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Regeneration of renal tubules at an artificial polyester interstitium

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Kurzfassung

Eine der großen Herausforderungen in der Biomedizin ist die Regeneration von Tubulusstrukturen beim chronischen Nierenversagen mit Hilfe von Stammzellen und Verfahren des Tissue engineering. Allerdings gibt es bis heute kaum gesichertes zellbiologisches Wissen, wie eine solche Regeneration therapeutisch gesteuert werden kann. Von großem wissenschaftlichem Interesse sind deshalb Vorgänge zur Entwicklung, die aus Stammzellen funktionelle Tubuli entstehen lassen. Für diese Arbeiten wird ein besonders leistungsfähiges Kultursystem benötigt.

Für die Generierung von renalem Parenchym wurde eine innovative Kulturtechnik entwickelt. Dazu werden Stamm-/Progenitorzellen der Niere von neonaten Kaninchen zwischen Schichten aus Polyestervliesen gehalten. Dieses artifizielle Interstitium ersetzt die Beschichtung mit Proteinen der extrazellulären Matrix. Eine räumliche Entwicklung von Tubulusstrukturen wird mit der Perfusionskultur, chemisch definiertem Iscove's Modified Dulbecco's Medium und Aldosteron (1×10^{-7} M) innerhalb von 13 Tagen erreicht.

Elektronenmikroskopische Daten zeigen, dass die generierten Tubuli ein polar differenziertes Epithel ausbilden, welches auf der luminalen Seite mit Tight junctions und am basalen Aspect eine intakte Basallamina aufweist. Immunhistochemische Befunde ergeben eine intensive Markierung für Cyclooxygenase 2, Troma I, Cytokeratin 19, Occludin, Na/K-ATPase, Laminin und Kollagen Typ III.

Die Daten zeigen, dass mit der vorgestellten Technik unter klar definierten in vitro Bedingungen die Regeneration von renalen Tubuli in Verbindung mit innovativen Vliesen und drug delivery Systemen untersucht werden kann.

Abstract

Tissue engineering using stem/progenitor cells to repair kidney function is thought to hold great promise in regenerative medicine. However, up to date sound cell biological knowledge about nephron renewal is lacking. In the focus of interest are mechanisms stimulating the development from stem/progenitor cells into structured tubules. For these investigations a powerful culture system is of essential importance.

An advanced technique stimulates renal stem/progenitor cells from neonatal rabbit kidney to develop numerous tubules between layers of polyester fleece. The resulting artificial interstitium substitutes coating by extracellular matrix proteins. Spatial formation of tubules is obtained after 13 days of perfusion culture using chemically defined Iscove's Modified Dulbecco's Medium containing aldosterone (1×10^{-7} M).

Transmission electron microscopy (TEM) shows that generated tubules contain a polarized epithelium with a tight junctional complex at the luminal side and an intact basal lamina at the basal aspect. Immunohistochemistry shows an intense label for cyclooxygenase 2, Troma I, cytokeratin 19, occludin, Na/K-ATPase, laminin and collagen type III.

In conclusion, regeneration of renal tubules can be simulated for the first time under advanced in vitro conditions. Applying this model stimulating influences on renal tubule development in combination with innovative fleece materials and drug delivery can be tested.

1 Spatial regeneration

The versatile processes promoting the development of stem/progenitor cells into renal tubules are under intensive research. On the one hand the complex spatial micro-architecture of the kidney has to be considered and on the other hand multiple growth factors triggering the development of stem/progenitor cells have to be tested.

A major problem is that the micro-architecture of the kidney hinders the performance of experiments in situ. For that reason culture experiments have to be made. However,

featuring tubulogenesis under in vitro conditions is more difficult than expected.

For example, renal tubule segments were isolated and placed at the bottom of a culture dish. Addition of a culture medium containing fetal bovine serum does not result in the elongation of the isolated tubule. In contrast, cells start to migrate, leave the tubule and spread on the bottom of the culture dish.

Offering a three-dimensional environment isolated cells collected from the urine or MDCK cells were coated by extracellular matrix proteins. After initiation of culture cells migrate to form first cords. During longer culture spa tial formation of tubules is observed. However, the relatively thick layer of three-dimensional coating by extracellular matrix proteins is problematic, since it does not promote an optimal exchange of nutrition and respiratory gas. In addition, during long term culture the coating leads to the formation of unstirred layers of medium. This again causes a deleterious accumulation of metabolites, which in turn hinders arise of a physiological environment.

2 Simulation of an artificial interstitium

For the development of stem/progenitor cells into renal tubules the environment has to fulfill certain physiological requirements. In the kidney developing tubules are embedded in an interstitium consisting of both extracellular matrix fibers and specific nutritional fluid. Thus, to promote the generation of tubules an artificial interstitium was created in analogy to the situation within the kidney.

The technical solution is to culture stem/progenitor cells between layers of polyester fleece. The fibers of the fleece simulate mechanical properties of extracellular matrix, while the space between the fibers is easily accessible for the transport of nutrition and respiratory gas. In addition, the interface between two fleece layers is highly biocompatible promoting the spatial development of tubules over long periods of time.

2.1 Stem/progenitor cells within fleeces

To investigate the regeneration of tubules under in vitro conditions a suitable source of renal stem/progenitor cells is needed.

Due to the limited size of embryonic mouse or rat specimens, neonatal rabbit kidney is selected as a favorite model. Even after birth the embryonic cortex of the organ contains numerous stem cell niches.

The embryonic tissue layer beyond the organ capsule is easily accessible for isolation. Stripping off the capsule with fine forceps a thin layer of embryonic tissue adheres to the explant. By this simple isolation method a layer of up to 1 cm^2 in square can be harvested. Up to date no other species is known for the isolation of tissue containing renal stem/progenitor cells in such an amount necessarily needed for subsequent culture and cell-biological analysis.

After isolation the embryonic tissue is placed between layers of polyester fleece (I7, Walraf, Grevenbroich, Germany) [1]. For mounting a base ring of a Minusheet® tissue carrier (Minucells and Minutissue, Bad Abbach, Germany) with 13 mm inner diameter is used. First a polyester fleece measuring 13 mm in diameter is transferred to the tissue carrier. Then the basic sandwich set-up containing renal stem/progenitor cells is mounted. Finally, a polyester fleece with 13 mm in diameter is placed on top as a cover. This specific arrangement results in a basic sandwich set-up with the isolated tissue in the middle and layers of polyester fleece covering the upper and lower sides.

For perfusion culture the tissue carrier is used in a container with horizontal flow characteristics. To maintain a constant temperature of 37°C, the perfusion culture container is placed on a thermoplate (Medax-Nagel, Kiel, Germany) and covered with a transparent lid.

2.2 Offering continuously fresh medium

For a culture period of 13 days always fresh medium is transported by a rate of 1.25 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). Applying a biological buffer such as HEPES or Buffer All medium has a pH of 7.4 and is saturated under atmospheric air to 160 mmHg oxygen during transportation.

The high content of oxygen in the medium is reached by a long thin-walled silicone tube, which is highly gas-permeable guaranteeing optimal diffusion between culture medium and surrounding atmosphere.

For the generation of renal tubules chemically defined Iscove´s Modified Dulbecco´s Medium (IMDM, GIBCO/Invitrogen, Karlsruhe, Germany) is used. Infections are prevented by an antibiotic-antimycotic cocktail (1%, GIBCO). To induce tubulogenic development aldosterone (1 x 10-7 M, Fluka, Taufkirchen, Germany) is administered to the medium.

3 Registration of development

After a culture period of 13 days the tubules are matured so far that cell biological analysis can be performed. By tearing off the layers of the fleece the artificial polyester interstitium is opened. The area for tubule formation is 5 mm in diameter and up to 250 µm in height. For whole mount label the specimens are fixed in ethanol and stained

by fluorescent Soybean Agglutinin (SBA). While the isolated stem/progenitor cells within their niches do not exhibit cellular SBA-label, acquisition of SBA-binding after a 13 days culture period in IMDM containing aldosterone illustrates the development of structured tubules.

To obtain exact information about the number of generated SBA-labeled tubules a WCIF ImageJ program (Bethesda, Maryland, USA) is used for counting (Fig. 1). Applying this technique an individual example depicts that 86 tubules are detected within a microscopic opening of 388 x 733 µm. Fluorescence microscopy further demonstrates that tubules are growing in a spatial arrangement.

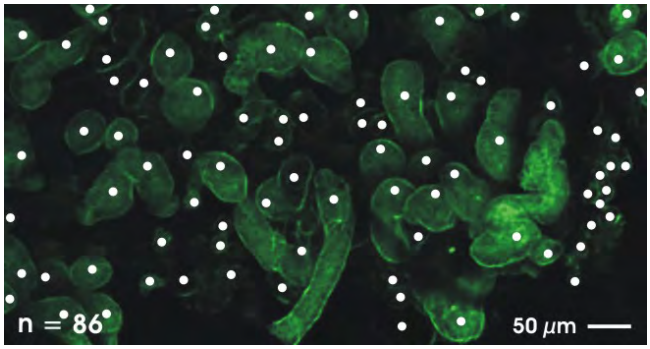

Figure 1 View on 86 SBA-labeled tubules generated at the artificial polyester interstitium.

Part of the tubules illustrates a straightforward growth, while others reveal a dichotomous branching or curling. When the tubules are not leaving the optical plain, it is possible to follow their longitudinal growth over a distance between 300 and 400 µm. SBA-label further shows that generated tubules contain polarized cells, a visible lumen and a basal lamina. In so far the used I7 polyester fleece is providing a perfect surrounding for the development of renal stem/progenitor cells.

Since coating by extracellular matrix proteins was not performed, it is possible for the first time to analyze generated tubules by scanning electron microscopy (SEM, Fig. 1). The surrounding of generated tubules is not stacked by proteins derived from a coating process. For that reason interactions between developed tubules and surrounding fleece fibers can be visualized.

The overall view demonstrates that tubules develop in a parallel fashion, while others exhibit a dichotomous branching. All of the tubules are covered by a continuously developed basal lamina.

Transmission electron microscopy (TEM) reveals more insights in generated tubules [2]. The tubules contain an isoprismatic epithelium surrounding a lumen. In the center of a cell a large nucleus can be recognized. Within the cytoplasm lysosomal elements are found. Vacuoles are filled to a various degree with electron-dense material suggesting that the containing material has been phagocytosed.

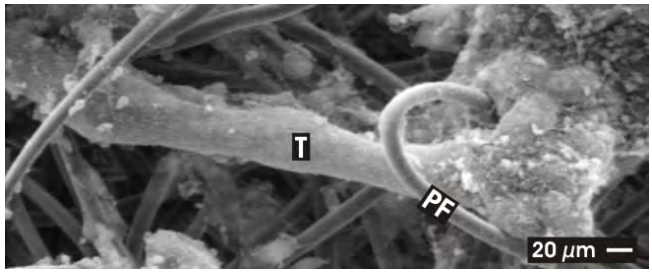

Figure 2 SEM analysis views tubules (T) developed between polyester fibers (PF) of the artificial interstitium.

Neighboring epithelial cells are in close contact to each other. Higher magnification of TEM demonstrates that the luminal and lateral plasma membranes are separated by a tight junctional complex. At the basal aspect of the epithelium a complete lamina is found.

To obtain detailed information about features of differentiation cryosections of generated tubules were analyzed by immunohistochemistry. Positive reaction for cyclooxygenase 2 (Cox2), Troma I (cytokeratin Endo-A), cytokeratine 19 and E-cadherin exhibits an intense label on all tubule cells. Label for occludin demonstrates the development of a tight junctional belt recognized as faint reaction in the luminal portion of generated tubules. Bright reaction for Na/K-ATPase α5 is detected at the basolateral plasma membrane of generated tubules. Strong reaction for laminin γ1 indicates the presence of this typical protein in the basal lamina. Reaction for collagen type III is contained in both the basal lamina of generated tubules and in the surrounding interstitial space.

Finally, increasing the layers of renal stem/progenitor cells by piling like bricks results in the formation of multiple tubules orientated in parallel rows. This technique makes it possible to extend the spatial environment for tubular growth and to generate renal superstructures urgently needed for biomedical research and kidney regeneration [3].

4 References

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