA) and examined with an Axiovert 35 microscope (Zeiss, Oberkochen/Germany) or a confocal laser scan microscope (MRC 500 with BHS-filter, Bio-Rad, Munich/Germany; in combination with a Zeiss IM 35 microscope). Results were documented using Agfa PAN 25 film material.

For antigen detection by the immunoperoxidase method, a modified procedure described by Kujat et al. [17] was applied. Cryosections were fixed following a two-step fixation protocol. First the sections were incubated for 30 s in a solution of 4.2% paraformaldehyde (Merck), 16% picric acid (Fluka, Buchs/Switzerland), 0.002% cobalt chloride and 0.1% glutaraldehyde (Serva, Heidelberg/Germany) in PBS, pH 7.2. Subsequently, the sections were immersed for 15 min in a solution which included all the reagents listed above except glutaraldehyde. Following a washing step (0.1 M Tris-[hydroxymethyl]-aminomethan, Sigma; 0.8% NaCl, 0.002% Triton X-100, Pierce, Rockford, IL/USA; pH 7.4) the samples were blocked by incubation in 0.1 M Tris-buffer, pH 7.4, 25% fetal calf serum, 1% NaCl, 1% Triton X-100 for 45 min. Primary antibodies (undiluted culture supernatants) were applied overnight. Biotin-SP conjugated donkey anti-mouse Ig (Dianova) was diluted 1:600 in blocking solution prior to application. Then the sections were washed and incubated for 30 min in phenylhydrazine solution (Sigma) including 0.0006% H_2O_2 (Merck) in order to block endogenous peroxidases. After that an ABC-detection complex was applied according to the manufacturer's instructions (Vectastain, Vector, Burlingame, CA/USA). The enzyme reaction was started by adding the substrate solution (0.5 mg/ml diaminobenzidine, 0.1 M Tris, pH 7.4, 0.002% cobalt chloride, 0.04% nickel chloride, 0.012% H_2O_2) and stopped by rinsing in wash buffer. The sections were dehydrated in a graded ethanol series and by a final incubation for 10 min in xylol (Merck). Finally, the sections were embedded in DePeX (Serva).

Production of endothelium-specific monoclonal antibodies

The production and characterization of the monoclonal antibody EnPo 1 (mouse antibody, IgG_1) has been described in detail by Kloth et al. [14]. The antibody EC1 was raised following the same protocol by immunizing BALB/c mice with kidney homogenate. Rabbit kidneys were homogenized in Tris-buffer (0.01 M Tris-[hydroxymethyl]-aminomethan, 0.15 M NaCl, 9×10^{-3} M EDTA, 1×10^{-5} M leupeptin, 1×10^{-4} M phenylmethansulfonyl fluoride; Sigma) with a Potter homogenizer (150 rpm, 5 min). The homogenate was diluted 1:2 with complete Freund's adjuvant and used for intraperitoneal immunization. Following two further boosts, the fusion of spleen and myeloma cells (clone: x63 Ag8.653) was carried out as described by Köhler and Milstein [16]. Antibody-producing fusion products were cloned by limiting dilution. For screening, kidney cryosections were used with the indirect immunofluorescence method as explained above. Hybridoma cells were cultured in RPMI 1640 supplemented with 10^{-5} M 2-mercaptoethanol and 10% fetal calf serum at 37 °C in a Heraeus incubator (humid 5% CO_2/95% air atmosphere). EC1 is an antibody of the IgG_1 class with kappa light chains as determined by means of a commercial isotyping kit (Holland Biotechnology, Leiden/The Netherlands).

Results

Characterization of the monoclonal antibody EC1

For the investigation of renal vascular development two different endothelium-specific antibodies were raised. Commercially available anti-Factor 8 antibodies, useful tools for the detection of vascular elements in human and bovine tissue, were not suitable in the rabbit system due to unspecific cross-reactions with other tissue components. The detailed characterization of the newly developed monoclonal antibody EnPo 1 has been described earlier

Tab. 1. Reaction pattern of EC1 and EnPo 1 within different rabbit organs.

Organ	Cell type	EC1	EnPo 1
Brain	continuous endothelium of large vessels	+++	+
	fenestrated endothelium of Plexus choroideus	++	++
Kidney	large vessels	+++	+
	small vessels	++	++
	afferent and efferent arterioles	++	++
	glomerular endothelium	++	++
	podocytes	−	+++
	developing vasculature in the outer cortex	++	++
	developing podocytes of the S-shaped body	−	+++
Liver	large vessels	++	+
	sinusoids	+	+
	central veins	++	+
	small vessels surrounding bile ducts	++	++
Lung	arteries and veins, all sizes	+++	+
	capillaries	++	++
Muscle	large vessels	++	+
	small vessels and capillaries	++	++
Small intestine	large vessels	++	+
	small vessels and capillaries	++	++
Spleen	trabecula and central vessels	+++	+
	small vessels and capillaries	++	++

+: Weak reaction.— ++: Clear labeling.— +++: Intensive labeling.— −−: No reaction. Both antibodies are of the IgG_1 subclass with kappa light chains.
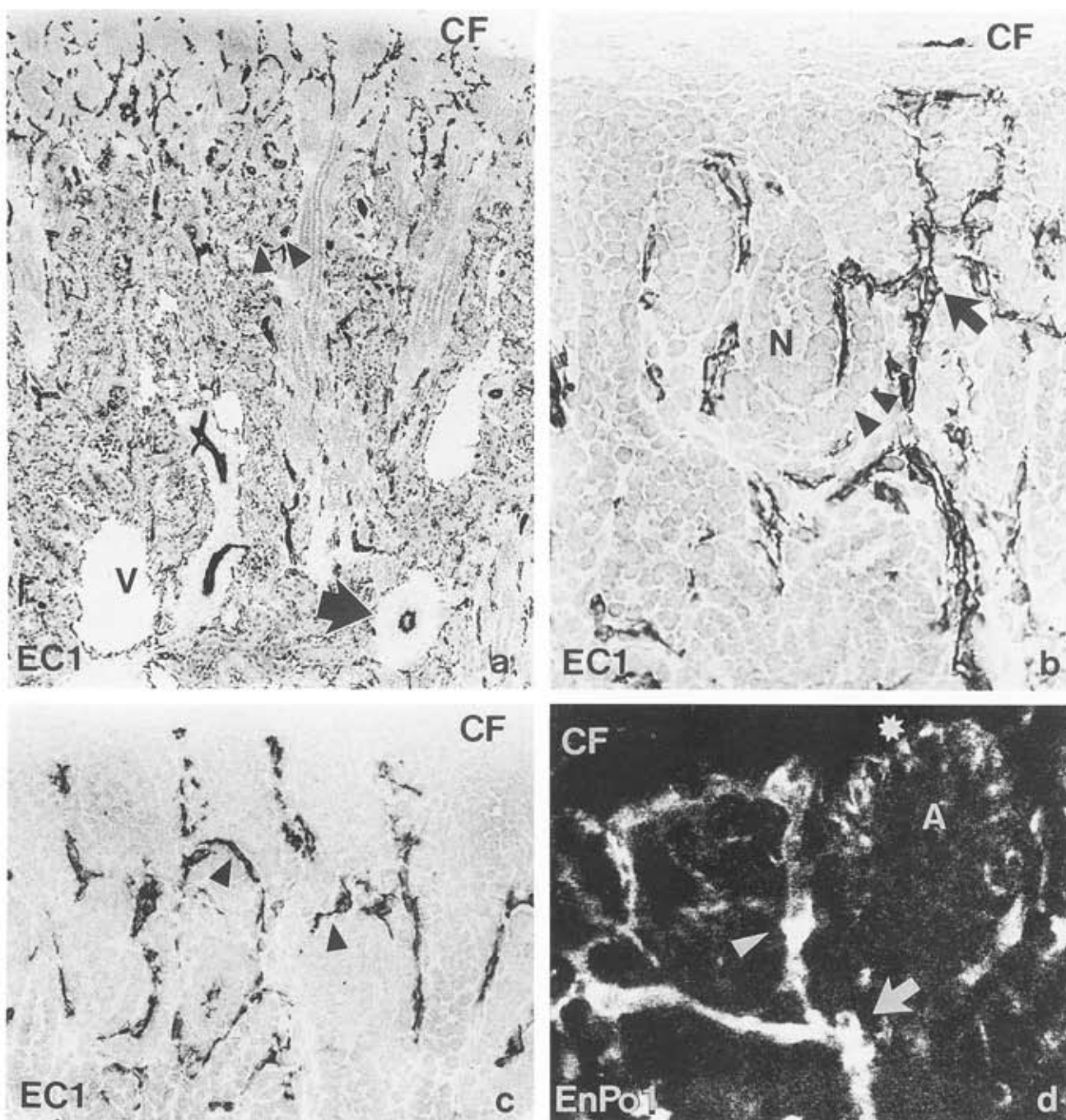
Fig. 2. Reaction pattern of the monoclonal antibodies EC1 and EnPo 1.—**a.** EC1 is specific for endothelial cells. Within the neonatal rabbit kidney EC1 labeled large arcuate arteries (*arrow*) and veins (V) with the same intensity as it did small vessels surrounding proximal tubules (*arrowheads*) and developing capillaries just beneath the capsula fibrosa (CF).— **b.** First small capillaries abundant within the S-shaped bodies (N) were intensively marked by EC1, while podocyte precursor cells (*arrowheads*) remain unlabeled. Capillaries found within the S-shaped bodies were connected with the differentiated vasculature (*arrow*) as well as with vessel-like structures protruding towards the capsula fibrosa (CF). These vessel-like structures were abundant within tissue regions which had been described previously as being avascular.—**c.** The EC1 labeling of developing capillaries in the outermost cortex showed a characteristic pattern which was found in all corticomedullary sections of the neonatal kidney. Capillaries (*arrowheads*) are oriented parallel to the capsula fibrosa (CF). Small vessel-like structures were found protruding like branches from these parallel-oriented bands. —**d.** This figure represents a projection of 10 optical sections gained by laser scanning microscopy. The monoclonal antibody EnPo 1 showed a different labeling pattern than EC1. Besides intensively marked small vessel-like structures within the outermost cortex region (*arrowhead*) EnPo 1 reacted with a distinct subpopulation of mesenchymal cells (*asterisk*). Only the mesenchyme surrounding the tip of the collecting duct ampulla (A) was positive for EnPo 1. Labeled mesenchymal cell islets were in contact with capillary-like structures which were connected with more differentiated vasculature (*arrow*).—CF Capsula fibrosa.— 50× (**a**), 300× (**b**), 150× (**c**), 500× (**d**).

[14]. EnPo 1 showed a strong reactivity with small capillaries and renal podocytes, while the endothelium of large vessels was only weakly labeled in rabbit renal tissue (Tab. I). The EnPo 1 antigen was also expressed by podocyte precursor cells located in the lower cleft of the S-shaped body. In order to discriminate between endothelium and podocytes, another monoclonal antibody was produced exhibiting exclusive specificity for endothelial cells. EC1 reacted with vessels throughout all the rabbit organs tested (Tab. I). Large and small vessels as well as arteries and veins were labeled with the same intensity (Fig. 2a). Up to now the EC1 antigen has been detected in rabbit tissue only.

Within the outer cortex region of neonatal rabbit kidneys EC1 reacted with the small capillaries surrounding the S-shaped bodies and vessel-like structures protruding towards the capsula fibrosa (Fig. 2b). Neither mesenchymal cells nor podocytes were labeled with EC1. A surpris-

ingly high degree of regular spatial organization of endo-
thelial cells was observed within this tissue zone. In tan-
gential sections through the renal cortex corticis endothe-
lial cells arranged in a honeycomb-like structure could be
demonstrated (Fig. 3). These webs were detectable by
both antibodies but exclusively within the developing tis-
sue region.

Comparison of conventional and perfusion cell culture techniques under serum-free conditions

The developmentally active zone of the neonatal rabbit
kidney could be prepared easily by stripping off the capsu-
la fibrosa [22]. In culture, cells of the collecting duct am-
pullae will grow out, forming a closed collecting duct epi-
thelium on top of the renal explant. All tissue components
are enclosed by the collecting duct epithelium on one side
and the capsula fibrosa on the other side. In order to en-
sure a proper and reproducible orientation of the explants
only sections showing both epithelium and capsula fibrosa
were analyzed. The combination of EC1 and EnPo 1 for
the detection of endothelial cells and podocytes in renal
tissue explants allowed us to investigate changes induced
in both cell populations during prolonged serum-free cul-
ture.

Renal tissue explants were cultured under serum-free
conditions using conventional culture plates as well as per-
fusion culture equipment (Fig. 1) [23]. A comparison of
tissue maintenance using morphological criteria revealed
poor preservation of all tissue components in explants cul-
tured under stationary conditions for 14 days without me-
dium supplements (Figs. 4a, b). Only few intact tubular
structures were found. In general, the cells showed irregu-
lar shapes with pycnotic nuclei, giving the impression of
widely disintegrated tissue. Within the perfused explants
tissue maintenance was greatly improved (Figs. 4c, d): be-
tween the well developed collecting duct epithelium and
the capsula fibrosa a matrix of regularly shaped cells was
present. Only a few cells with condensed, pycnotic nuclei
were seen. Intact tubular structures were observed fre-
quently. The antibodies EnPo 1 and EC1 were used to
identify endothelial cells and podocytes within the ex-
plants. Following stationary culture, only diffusely labeled
cell clusters were detectable by EC1 (Fig. 4a). EnPo 1-po-
sitive clusters were densely labeled (Fig. 4b). It should be
emphasized that endothelial cells expressing the EnPo 1
antigen were scarce in these explants. There was no colo-
calization of EnPo 1-positive clusters and EC1 labeling in
consecutive cryosections. Thus, only podocytes were de-
tected by EnPo 1. EnPo 1 antigen expression by the endo-
thelium seems to be severely disturbed under stationary
culture conditions. The EC1 antigen was detectable, but
the endothelial cells had lost their spatial organization.
The orderly arrangement of labeled cells as observed in
neonatal kidneys (Figs. 2, 3) and freshly prepared explants
was completely destroyed under stationary culture.

In explants continuously perfused with fresh medium
for 13 days, large, intensively labeled areas were detect-
able by EC1 (Fig. 4c). These areas were arranged parallel
to the capsula fibrosa forming a broad streak of positive

cells. Single cell clusters which did not colocalize with
EC1-positive cells were observed following the applica-
tion of EnPo 1. Broad streaks of cells colocalizing with
EC1-positive areas were only weakly labeled by EnPo 1.
These results indicate that under perfusion without any
medium supplements EnPo 1 antigen was expressed in
large amounts by the podocytes only. Nevertheless, tissue
preservation as well as EC1 antigen expression were
markedly improved within perfused tissue. EC1-labeled
cells were arranged in continuous streaks which were not
detectable in stationary cultured tissues.

Comparison of conventional and perfusion cell culture techniques using medium supplemented with hormones

In further experiments culture medium was supplemented
with a hormone combination of aldosterone and 1,25-hy-
droxyvitamin D3. In stationary cultures, hormone admin-
istration resulted in a remarkable improvement in tissue
preservation (Figs. 5a, b). Tubular structures were found
frequently, and the main part of the surrounding tissue did
not show the high degree of disintegration observed in ex-
plants cultured without any supplements (Figs. 4a, b).
EC1-labeled cells were arranged in streaks running paral-
lel and perpendicular to the capsula fibrosa. When consec-
utive sections were analyzed some cells of these streaks
showed weak EnPo 1 labeling. EC1-negative areas of po-
docyte clusters were strongly reactive with EnPo 1
(Fig. 5b).

Perfused renal explants supplied with the hormone
combination showed excellent tissue preservation mani-
fested by numerous tubular structures embedded in a
well-preserved cellular lattice (Figs. 5c, d). Streaks of
EC1-positive cells were found running parallel to the cap-
sula fibrosa. Branches growing out from these streaks and
towards the former organ capsule were numerous
(Fig. 5c). These streaks were also positive for EnPo 1
(Fig. 5d), but the EnPo 1 antigen is expressed more weak-
ly under culture conditions than in undisturbed tissue
(Fig. 2d). However, the arrangement of cell streaks and
branches expressing endothelial antigens was a coarse
image of the in vivo situation (Fig. 2c). In culture, the

Fig. 3. Tangential sections through the cortex corticis of neona-
tal rabbit kidney.—**a, b.** In tangential cryosections of the outer-
most renal cortex both antibodies reveal a honeycomb-like label-
ing pattern. Developing capillaries build a highly organized net-
work closely beneath the capsula fibrosa.—**c.** This network con-
sists of spindle-shaped cells as demonstrated in phase-contrast
images (**a** and **c** show the same section).—**d.** Tangentially cut ex-
plants cultured in the presence of aldosterone and vitamin D3 un-
der permanent perfusion showed the same labeling pattern. EC1-
positive cells forming a highly organized network are found in
perfused, hormone-treated tissue only.—300× (**a–c**), 200× (**d**).
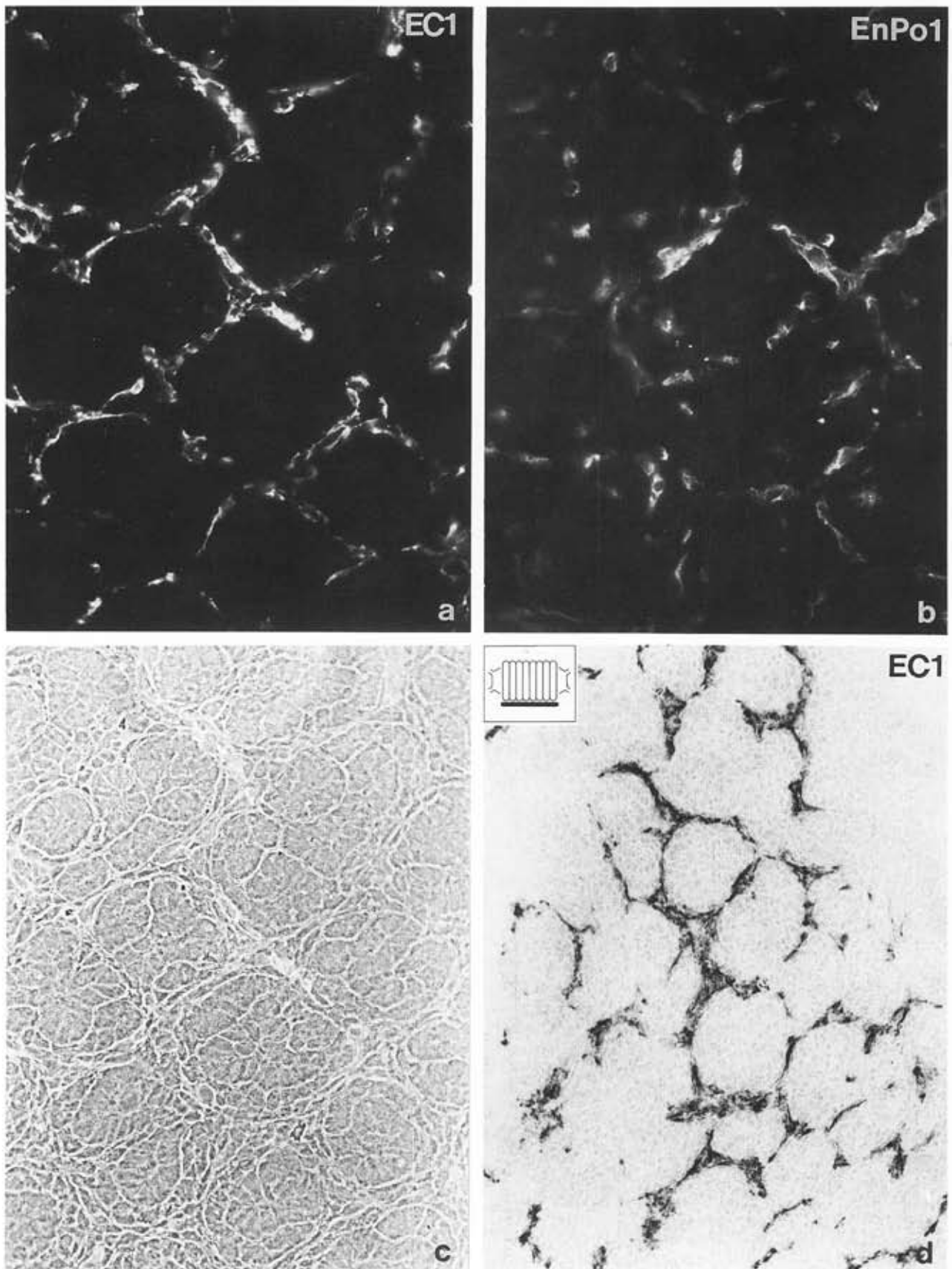
small vessel-like structures growing towards the capsula fibrosa (Fig. 2c) have expanded forming broad branches of EC1/EnPo 1-positive cells. In tangential sections cut through the outermost cortex zone of the kidney, highly organized endothelial cell webs could be demonstrated by EnPo 1 and EC1 labeling (Figs. 3a, b). Within the explant sections well-preserved endothelial webs were found exclusively in hormone-treated, perfused tissue (Fig. 3d).

The combination of the perfusion technique and hormone application led to a clear-cut improvement in tissue preservation and antigen expression. Furthermore, the orderly arrangement of tissue components was maintained only by the interaction of an improved culture technique and hormone stimulus.

Development of endothelial structures in renal explants perfused with VEGF

VEGF is the only known growth factor exhibiting growth-promoting activity exclusively for endothelial cells [3, 29]. The application of VEGF on renal explants kept under perfusion culture resulted in the formation of broad EC1-positive streaks running parallel to the former organ capsule (Fig. 6a). EC1-labeled cell rows arborizing from these streaks extended towards the capsula fibrosa. EnPo 1 labeling was not detectable on cells labeled by EC1 (Fig. 6b). The overall tissue preservation was excellent. VEGF application alone did not result in an arrangement of endothelial cells similar to that in the in vivo situation, but the growth-promoting effect which was observed following aldosterone and vitamin D3 administration was achieved as well. Tissue treated with a combination of VEGF, aldosterone and vitamin D3 under perfusion culture condition showed honeycomb-like structures as well as cell streaks and branches which seem to be broader than with VEGF alone (Figs. 6c, d).

Discussion

The maintenance of morphological characteristics, cell viability and proliferative activity are parameters widely used in determining the success of a cell culture method [1, 2, 4, 28]. The comparison of tissue kept in stationary culture with perfused renal explants revealed profound differences with respect to morphological and immunohistological criteria. Perfused explants showed a remarkably high degree of tissue preservation, while conventionally cultured explants exhibited only poorly preserved cell and organ structures. It should be emphasized that serum supplements were used neither for explant preparation nor during the culture period of 14 days. Thus, improved tissue maintenance was achieved by a constant supply with fresh medium and the continuous drainage of metabolites.

It is a well known fact that primary cell cultures show a high tendency of dedifferentiation [7–9]. Molecules expressed in considerable amounts by cells of an intact tissue are down-regulated following artificial tissue disintegration, while others not present in vivo appear under culture. Thus the expression of characteristic marker molecules which can be modulated by environmental factors is another criterium for optimized culture conditions. In this study, two monoclonal antibodies were used which reacted strongly with rabbit endothelial cells (Tab. I). EC1 showed exclusive specificity for endothelial cells, while EnPo 1 served as a marker for endothelial cells and podocytes [14]. Broad streaks of EC1-antigen-expressing cells were observed following perfusion culture. Due to the organized arrangement of these streaks in explants perfused with hormone-supplemented medium, it is assumed that these structures developed by proliferation from small capillaries and EC1/EnPo 1-positive cell rows found in the outermost kidney cortex. The high degree of spatial endothelial organization observed in vivo was maintained only when hormones and perfusion culture were applied in combination. Permanent medium exchange proved to be a prerequisite for excellent tissue preservation under prolonged serum-free culture. In explants kept under stationary culture conditions without hormone application, neither a comparable tissue organization nor a high EC1 antigen expression was observed. These samples revealed only scattered EC1 labeling. This might be due rather to the advanced tissue disintegration than to antigen down-regulation.

The EnPo 1 antigen is expressed in the highest amounts by podocytes and small vessels of the kidney. Within conventionally cultured renal explants which were not treated with hormone, only podocytes but not endothelial cells reacted with EnPo 1. While in experiments without hormone administration it is not clear whether this is due to tissue disintegration, hormone-treated but not perfused material clearly showed that a down-regulation of the antigen took place under culture. This effect could be reversed to some extent by the combination of medium perfusion and hormone application. Thus, we could demonstrate an improved tissue preservation in perfused explants with respect to morphological criteria as well as the maintenance of antigenicity.

Fig. 4. Renal explants cultured for 14 days without medium supplements.— **a, b.** Consecutive sections of a tissue explant cultured for 14 days under conventional stationary conditions. Tissue preservation of these explants is poor. The capsula fibrosa is hardly detectable, no single cells can be distinguished, and no tubular structures are preserved.—**a.** Some clusters of scattered cells were detected by EC1.—**b.** EnPo 1 intensively labeled some dense areas but no vessel-like structures.—**c, d.** Using the perfusion culture technique much better tissue preservation is achieved. No medium additives have been used, but intensive immunostaining can be observed between the capsula fibrosa (CF) and a well developed epithelium (E).—**c.** EC1-positive cells are organized in broad streaks.—**d.** Analysis of consecutive tissue sections revealed that EC1-positive bands are only weakly labeled by EnPo 1 (*arrow*), while dark EnPo 1-marked cell clusters are completely negative for EC1 (*asterisk* in **c**).—200× (**a, b**), 300× (**c, d**).
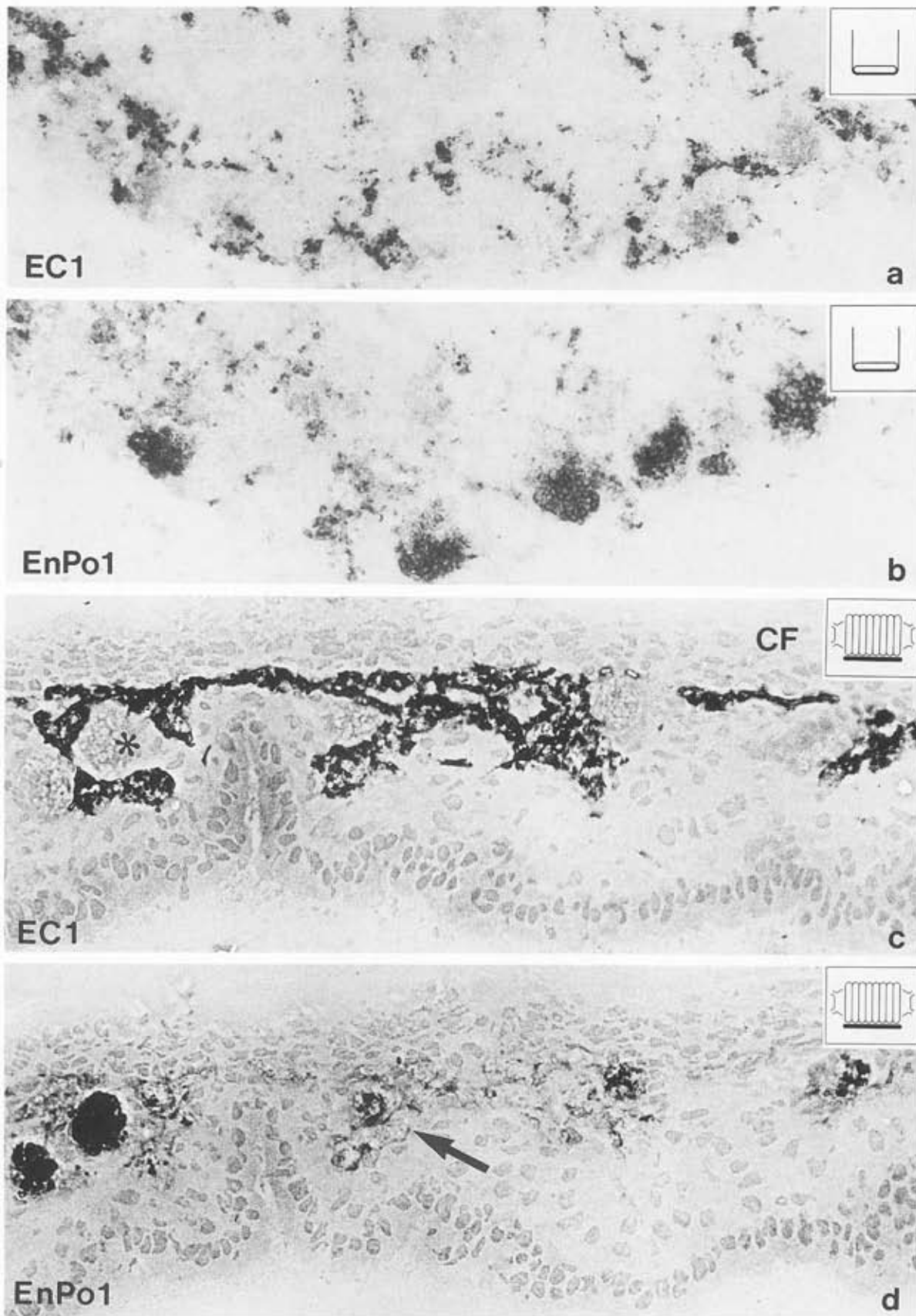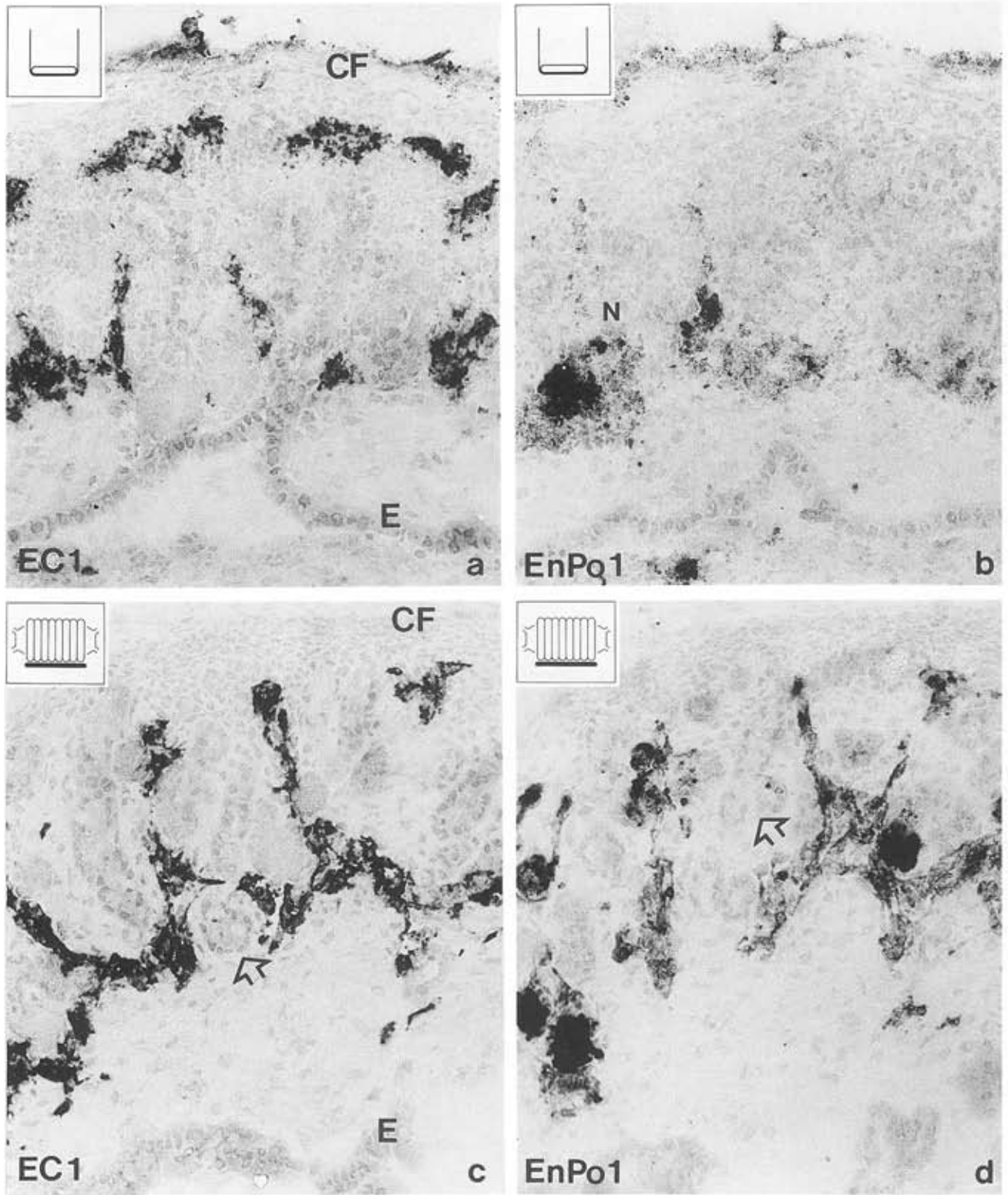
Numerous factors influencing renal vascular development have been described in recent years [13, 32, 34, 38]. Growth and migration-promoting factors as well as components of the extracellular matrix [6, 10, 36, 39] are thought to be involved in the highly coordinated process of renal organogenesis. Besides making a detailed analysis of the in vivo situation possible, different cell culture approaches have been of excellent use in elucidating parts of this complex process in the past. We have established a tissue culture model which allows the investigation of developmental processes under controlled culture conditions. First we were interested whether the high degree of tissue organization within renal cortex explants can be maintained under in vitro conditions by the application of defined growth and differentiation factors.

Aldosterone has been reported to stimulate the Na/K-ATPase activity [18, 21, 25], while vitamin D is known as a differentiation factor. The stimulating activity of aldosterone markedly influences the differentiation of collecting duct epithelial cells [24]. Thus, we were interested to know whether endothelial cells were also sensitive to aldosterone treatment. Vitamin D3 was added in order to stimulate endothelial cell proliferation and differentiation. Under conventional culture conditions better tissue preservation was achieved following the application of this hormone combination, although no great degree of endothelial tissue organization was maintained. Utilization of the perfusion culture technique in conjunction with hormone administration resulted in the maintenance of a coarse image of the endothelial tissue organization observed in vivo.

However, both hormones are known to interact with a variety of different cell types. In the case of aldosterone, unspecific stimulating effects have been reported [19]. Therefore, the experiments were repeated administering VEGF, a peptide which exclusively promotes endothelial cell growth [3, 4, 29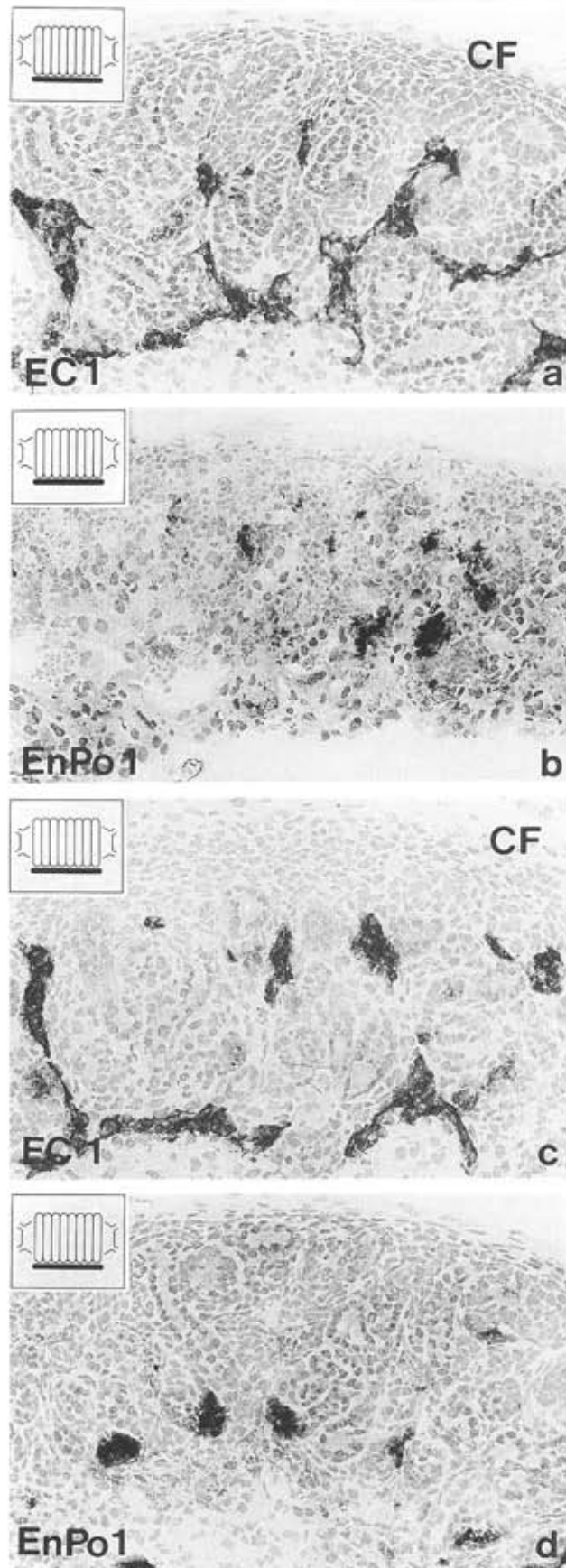]. While tissue integrity and antigenici-ty were well-preserved in these cultures, the high degree of endothelial cell organization observed in vivo and in aldosterone/vitamin D3-treated cultures was not achieved. We have to conclude that endothelial cell growth promotion by itself did not result in the expression and maintenance of a well-developed endothelial network. Furthermore, it remains subject to further investigation whether a direct steroid stimulus on the differentiating endothelium leads to the results described above. It is thinkable that other cell populations abundant in our organotypic culture system might be triggered by the applied hormones to produce factors responsible for the maintenance of endothelial tissue organization. Recently, it has been reported that the development of collecting duct epithelium in vitro is considerably affected by aldosterone application [24]. The interaction of the collecting duct ampullae with the surrounding mesenchymal cells leads to the coordinated development of nephrons and the collecting duct system (reviewed in [35]). Whether the inductive process might also participate in the development of the renal vascular network is not clear. The in vitro system presented in this study appears to be an appropriate tool for the detailed analysis of growth and differentiation factors involved in the developmental processes taking place within the neonatal kidney cortex.

References

[1] Avner, E. D., W. E. Sweeney Jr., N. P. Piesco, D. Ellis: Growth factor requirements of organogenesis in serum-free metanephric organ culture. In Vitro Cell. and Dev. Biol. **21**, 297–304 (1984).

[2] Bard, J. B., A. S. A. Ross: LIF, the ES-inhibition factor, reversibly blocks nephrogenesis in cultured mouse kidney rudiments. Development **113**, 193–198 (1991).

[3] Breier, G., U. Albrecht, S. Sterrer, W. Risau: Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development **114**, 521–532 (1992).

[4] Chaillier, P., J. Ferrari, N. Brière: Fetal mouse kidney maturation in vitro: Coordinated influences of epidermal growth factor, transferrin and hydrocortisone. Anat. Embryol. **184**, 319–329 (1991).

[5] Chaudhari, A., M. A. Kirschenbaum: A rapid method for isolating rabbit renal microvessels. Am. J. Physiol. **254** (Renal Fluid Electrolyte Physiol. **23**), F291–F296 (1988).

[6] Ekblom, P., I. Thesleff, A. Miettinen, L. Saxén: Organogenesis in a defined medium supplemented with transferrin. Cell Diff. **10**, 281–288 (1981).

[7] Gandolfi, A. J., K. Brendel: In vitro system for nephrotoxicity studies. Toxicol. in Vitro **4**, 337–345 (1990).

[8] Gstrauntaler, G., D. Steinmassel, W. Pfaller: Renal cell cultures: A tool for studying tubular function and nephrotoxicity. Toxicol. Lett. **53**, 1–7 (1990).

[9] Horster, M.: Tissue culture in nephrology: potential and limits for the study of renal disease. Klin. Wochenschr. **58**, 965–973 (1980).

Fig. 5. Renal explants cultured in the presence of aldosterone and vitamin D3.—**a, b.** Hormone application (aldosterone: 10^{-7} M, 1.25 hydroxyvitamin D3: 10^{-9} M) supported tissue preservation under stationary culture conditions. Between the capsula fibrosa (CF) and developed epithelium (E) some tubules (N) can be observed.—**a.** Cell clusters labeled by EC1 did not show the extreme disintegration which has been observed under stationary culture without hormone supplements.—**b.** The EnPo 1 antigen expression is mainly restricted to large, darkly labeled cell clusters which do not colocalize with EC1-marked structures in consecutive sections.—**c, d.** A combination of the perfusion cell culture technique and the application of aldosterone in conjunction with vitamin D3 resulted in an endothelial organization which is a coarse image of the in vivo situation. Bands of labeled cells running parallel to the capsula fibrosa (CF) and epithelium (E) were detectable with both antibodies. Furthermore, marked branches were found protruding towards the surface of the tissue explant. Well-preserved tubules (*open arrows*) are numerous.—**d.** Large dark clusters of positive cells were found in EnPo 1-labeled tissue only. These structures were negative for EC1.—200×.

[10] Ingber, D. E., Y. Folkman: How does extracellular matrix control capillary morphogenesis? Cell **58**, 803–805 (1989).

[11] Kazimierczak, J.: Development of the renal corpuscle and the juxtaglomerular apparatus: a light and electron microscopic study. Acta Pathol. Microbiol. Scand. (A) **218**, 9–65 (1971).

[12] Klagsbrun, M., P. A. D'Amore: Regulators of angiogenesis. Annu. Rev. Physiol. **53**, 217–239 (1991).

[13] Klein, G., M. Ekblom, L. Fecker, R. Timpl, P. Ekblom: Differential expression of laminin A and B chains during development of embryonic mouse organs. Development **110**, 823–837 (1990).

[14] Kloth, S., D. Meyer, W. Röckl, A. Miettinen, J. Aigner, A. Schmidbauer, W. W. Minuth: Characterization of an endothelial protein in the developing rabbit kidney. Differentiation **52**, 79–88 (1992).

[15] Kloth, S., J. Aigner, A. Schmidbauer, W. W. Minuth: Interrelationship of renal vascular development and nephrogenesis. (submitted).

[16] Köhler, G., C. Milstein: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256**, 495–497 (1975).

[17] Kujat, R., C. Rose, K.-H. Wrobel: The innervation of the bovine ductus deferens: comparison of a modified acetylcholinesterase-reaction with immunoreactivities of cholintransferase as pan-neuronal markers. Histochemistry **99**, 231–239 (1993).

[18] Laplace, J. R., R. F. Husted, J. B. Stokes: Cellular responses to steroids in the enhancement of Na^+ transport by rat collecting duct cells in culture. J. Clin. Invest. **90**, 1370–1378 (1990).

[19] Le Grimellec, C., G. Friedlander, E. H. El Yandouzi, P. Zlatkine, M.-C. Giocondi: Membrane fluidity and transport properties in epithelia. Kidney Int. **42**, 825–836 (1992).

[20] Mantovani, A., F. Bussolino, E. Dejana: Cytokine regulation of endothelial cell function. FASEB J. **6**, 2591–2599 (1992).

[21] Marver, D., M. J. Schwartz: Identification of mineralcorticoid target sites in the isolated rabbit cortical nephron. Proc. Natl. Acad. Sci. USA **77**, 3972–3976 (1980).

[22] Minuth, W. W.: Neonatal rabbit kidney kidney cortex in culture as tool for the study of collecting duct formation and nephron differentiation. Differentiation **36**, 12–22 (1987).

[23] Minuth, W. W., G. Stöckl, S. Kloth, R. Dermietzel: Construction of an apparatus for perfusion cell cultures which enables in vitro experiments under organotypic conditions. Eur. J. Cell Biol. **57**, 132–137 (1992).

[24] Minuth, W. W., W. Fietzek, S. Kloth: Aldosterone modulates the development of PNA binding cell isoforms within renal collecting duct epithelium kept in perfusion culture. Kidney Int. **44**, 537–544 (1993).

[25] Muto, S., G. Giebisch, S. Sansom: Effects of adrenalectomy on CCD: evidence for differential response of two cell types. Am. J. Physiol. **253**, F242–F252 (1987).

Fig. 6. Influence of VEGF on endothelial development in renal cortex explants. Renal explants were cultured for 13 days under permanent perfusion without serum supplements. — **a, b.** In the presence of VEGF (5 ng/ml) small bands of EC1-positive cells with branches reaching towards the capsula fibrosa (CF) were detectable. — **b.** EnPo 1-labeled sections did not show band-like structures: only clusters of marked cells could be observed. — **c, d.** A combination of VEGF, aldosterone and vitamin D3 resulted in the expression of broad bands and branches detectable by EC1. — **d.** The EnPo 1 antigen was expressed by the podocytes only. No band-like structures were detectable within these explants by EnPo 1. — 150×.

[26] Okamura, K., A. Morimoto, R. Hamanaka, M. Ono, K. Kohno, Y. Uchida, M. Kuwano: A model system for tumor angiogenesis: Involvement of transforming growth factor-α in tube formation of human microvascular endothelial cells induced by esophageal cancer cells. Biochem. Biophys. Res. Commun. **186**, 1471–1479 (1992).

[27] Pepper, M. S., N. Ferrera, L. Orci, R. Montesano: Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angogenesis in vitro. Biochem. Biophys. Res. Commun. **189**, 824–831 (1992).

[28] Perantoni, A. O., C. L. Williams, A. L. Lewellyn: Growth and branching morphogenesis of rat collecting duct anlagen in the absence of metanephrogenic mesenchyme. Differentiation **48**, 107–113 (1991).

[29] Plate, K. H., G. Breier, H. A. Weich, W. Risau: Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. Nature **359**, 845–848 (1992).

[30] Picker, L. J., R. A. Warnock, A. R. Burns, C. M. Doerschuk, E. L. Berg, E. C. Butcher: The neutophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. Cell **66**, 921–933 (1991).

[31] Raij, L.: Mechanisms of vascular injury: The emerging role of the endothelium. J. Am. Soc. Nephrol. **2**, S2–S8 (1991).

[32] Risau, W., P. Ekblom: Production of a heparin-binding angiogenesis factor by the embryonic kidney. J. Cell Biol. **103**, 1101–1107 (1986).

[33] Rosenstein, J. M., J. M. Krum, L. A. Sternberger, M. T. Pulley, N. H. Sternberger: Immunocytochemical expression of the endothelial barrier antigen (EBA) during brain angiogenesis. Dev. Brain Res. **66**, 47–54 (1992).

[34] Sariola, H., M. Saarma, K. Sainio, U. Arumäe, J. Palgi, A. Vaahtokari, I. Thesleff, A. Karananov: Dependence of kidney morphogenesis on the expression of nerve growth factor receptor. Science **254**, 571–573 (1991).

[35] Saxén, L.: Organogenesis of the kidney. In: Barlow, P. W., P. B. Green, C. C. Wyhi (eds.): Developmental and Cell Biology Series. Cambridge University Press. Cambridge 1987.

[36] Sorokin, L., P. Ekblom: Development of tubular and glomerular cells of the kidney. Kidney Int. **41**, 657–664 (1992).

[37] Stoolman, L. M.: Adhesion molecules controlling lymphocyte migration. Cell **56**, 907–910 (1989).

[38] Vainio, S., M. Jalkanen, M. Bernfield, S. Saxén: Transient expression of syndecan in mesenchymal cell aggregates of the embryonic kidney. Dev. Biol. **152**, 221–232 (1992).

[39] Weller, A., L. Sorokin, E.-M. Illgen, P. Ekblom: Development and growth of mouse embryonic kidney in organ culture and modulation of development by soluble growth factor. Dev. Biol. **144**, 248–261 (1991).