Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomaterials 26 (2005) 6588–6598
Biomaterials
www.elsevier.com/locate/biomaterials

Growth of embryonic renal parenchyme at the interphase of a polyester artificial interstitium

Will W. Minuth*, Lucia Denk, Sabine Heber

Department of Molecular and Cellular Anatomy, University of Regensburg, University Street 31, D-93053 Regensburg, Germany

Received 2 December 2004; accepted 4 April 2005
Available online 20 June 2005

Abstract

The construction of an artificial kidney module by tissue engineering or the application of cell-based therapies for the treatment of renal failure requires exact information regarding the cellbiological mechanisms of parenchyme development in combination with different kinds of biomaterials. To learn more about these processes tissue cultures are frequently used experimental tools. However, apart from experiments with early kidney anlagen there is a lack of suitable in-vitro models regarding the generation and long-term maintenance of renal tubules. In the present paper we like to demonstrate an advanced culture technique, which allows to generate tubular elements derived from renal stem cells. For the growth of tubules it is essential to fine-tune the interface between the embryonic tissue and the dead fluid space within a perfusion culture container by offering a polyester artificial interstitium. Culture was performed in IMDM supplemented with hormones and growth factors but using serum-free conditions over 14 days. Formation of tissue was then analysed by immunohistochemistry and two-dimensional (2D) electrophoresis. Culture in pure IMDM leads to a complete loss of tissue formation. In contrast, application of aldosterone (A) induces the development of numerous polarised tubules. Surprisingly, addition of epidermal growth factor (EGF), a cocktail of insulin, transferrin and selenium (ITS), retinoic acid (RA), cholecalciferol ($VitD_3$) or bovine pituitary extract (BPT) does not further improve development of tubules, but leads to intensive cell clustering and a decrease of tubule formation. 2D Western blots of developing tissue probed with soybean agglutinin (SBA) reveal a unique pattern of newly detected proteins. It is found that growth factors do not support but abolish protein spots upregulated by aldosterone. It remains to be investigated, which cellbiological effect stimulates the embryonic cells to develop tubules in competition to cell clusters at the interphase of an artificial interstitium.

Keywords: Artificial interstitium; Polyester; Kidney; Tubules; Stem cells; Tissue engineering

1. Introduction

New concepts in regenerative medicine such as the construction of an artificial kidney module by tissue engineering or treatment of acute and chronic renal failures by using cell-based therapies need profound knowledge of parenchyme development [1–3]. Research in this field is hindered by the limited regeneration of renal parenchyme. Tissues within the human organism differ strongly in their capacity for cellular regeneration in the case of acute or chronic damage [4]. For example, liver parenchyme belongs to tissues with a high capacitiy for regeneration [5], glomeruli and tubules show a only limited capacity for repair, while complete renewal of tissue is lacking [6]. It is unknown, if this limitation is caused by a lost capability for cell division or by a lack of necessary growth factors acting on the site of tissue damage [7]. Candidates for essential stimuli of parenchyme regeneration are growth factors such as HGF, EGF and IGF-1 [8].

Renewal of tissue needs a source of cells. For years stem cells have been in the focus of research [9–12]. However, reviewing the recent literature it appears that

*Corresponding author. Tel.: +49 0 941 943 2876; fax: +49 0 941 943 2868.
E-mail address: will.minuth@vkl.uni-regensburg.de (W.W. Minuth).

0142-9612/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2005.04.029

application of hematopoetic stem cells did not fullfill all the expectations set in this therapeutical protocol. Considering the unique development of the kidney organ-specific stem cells may appear more promising [13]. However, independent of the source of cells it is most important to learn more about the processes involved in the regeneration of a functional parenchyme. Compared to the hematopoetic system or to other organs like liver, intestine, testis or skin the knowledge how to generate functional renal parenchyme derived from renal stem cells is up to date inferior [14].

In an earlier publication we showed the feasibility to generate renal tubules derived from embryonic tissue without embedding in extracellular matrix proteins [15]. Development of tissue takes place at the interphase of a polyester artificial interstitium in a perfusion culture container. In the present experiments we like to demonstrate for the first time the competitive development between polarised renal tubules and spreading of cells forming clusters. We found that application of aldosterone supports development of tubules, while application of growth factors prevents parenchyme development. Furthermore, we registered a new group of proteins in two-dimensional (2D) Western blots probed with Soybean agglutinin (SBA). The results show that hormonal supplement produces a unique pattern of SBA-positive proteins. Individual proteins are upregulated by aldosterone, while others are suppressed after application of growth factors.

2. Materials and methods

2.1. Isolation of embryonic explants containing renal progenitor cells

One-day old New Zealand rabbits were anesthetised with ether and killed by cervical dislocation. Both the kidneys were removed immediately. Each kidney was dissected in two parts. By stripping off the capsula fibrosa with fine forceps a fully embryonic tissue layer is harvested containing numerous CD ampullae, S-shaped bodies and mesenchyme [16].

2.2. Generation of renal tubules in perfusion culture

For long-term culture a tissue holder with 14 mm outer diameter was placed in a gradient container (Fig. 1, Minucells and Minutissue, Bad Abbach, Germany) as earlier described [15,16]. To minimise the dead fluid volume within the culture container the freshly isolated embryonic renal tissue was cultured between a layer of highly porous biocompatible polyester fleece (Walraf, Grevenbroich, Germany) as an artificial interstitium on top of the holder. Thus, the embryonic tissue and the polyester material were in close contact. Fresh serum-free IMDM (Iscove's Modified Dulbecco's Medium including phenolred, GIBCO/Invitrogen, Karlsruhe, Germany, No. 21980-032) was continuously perfused for 14 days

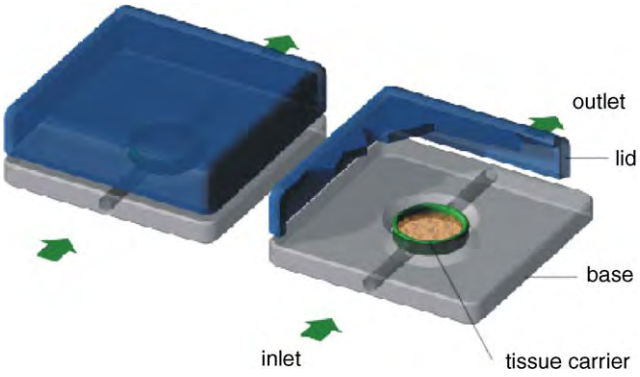

Fig. 1. Schematic illustration of a surface and insight view of a perfusion culture container. The space between the lid and base is filled with an artificial interstitium made of polyester fibers.

at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). Aldosterone (A, 1×10^{-7} M, Sigma, Taufkirchen, Germany), epidermal growth factor (EGF, 0.1 ng/ml, GIBCO/Invitrogen, No. 13247-051), insulin–transferrin–selenium–G supplement (ITS G, 1%, GIBCO/Invitrogen, No. 41400-045), cholecalciferol ($VitD_3$, 1×10^{-8} M, Calbiochem, Merck Biosciences, Schwalbach, Germany, No. 679103), retinoic acid (RA, 0.1 μmol/l, Sigma, No. R-2625), bovine pituitary extract (BPT, 20 μg/ml, GIBCO/Invitrogen, No. 13028-014) and 1% antibiotic–antimycotic solution (GIBCO/Invitrogen, No. 15240-062) was added to individual series of culture media. Furthermore, up to 50 mmol/l HEPES (GIBCO/Invitrogen, No. 15630-056) was used in the medium to maintain a constant pH of 7.4 in perfusion culture under atmospheric air containing 0.3% CO_2. To maintain a constant temperature of 37 °C, the container was placed on a thermo plate (Medax, Kiel, Germany) and covered by a transparent lid.

2.3. Amount of cultured constructs

A total of 130 tissue constructs was generated for the presented experiments. The mean of generated structure size is given in the text.

2.4. Lectin- and antibody labelling

Cryosections of the generated tubules (20 μm) were fixed in ice-cold ethanol and then washed with phosphate buffered saline (PBS). After incubation in blocking solution (PBS + 1% bovine serum albumine (BSA) + 10% horse serum) for 30 min the specimens were exposed to fluorescein- isothiocyanate (FITC)-conjugated Soybean agglutinin (SBA, Vector Laboratories, Burlingame, USA) diluted 1:2000 in PBS for 45 min as described earlier [17]. Following several washes in PBS tissue was then embedded with Slow Fade Light Antifade Kit (Molecular Probes, Eugene, USA) and analysed using an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems, San Jose, CA, USA). For histological control sections were stained with toluidine blue.

For antibody labelling the specimens were fixed in ice-cold ethanol. After washing with PBS the sections were blocked

with PBS containing 1% BSA and 10% horse serum for 30 min. Mab (monoclonal antibody) anti-Na/K-ATPase, anti-TROMA-1 (Development Studies Hybridoma Bank, University of Iowa; Department of Biological Sciences, Iwoa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD), mab antioccludin (Zymed, San Francisco, USA), mab anticytokeratine 19 (gift from Dr. R. Moll, Marburg, Germany) and mab antilaminin γ1 (kindly provided by Dr. L. Sorokin, Lund, Sweden) were applied as primary antibodies for 1 h in blocking solution. The specimens were incubated for 45 min with donkey-antimouse-IgG-fluorescein-isothiocyanate (FITC)- or biotin-conjugated secondary antibodies diluted 1:200 in PBS containing 1% BSA (Jackson Immunoresearch Laboratories, West Grove, USA). In the case of the biotin-conjugated antibody the specimens were further processed with a Vectastain Elite ABC reagent followed by application of Vector NovaRED (Vector Laboratories, Burlingame, USA). The sections were then analysed using an Axioskop 2 plus microscope or a laser-scanning microscope Zeiss LSM 510 meta (Zeiss, Oberkochen, Germany). Images were made by a digital camera and thereafter processed with Photoshop 5.5 (Adobe Systems, San Jose, USA).

2.5. 2D electrophoresis

Tissue samples were homogenised in lysis buffer (pH 7.5) containing 8 M urea, 4% CHAPS, 40 mM TRIS-HCl, 2 mM DTT and 0.5% carrier ampholytes. After determination of solubilised protein content a 150 µg sample was loaded on a gel tube for isoelectric focusing. The gel tubes were run for 14 h using increasing voltage (100–1000 V) in a model 175 tube cell apparatus (Bio-Rad Laboratories, Hercules, USA). The focused gel tubes were equilibrated in a buffer containing 2% SDS, 10% glycerin, 125 mM TRIS-HCl, 1% ß-mercaptoethanol and 1 mM EDTA and thereafter laid on the surface of 1 mm thick 10% Laemmli gels in order to separate proteins by SDS–PAGE at 100 V and 120 mA for 2 h. For control gel plates were occasionally stained with Serva Blue R (SERVA, Heidelberg, Germany).

2.6. Western blotting and SBA probing

In order to detect SBA-labelled molecules 2D separated proteins were electrophoretically transferred to nitrocellulose transfer membranes (Millipore, Bedford, USA). After exposure to TBS containing 0.01% Tween for 5 min the membrane was treated in 50 mmol citric acid/Na_2HPO_4 buffer pH 5.3 containing 0.015% H_2O_2. Then biotinylated Soybean Agglutinin (SBA) diluted 1:100 was incubated for 45 min at room temperature. After several washing steps the membrane was developed by a Vectastain Elite ABC Kit (Vector Laboratories, USA, No. PK-6100) including a DAB kit following manufacturer's instructions. The reaction was stopped by washing the membrane in tap water. Blots were documented with a Scan Jet 6200 C scanner (Hewlett Packard, Greely, USA). In the figures individual experiments are shown. Assessment of apparent molecular weight (kD) and isoelectric point (IEP) was performed with 2D SDS–PAGE standard (Bio-Rad Laboratories, Hercules, USA) and 2D protein molecular weight marker mix (PIERCE, Perbio Science, Bonn, Germany), which were run in parallel experiments. Following marker proteins (kD/IEP) were used: 2D SDS–PAGE standard: Hen egg white conalbumin type I 76/6.0–6.6, BSA 66/5.4–5.6, bovine muscle actin 43/5.0–5.1, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase 36/8.3–8.5, bovine carbonic anhydrase 31/5.9–6.0, soybean trypsin inhibitor 21.5/4.5 and equine myoglobine 17.5/7.0. 2D protein molecular weight marker mix: apo-transferrin (human plasma) 80/6.2, glutamic dehydrogenase (bovine liver) 56/6.5–6.9, actin (bovine muscle) 43/5.2, carbonic anhydrase (bovine erythrocytes) 29/6.3, myokinase (chicken muscle) 22.5/8.7, trypsin inhibitor (soybean) 20/4.5 and myoglobin (horse skeletal muscle) 17/7.0–7.4.

3. Results

3.1. Culture of renal tubules

The first series of experiments was performed to analyse the growth behaviour of renal tubules derived from embryonic tissue in culture media supplemented with aldosterone and different kinds of growth factors. We applied an advanced perfusion culture technique consisting of a container filled with a polyester artificial interstitium (Fig. 1). The standard medium for the generation of renal tubules was serum-free IMDM (I) containing HEPES (H) as biological buffer system. By morphological and histochemical methods we elaborated, in how far hormonal supplement supports the 3D extension of renal tubules. For histochemical detection we used SBA, which recognises in the kidney terminal *N*-acetylgalactosamine (GalNAcα1) residues on glycoproteins as described before [15,17].

IH: In a first experiment embryonic tissue derived from neonatal rabbit kidney was cultured in IMDM containing HEPES (IH) for 14 days (Fig. 2a and b). Cryostat sections stained with toluidine blue showed a desintegration of tissue, only thin rows with a few little cell clusters were developed (Fig. 2a). In none of the samples polarised tubular structures could be observed. Labelling the cultured tissue with SBA demonstrated single labelled cells growing on the surface of the polyester fibers (Fig. 2b). SBA-positive tubular structures could not be observed.

IH–A: In a second experiment embryonic renal tissue was cultured in IH medium containing aldosterone (IH–A) for 14 days. Toluidine blue staining of cryostat sections showed the development of compact tissue at the interface of the polyester artificial interstitium used in the perfusion culture container (Fig. 2c). A network of numerous polarised renal tubules was formed as earlier shown [15]. Incubation of the generated tissue with lectin demonstrated that numerous tubules were positive for SBA, while part of tissue could not be labelled with the lectin (Fig. 2d). Overgrowth of cells on polyester fibers and clustering was barely observed.

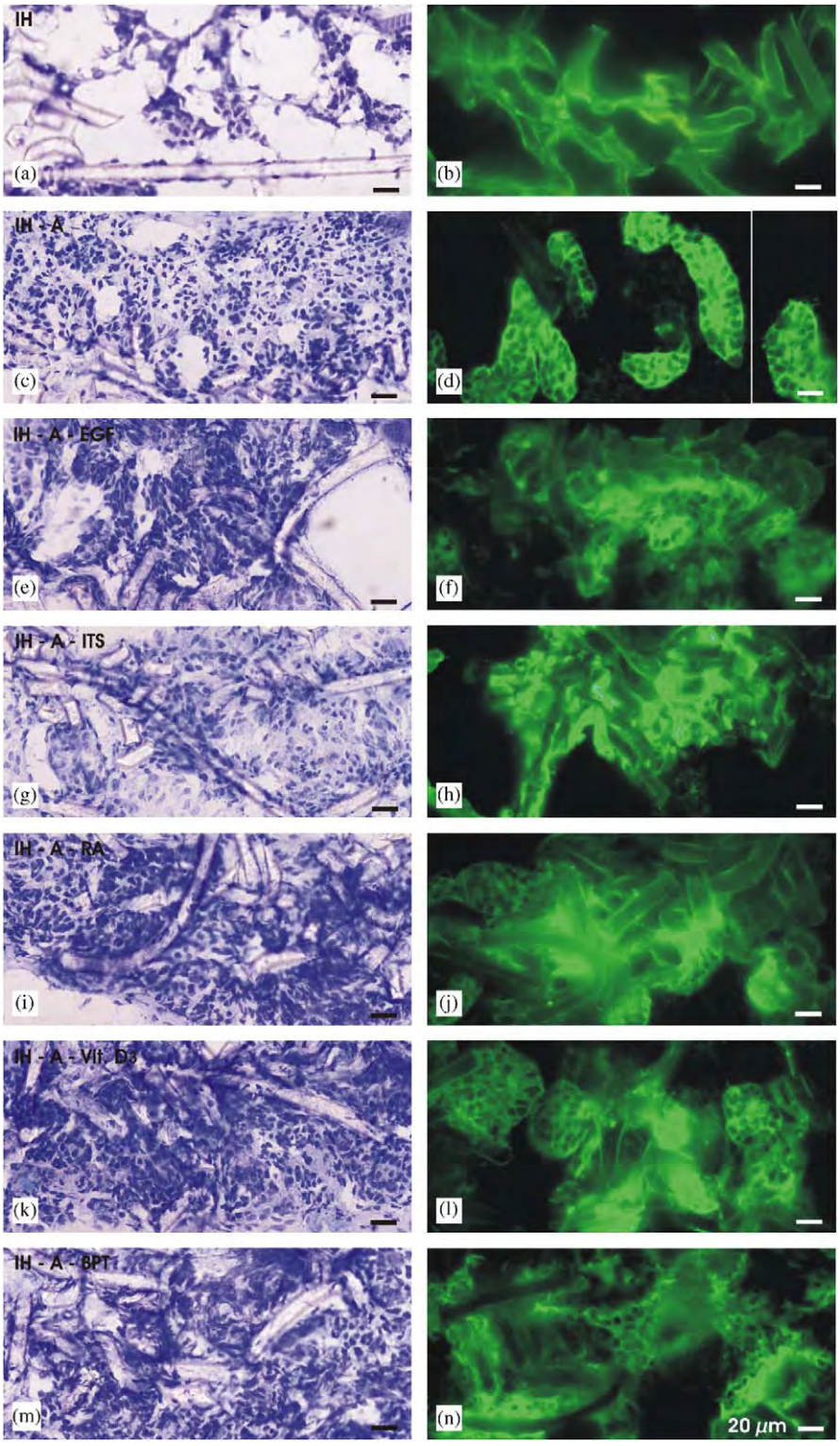

Fig. 2. Morphological view (a, c, e, g, i, k, m) and fluorescent SBA-label (b, d, f, h, j, l, n) on cultured embryonic renal tissue after 14 days of culture at the interphase of an artificial interstitium. Culture of specimens in IMDM containing HEPES (IH; a, b). Development of specimens in IH containing aldosterone (IH–A) reveals numerous tubules (c, d). Administration of additional EGF (IH–A–EGF) demonstrates an intensive overgrowth of cells on fibers and cluster formation (e, f). Addition of ITS (IH–A–ITS) indicates broad cell clustering and to a low degree formation of tubules (g, h). Presence of RA (IH–A–RA) shows overgrowth on cells on fibers and only little tubule formation (i, j). Application of $VitD_3$ (IH–A–$VitD_3$) demonstrates most intensive cell clustering (k, l). Use of BPT (IH–A–BPT) illuminates most intensive overgrowth of cells on polyester fibers (m,n).

The next experiment was performed to analyse, if the promoting effect on tubule development induced by aldosterone can be further increased by administration of different kinds of growth factors.

IH–A–EGF: Beside aldosterone EGF was applied in the culture medium to possibly increase the length of generated tubules [18]. Generated tissue after toluidine blue staining showed areas with very densly packed cells and regions with spongy cell aggregation. In the densly packed tissue areas many cells demonstrated an overgrowth on the polyester fibers (Fig. 2e). It could be observed that the cells lost their polarity and formed only few tubular structures. As compared to the IH–A series (Fig. 2c and d) an unexpectedly low number of tubules could be detected (Fig. 2e). Labelling the tissue with SBA further shows that many SBA-positive cells have overgrown the fiber network (Fig. 2f). The cells growing on the polyester form tubular structures around the fibers. Numerous clusters of cells can be recognised filling especially the space between the fibers.

IH–A–ITS: Recent literature demonstrated that insulin (I), transferrin (T) and selenium (S) are successfully used additives for the culture of tissue anlagen [19]. Consequently, in the present set of experiments embryonic tissue derived from rabbit kidneys was cultured in IMDM containing aldosterone and a cocktail of ITS. Cryostat sections showed at the first sight that a compact tissue formation is present (Fig. 2g). Conspicous is the overgrowth of numerous cells including the polyester fibers of the artificial interstitium. The formation of tubules was reduced, while development of cell clusters is increased. Labelling the tissue with SBA indicated the development of a few polarised tubules but to a high degree the overgrowth of cells on fiber material (Fig. 2h). Obvious is the high number of cells forming clusters between the fiber network.

IH–A–RA: Retinoic acid (RA) was described as a modulator of parenchyme development [20]. Consequently, we tested the effect of RA in the present perfusion culture experiments. Cross sections of the generated tissue revealed that only few tubules can be recognised beside numerous cell clusters (Fig. 2i). Most impressive is the dense packing of clusters and the intensive overgrowth of cells on the polyester fibers. Labelling the tissue by SBA revealed only few tubular elements, but to a high degree cell clustering and an intensive growth of cells on the fiber surface (Fig. 2j).

IH–A–VitD_3: Cholecalciferol was used as a factor influencing renal tissue development [21]. Toluidine blue stained sections of raised tissue demonstrated the development of densly packed areas of cells in the space between the fiber network (Fig. 2k). Numerous cells showed an overgrowth on the fibers. Obvious are round cell clusters with a diameter of more than 100 μm residing in the vicinity of the fibers. SBA-labelling showed only few developed tubules. In contrast, numerous cells have overgrown the fibers to form big cell clusters in the vicinity of the fibers (Fig. 2l).

IH–A–BPT: BPT was frequently applied as supplement for cell culture [22]. The present experiments showed after toluidine blue staining an intensive overgrowth of cells on the polyester fibers. In addition numerous cell clusters are present (Fig. 2m). Also SBA-labelling revealed an intensive overgrowth of cells on the fibers together with a broad clustering of cells (Fig. 2n). Formation of tubules was barely observed.

Summing up the histochemical data it can be shown that adminstration of aldosterone to the culture medium supports the development of tubular structures at the interphase of an artifial interstitium made by a polyester fleece (Table 1). In contrast, further application of EGF, ITS, RA, VitD_3 or BPT to IMDM containing aldosterone does not improve the development of tubules but induces an intensive overgrowth of cells on the fiber material and development of numerous cell clusters.

3.2. 2D electrophoresis of SBA-labelled proteins

The morphological data of the cultured tissue demonstrated that application of aldosterone and growth factors leads to different growth patterns of tissue development (Fig. 2). Since individual cellular and extracellular proteins in embryonic kidney can be labelled with SBA [17], we analysed SBA-positive proteins by 2D electrophoresis in the different experimental series.

The amount of solubilised protein from the embryonic tissue determined after isolation was 4.1 μg protein/μl. Analysing a 2D Western blot of a 150 μg soluble protein specimen of freshly isolated tissue revealed 330 protein spots (Fig. 3). This high number of spots is only found in freshly isolated but not in cultured tissue. As shown later part of these protein spots is up-regulated, while others are down-regulated to a different degree in the individual series of experiments cultured for 14 days.

3.2.1. Up-regulated SBA-positive protein spots

IH: 2D electrophoresis of 150 μg protein, content of specimen 5.7 μg protein/μl. 2D Western blot probed with SBA showed numerous protein spots. Most apparent are proteins in a molecular weight range between 200 and 20 (Fig. 4a).

IH–A: 2D electrophoresis of 150 μg protein, content of specimen 4.5 μg protein/μl. 2D Western blot probed with SBA demonstrated the smallest protein content in this experimental series (Table 1). In contrast, addition of aldosterone elucidates the most prominent SBA-labelled protein spots of all experimental series. As compared to IH series (Fig. 4a) aldosterone up-regulates individual proteins (Fig. 4b). Impressive spots are 69/5.7, 64/6.6, 48/6.2, 45/5.8, 31/7.5, 28/7.3 and 27/7.4.

Table 1
Generated tubules versus overgrowth of cells on polyester fibers and cluster formation

	Development of tubules	Tubules and clustering	Clustering	Protein content (μg/μl)
After isolation	+ + +	–	–	4.1
IH	–	+	+	5.7
IH–A	+ + +	–	–	4.5
IH–A–EGF	+	+ +	+ + +	5.1
IH–A–ITS	+	+ +	+ + +	6.5
IH–A–RA	+	+ + +	+ + +	5.3
IH–A–VitD$_3$	+	+ + +	+ + +	5.1
IH–A–BPT	+	+ + +	+ + +	5.1

+ + + intensive, + + mediocre, + low, –missing of tubular development and/or cell clustering. Protein content of solubilised proteins is given as a mean of 3 measurements.

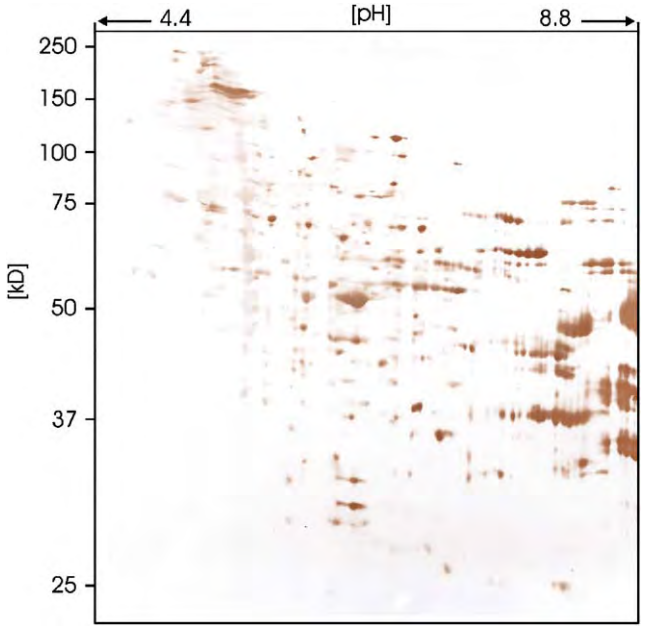

Fig. 3. SBA-labeled protein pattern in 2D Western blots of embryonic renal tissue after isolation. 330 protein spots are detected. SBA-labelled proteins are found in a pH between 4.4 and 8.8.

IH–A–EGF: 2D electrophoresis of 150 μg protein, content of specimen 5.1 μg protein/μl. 2D Western blot probed with SBA revealed less prominent protein spots after EGF treatment (Fig. 4c) as compared to IH–A–series (Fig. 4b). The following spots were found to be up-regulated: 62/7.8, 48/6.2, 45/5.8 and 37/6.1.

IH–A–ITS: 2D electrophoresis of 150 μg protein, content of specimen 6.5 μg protein/μl. 2D Western blot probed with SBA elucidated an unexpected weak pattern of spots after ITS treatment (Fig. 4d) as compared to the IH–A–series (Fig. 4b). As shown before a group of spots is upregulated. Remarkable spots are 69/5.7, 33/6.5, 31/7.0 and 28/6.9.

IH–A–RA: 2D electrophoresis of 150 μg protein, content of specimen 5.3 μg protein/μl. 2D Western blot probed with SBA demonstrated only one up-regulated protein spot: 62/7.2 (Fig. 4e).

IH–A–VitD$_3$: 2D electrophoresis of 150 μg protein, content of specimen 5.1 μg protein/μl. One protein spot (37/7.4) with high and one spot (37/7.2) with lower SBA-label was found (no figure).

IH–BPT: 2D electrophoresis of 150 μg protein, content of specimen 5.1 μg protein/μl. 2D Western blot probed with SBA demonstrates that 2 spots are up-regulated: 62/8.2, 31/7.5 (Fig. 4f).

3.2.2. *Down-regulated SBA-positive protein spots*

IH–A–EGF: 2D electrophoresis of 150 μg protein, content of specimen 5.1 μg protein/μl. 2D Western blot probed with SBA revealed 3 down-regulated proteins: 64/6.6, 64/6.8, 28/7.2 (Fig. 5c).

IH–A–ITS: 2D electrophoresis of 150 μg protein, content of specimen 6.5 μg protein/μl. 2D Western blot probed with SBA elucidated 2 down-regulated protein spots: 52/7.5, 43/7.2 (Fig. 5d).

IH–A–RA: 2D electrophoresis of 150 μg protein, content of specimen 5.3 μg protein/μl. 2D Western blot probed with SBA demonstrated 3 down-regulated protein spots: 67/7.1, 67/7.4, 52/7.5 (Fig. 5e).

IH–A–VitD$_3$: 2D electrophoresis of 150 μg protein, content of specimen 5.1 μg protein/μl. Except protein spots (37/7.4 and 37/7.2) no SBA-label was found (no figure).

IH–BPT: 2D electrophoresis of 150 μg protein, content of specimen 5.1 μg protein/μl. 2D Western blot probed with SBA demonstrated that numerous spots are down-regulated: 68/7.1, 67/7.5, 62/7.3, 48/6.2, 30/6.6, 28/7.3, 27/7.4 (Fig. 5f).

Comparing the 2D electrophoresis data in the different series of experiments it can be summarised (Table 2), that aldosterone up-regulates the following SBA-labelled proteins: 69/5.7, 64/6.6, 48/6.2, 45/5.8, 31/7.5, 28/7.3 and 27/7.4 (Figs. 4b and 5b). In contrast, BPT, e.g. was able to up-regulate (Fig. 4f) but also to down-regulate the expression of proteins (Fig. 5f). Application of EGF (Figs. 4c and 5c), ITS (Figs. 4d

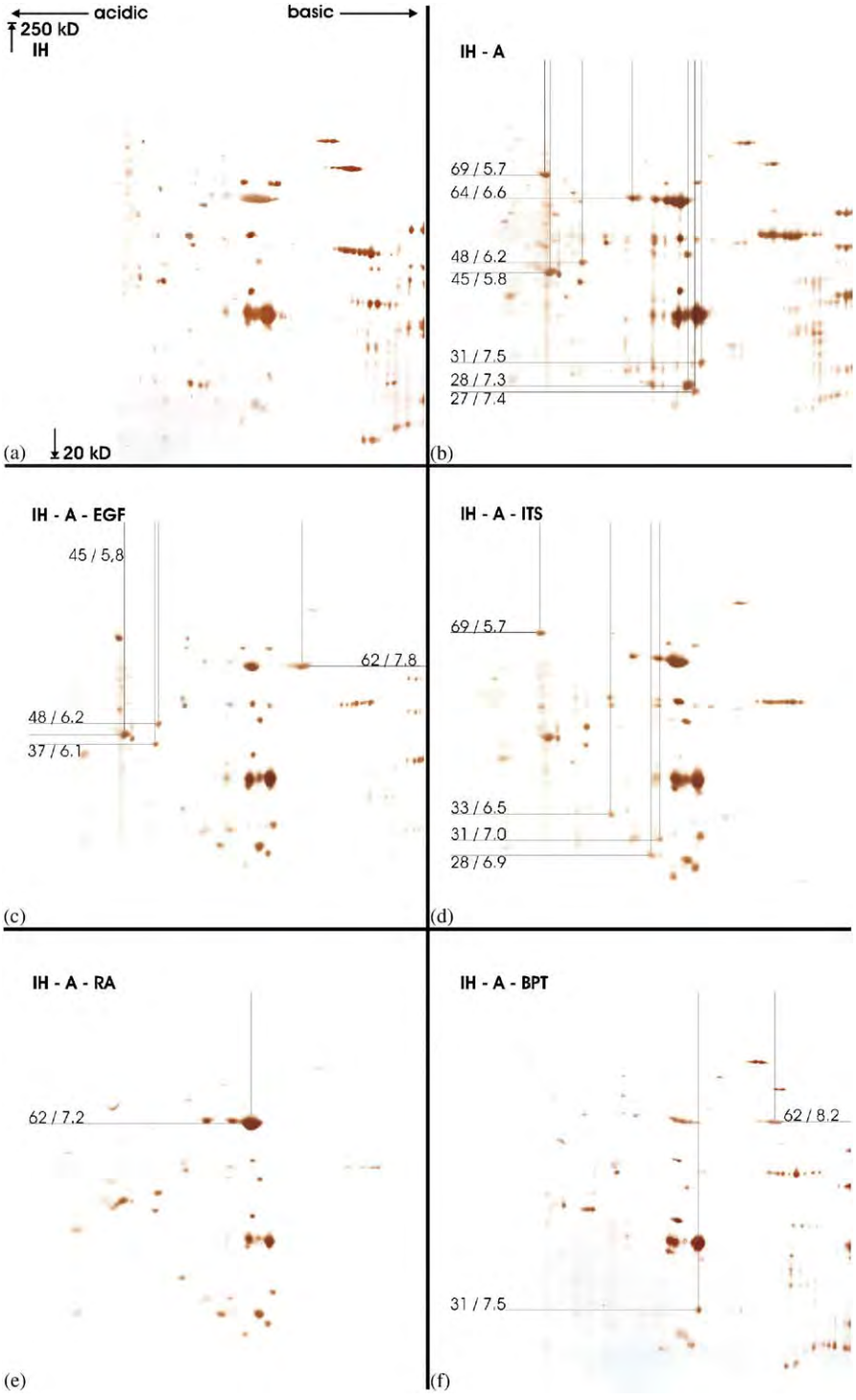

Fig. 4. Up-regulated proteins shown in 2D Western blots and SBA probing. Embryonic renal explants were cultured at the interphase of an artificial interstitium. Culture was performed in IH (a), IH–A (b), IH–A–EGF (c), IH–A–ITS (d), IH–A–RA (e), IH–A–BPT (f). Examples of differently expressed SBA-labelled proteins are illustrated by molecular weight and isoelectric point.

and 5d) and RA (Figs. 4e and 5e) and BPT (Figs. 4f and 5f) showed an individual pattern and may represent in part the formation of cell clusters (Fig. 2f,h,j,l,n). However, a distinct single protein spot indicating the clustering of cells could not be observed.

4. Discussion

While numerous data are available showing the primary cellbiological events during development of the kidney anlage, knowledge about regeneration, organ

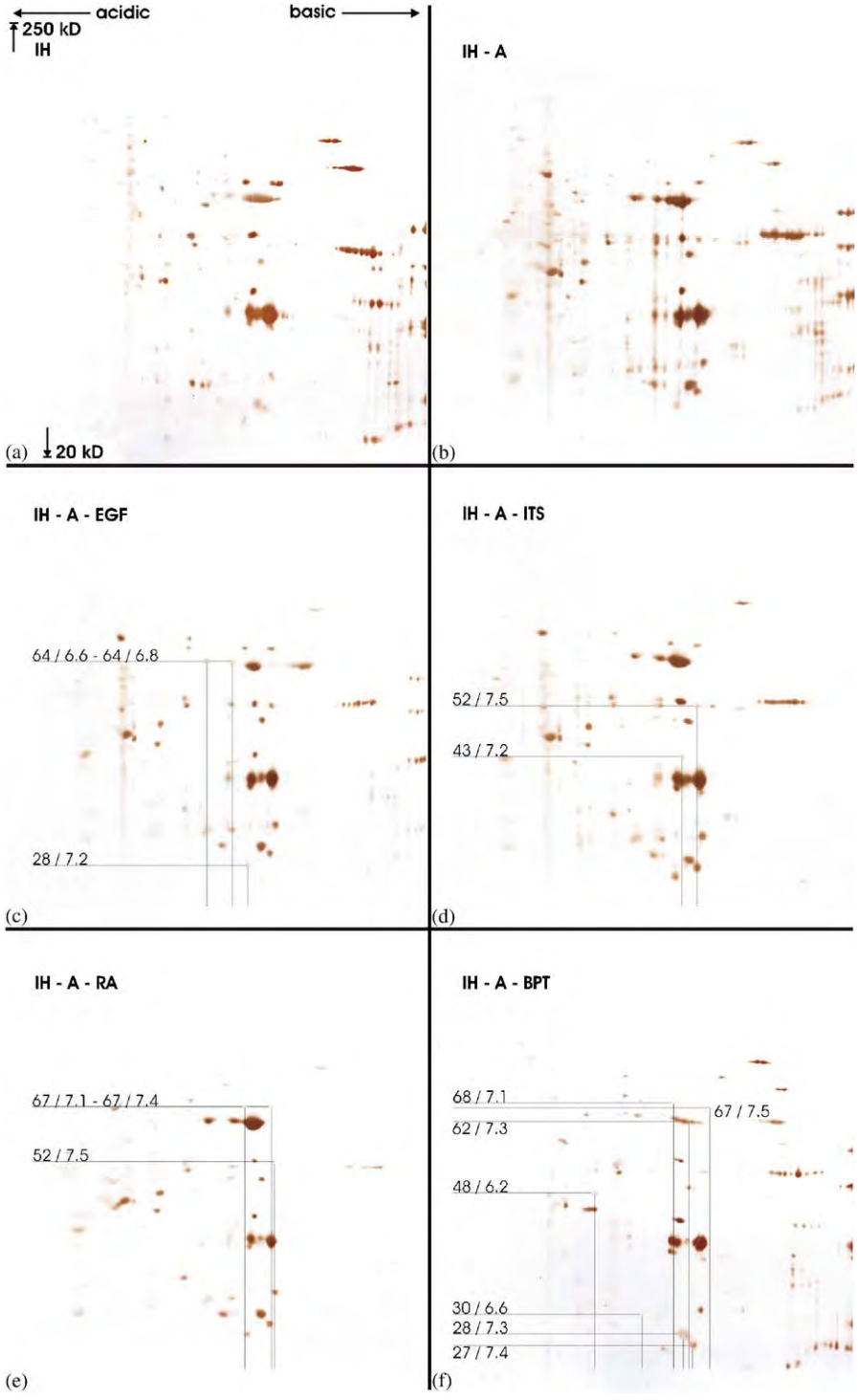

Fig. 5. Down-regulated proteins shown in 2D electrophoresis following Western blot and SBA probing. Embryonic renal explants were cultured at the interphase of an artificial interstitium. Culture was performed in IH (a), IH–A (b), IH–A–EGF (c), IH–A–ITS (d), IH–A–RA (e), IH–A–BPT (f). Examples of differently expressed SBA-labelled proteins are illustrated by molecular weight and isoelectric point.

growth and terminal differentiation of renal parenchyme is barely available [23–25]. To investigate nephron development culture of mesenchyme in transfilter experiments is commonly used [26]. However, life-span and developmental capacity of this kind of culture are limited. An alternative approach is the culture of renal cells with defined tubular origin at the bottom of a culture dish or on a filter. The spreading of cells in serum-containing medium results in a flat outgrowth [27,28]. However, embedding of cells in collagen allows

Table 2
Up- and down-regulated proteins (kD/IEP) in embryonic renal tissue after a 14-day culture period

Series	Up-regulated proteins	Down-regulated proteins
IH	—	—
IH–A	69/5.7, 64/6.6, 48/6.2, 45/5.8, 31/7.5, 28/7.3, 27/7.4	—
IH–A–EGF	62/7.8, 48/6.2, 45/5.8, 37/6.1	64/6.6, 64/6.8, 28/7.2
IH–A–ITS	69/5.7, 33/6.5, 31/7.0, 28/6.9	52/7.5, 43/7.2
IH–A–RA	62/7.2	67/7.1, 67/7.4, 52/7.5
IH–A–VitD_3	37/7.4, 37/7.2	Most
IH–A–BPT	62/8.2, 31/7.5	68/7.1, 67/7.5, 62/7.3, 48/6.2, 30/6.6, 28/7.3 27/7.4

the generation of 3D structures as earlier shown including cell lines such as MDCK cells [29–31]. Disadvantages are the use of serum-containing media, the limited amount of material available for cellbiological analysis and the low degree of nephron-specific differentiation.

The aim of the present experiments was to elaborate culture conditions to investigate the development of renal tubules derived from epithelial and mesenchymal stem cell populations in serum-free culture medium and in combination with a suitable biomaterial. Due to the limited size of embryonic mouse or rat specimens we selected the neonatal rabbit as a cellbiological model, since the embryonic cortex contains numerous stem cell niches in their original extracellular environment [16]. The embryonic tissue layer is easily accessible for isolation and can be harvested in sufficient amounts for tissue culture or cellbiological analysis.

First we determined the protein content of the cultured specimens (Table 1). One may expect that the amount of protein correlates with the number of tubules developed. However, our results indicate that this assumption is not correct. To start a culture protocol we consistently used explants 6 mm in diameter with constant thickness. Determination of protein content showed that the embryonic tissue after isolation contains in mean 4.1 μg/μl of solubilised protein. In contrast, culture of tissue in IH medium after 14 days demonstrated 5.7 μg protein/μl. Administration of aldosterone showed an unexpected low content of 4.5 μg protein/μl. In contrast, treatment of cultures with EGF, ITS, RA, VitD_3 or BPT revealed a higher contents ranging between 5.1 and 6.5 μg protein/μl. Thus, application of growth factors increases the amount of biomass between 24% and 58% during a 14 days culture period. It further indicated that the generated tissue is viable under described culture conditions.

In contrast, fluorescence microscopy elucidated that culture of embryonic explants in serum-free medium without hormonal supplement leads to tissue desintegration (Fig. 6a). Application of aldosterone stimulates the cells to form numerous polarised tubular structures (Fig. 6b). Immunohistochemistry further demonstrated that aldosterone generates renal collecting duct derived tubules with an apico-basal polarisation. This can be recognised by labelling the tubules with tissue specific antibodies. Mab anticytokeratine 19 (Fig. 7a) and anti-Troma-1 (Fig. 7b) label demonstrate collecting duct-specific features. In addition, mab antilaminin γ1 (Fig. 7c) shows development of a basement membrane, mab antioccludin (Fig. 7d) reveals development of tight junctions, while mab anti-Na/K ATPase (Fig. 7e) demonstrates the appearance of an important functional feature as found within the kidney.

Most important, application of growth factors such as insulin–transferrin–selenium–G supplement (ITS, Fig. 6c) in aldosterone-containing medium (IH–A) does not further improve development of tubular structures. Application of the mentioned growth factors stimulated the cells to grow out and to spread over polyester fibers. Under these conditions unexpected large cell clusters are formed between the fibers. Although all of the growth factors increased cell mass (Table 1), they did not support the development of tubular structures. It is a new finding that the development of cell clusters after growth hormone application is competive to the arise of tubular structures (Fig. 6b and c). Up to date we can only speculate about the reasons why development of tubules is promoted after aldosterone application and why development of cell clusters is inhibited after further growth factor administration. As seen by the content of protein (Table 1) and by histochemical labelling application of growth factors such as epidermal growth factor (EGF, Fig. 2e), insulin–transferrin–selenium–G supplement (ITS, Fig. 2g), retinoic acid (RA, Fig. 2i), cholecalciferol (VitD_3, Fig. 2k) or bovine pituitary extract (BPT, Fig. 2m) obviously accelerates growth of cells to an extreme extend. It appears that tubules cannot be formed in a coordinated progress when cell proliferation is too fast. A similar growth behaviour is found in isolated renal tubules maintained at the bottom of a culture dish [27,28].

Numerous publications demonstrated a pleiotropic action of aldosterone comprising non-genomic effects [32], promotion of growth [33] or differentiation [34] and renal injury [35]. The present experiments demonstrated

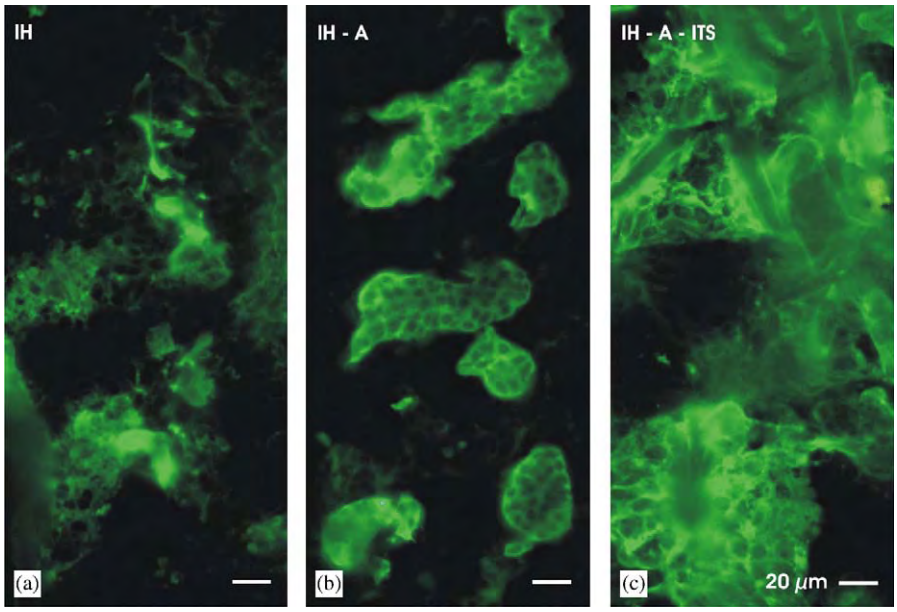

Fig. 6. SBA-labelled renal embryonic tissue cultured at the interphase of an artificial interstitium in IH (a), IH–A (b) and IH–A–ITS (c) after 14 days. IH medium reveals disintegration of tissue (a), while IH–A (b) shows development of numerous tubules. In contrast, IH–A–ITS (c) demonstrates intensive cell cluster formation and overgrowth of cells on polyester fibers.

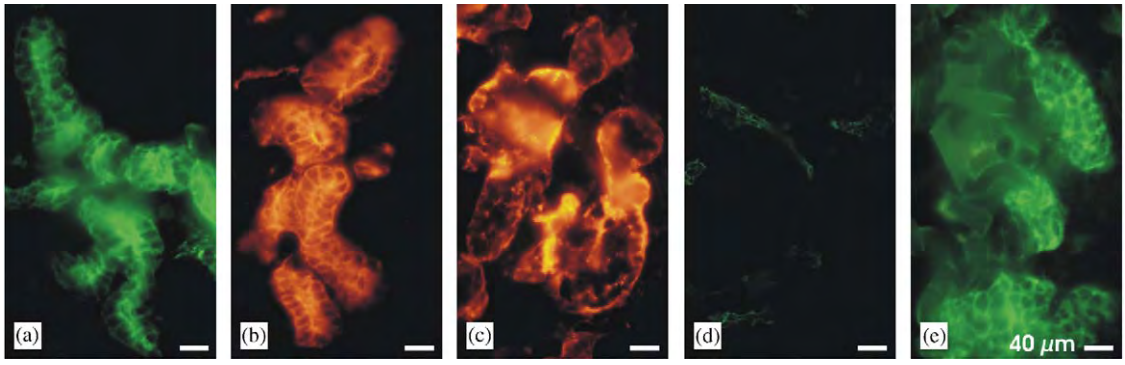

Fig. 7. Development of tissue-specific features in generated tubules cultured in IH–A for 14 days. Label with mab anticytokeratine 19 (a), Troma-1 (b), laminin γ1 (c), occludin (d) and Na/K ATPase (e).

for the first time that aldosterone triggers development of renal tubules (Fig. 6b), while application of various growth factors did not further support this process (Fig. 6c). 2D Western blots and SBA probing further showed the up-regulation of individual not yet further identified aldosterone-dependent proteins (Fig. 4, Table 2). Several of these proteins are down-regulated by the administration of growth factors (Fig. 5). In none of the cases the pattern induced by aldosterone is supported or even increased by the administration of growth factors. It further reflects the abolishment of aldosterone effect after administration of growth factors. Consequently, further experiments are under work to find different growth factors, which do not abolish but complete the action of aldosterone on SBA-labelled proteins.

5. Conclusions

By the present set of experiments we obtained first important information that culture of embryonic renal tissue at the interphase of an artificial interstitium made of polyester does not develop automatically into a network of structured tubules. Aldosterone stimulates the formation of tubules, while addition of various growth factors leads to a complete change in cell behaviour, since development of tubules is replaced by

cell cluster formation. Experiments are under work to elaborate this new insight in parenchyme development.

Acknowledgements

The technical assistance of Mr. Uwe de Vries is gratefully acknowledged.

References

[1] Humes HD, Weitzel WF, Barlett RH, Swaniker FC, Paganini EP, Luderer JR, Sobota J. Initial clinical results of the bioartificial kidney containing human cells in ICU patients with acute renal failure. Kidney Int 2004;66(4):1578–88.
[2] Hammerman MR. Treatment for end-stage renal desease: an organogenesis/tissue engineering odyssey. Transpl Immunol 2004;12(3–49):211–8.
[3] Atala A, Koh CJ. Tissue engineering applications of therapeutic cloning. Annu Rev Biomed Eng 2004;6:27–40.
[4] Ogle B, Cascalho M, Platt JL. Fusion of approaches to the treatment of organ failure. Am J Transplant 2004;4,6:74–7.
[5] Taub R. Liver regeneration: from the myth to mechanism. Nat Rev Mol Cell Biol 2004;5(10):836–47.
[6] Koh CJ, Atala A. Tissue engineering, stem cells, and cloning: opportunities for regenerative medicine. J Am Soc Nephrol 2004;15(5):1113–25.
[7] Negri AL. Prevention of progressive fibrosis in chronic renal diseases: antifibrotic agents. J Nephrol 2003;17(4):496–503.
[8] Ernst F, Hetzel S, Stracke S, Czock D, Vardas G, Lutz MP, Keller F, Jehle PM. Renal proximal tubular cell growth and differentiation are differently modulated by renotropic factors and tyrosine kinase inhibitors. Eur J Clin Invest 2001;31(12):1029–39.
[9] Dekel B, Reisner Y. Embryonic committed stem cells as a solution to kidney donor shortage. Expert Opin Biol Ther 2004;4(4):443–54.
[10] Rookmaaker MB, Verhaar MC, van Zonneveld AJ, Rabelink TJ. Progenitor cells in the kidney: biology and therapeutic perspectives. Kidney Int 2004;66(2):518–22.
[11] Oliver JA, Maarouf O, Cheema FH, Martens TP, Al-Awqati Q. The renal papilla is a niche for adult kidney stem cells. J Clin Invest 2004;114(6):795–804.
[12] Challen GA, Martinez G, Davis MJ, Taylor DF, Crowe M, Teasdale RD, Grimmond SM, Little MH. Identifying the molecular phenotype of renal progenitor cells. I Am Soc Nephrol 2004;15(9):2344–57.
[13] Ogle B, Cascalho M, Platt JL. Fusion of approaches to the treatment of organ failure. Am J Transplant 2004;4(6):74–7.
[14] Kim S, Park HJ, Han J, Choi CY, Kim B. Renal tissue reconstruction by the implantation of renal segments on biodegradable polymer scaffolds. Biotech Lett 2003;25:1505–8.
[15] Minuth WW, Sorokin L, Schumacher K. Generation of renal tubules at the interface of an artificial interstitium. Cell Physiol Biochem 2004;14:387–94.
[16] Minuth WW. Neonatal rabbit kidney cortex in culture as tool for the study of collecting duct formation and nephron differentiation. Differentiation 1987;36(1):12–22.
[17] Schumacher K, Strehl R, de Vries U, Groene HJ, Minuth WW. SBA-positive fibers between CD ampulla, mesenchyme and renal capsule. J Am Soc Nephrol 2002;13:2446–53.
[18] Weston CE, Feibelman MB, Wu K, Simon EE. Effects of EGF and IGF-1 on proliferation of cultured human proximal tubule cells after oxidant stress. Ren Fail 2004;26(1):13–20.
[19] Gaber AO, Fraga D. Advances in long-term islet culture. Cell Biochem Biophys 2004;40:49–54.
[20] Tulachan SS, Doi R, Kawaguchi Y, Tsuji S, Nakajima S, Masui T, Koizumi M, Toyoda E, Mori T, Ito D, Kami K, Fujimoto K, Imamura M. All-trans retinoic acid induces differentiation of ducts and endocrine cells by mesenchymal/epithelial interactions in embryonic pancreas. Diabetes 2003;52(1):76–84.
[21] Cao LP, Bolt MJ, Wei M, Sitrin MD, Chun Li Y. Regulation of calbindin-D9k expression by 1,25-dihydroxyvitamin D(3) and parathyroid hormone in mouse primary renal tubular cells. Arch Biochem Biophys 2002;400(1):118–24.
[22] Kudlow JE, Kobrin MS. Secretion of epidermal growth factor-like mitogens by cultured cells from bovine anterior pituitary glands. Endocrinology 1984;115(3):911–7.
[23] Inoue CN, Sunagawa N, Morimoto T, Ohnuma S, Katsushima F, Nishio T, Kondo Y, Iinuma K. Reconstruction of tubular structures in three-dimensional collagen gel culture using proximal tubular epithelial cells voided in human urine. In Vitro Cell Dev Biol Anim 2003;39(8–9):364–7.
[24] Rosario M, Birchmeier W. How to make tubes: signalling by the Met receptor tyrosine kinase. Trends Cell Biol 2003;13(6):328–35.
[25] Eisen R, Ratcliffe DR, Ojakian GK. Modulation of epithelial tubule formation by Rho kinase. Am J Physiol Cell Physiol 2004;286(4):C857–66.
[26] Saxen L, Lehtonen E. Embryonic kidney in organ culture. Differentiation 1987;36(1):2–11.
[27] Ash SR, Cuppage FE, Hoses ME, Selkurt EE. Culture of isolated renal tubules: a method of assessing viability of normal and damaged cells. Kidney Int 1975;7(1):55–60.
[28] Horster M. Primary culture of mammalian nephron epithelia: requirements for cell outgrowth and proliferation from defined nephron segments. Pflugers Arch 1979;382(3):209–15.
[29] Ito T, Williams JD, Al-Assaf S, Phillips GO, Phillipps AO. Hyaluron and proximal tubular cell migration. Kidney Int 2004;65(3):823–33.
[30] Williams MJ, Clark P. Microscopic analysis of the cellular events during scatter factor-induced epithelial tubulogenesis. J Anat 2003;203(5):483–503.
[31] Kondo S, Kagami S, Urushihara M, Kitamura A, Shimizu M, Strutz F, Muller GA, Kuroda Y. Transforming growth factor-beta1 stimulates collagen matrix remodelling through increased adhesive and contractive potential by human renal fibroblasts. Biochem Biophys Acta 2004;1693(2):91–100.
[32] Chun TY, Pratt JH. Non-genomic effects of aldosterone: new actions and questions. Trends Endocrinol Metab 2004;15(8):353–4.
[33] Van Den Meiracker AH, Huizenga AT, Boomsma F. Profibrotic effects of aldosterone. Ned Tijdschr Geneeskd 2004;148(31):1532–6.
[34] Minuth WW, Gilbert P, Gross P. Appearance of specific proteins in the apical plasma membrane of cultured renal collecting duct principal cell epithelium after chronic administration of aldosterone and arginine vasopressin. Differentiation 1988;38:194–202.
[35] Nishiyama A, Abe Y. Aldosterone and renal injury. Nippon Yakurigaku Zasshi 2004;124(2):101–9.