TISSUE ENGINEERING
Volume 10, Number 7/8, 2004

Controlled Respiratory Gas Delivery to Embryonic Renal Epithelial Explants in Perfusion Culture

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ABSTRACT

During generation of artificial tissues high levels of oxygen are usually available whereas after implantation into a recipient's body the implant is not vascularized immediately, which leads to low oxygen partial pressures within the implanted tissue. Under these conditions cells will experience an oxygen shortage, contrasting with the abundance of oxygen during culture. It is uncertain whether tissues can be trained to tolerate such an acute hypoxic situation so that nonphysiological stress reactions and tissue necrosis can be avoided. To investigate the effects of varying oxygen levels on embryonic renal tissue *in vitro* we have been developing a model system combining continuous medium renewal with the ability to control levels of oxygen and carbon dioxide by gas equilibration through gas-permeable tubing. Renal embryonic tissue from neonatal rabbit was cultured in serum-free Iscove's modified Dulbecco's medium at 45, 90, 115, and 160 mmHg oxygen partial pressure for 14 days under continuous medium exchange in such a setup. After a 14-day culture period tissue sections were analyzed by cell biological methods and compared with fresh tissue histology. Surprisingly, embryonic renal explants survive and maintain good morphology for 14 days under all O_2 conditions tested. Expression of cytokeratin 19 within the established epithelium remains unchanged, indicating a structurally intact tissue. However, Na/K-ATPase is clearly downregulated under low O_2 conditions, whereas COX-2 expression increases drastically. An antiparallel effect of decreased O_2 concentrations on glycoprotein expression can be demonstrated with the lectin *Dolichos biflorus* agglutinin. Scanning electron microscopy reveals oxygen-dependent changes in cellular surface differentiation of developed collecting duct epithelium.

INTRODUCTION

WITHIN THE HEALTHY ADULT BODY cells are exposed to varying oxygen levels, depending on the tissue location[1] (Fig. 1). Arterial endothelial cells, for example, are in direct contact with oxygen-rich blood, whereas hepatocytes receive lower levels of oxygen.[2–4] Chondrocytes within articular cartilage dwell in a low-oxygen environment, because this tissue is avascular.[5–7] Thus, all of the tissues within the organism are adapted to individual oxygen levels.

Furthermore, the extent of blood vessels and the resulting oxygen supply is different in healthy adult tissue compared with embryonic, fetal, neonatal, and wounded tissues.[8–11] Within those tissues relatively large areas exist that show no or incomplete development of functional blood vessels. As a consequence these avascular tissue areas must be supplied by diffusion of oxygen across long distances.[12,13]

Again different is the situation in which embryonic and adult cells are grown on a scaffold in order to generate an artificial tissue.[14,15] When an engineered tissue construct is implanted into a recipient's body, it is not vascularized immediately, and is sometimes even encapsulated with a layer of fibroblasts. Under these conditions cells will experience an acute oxygen shortage contrast-

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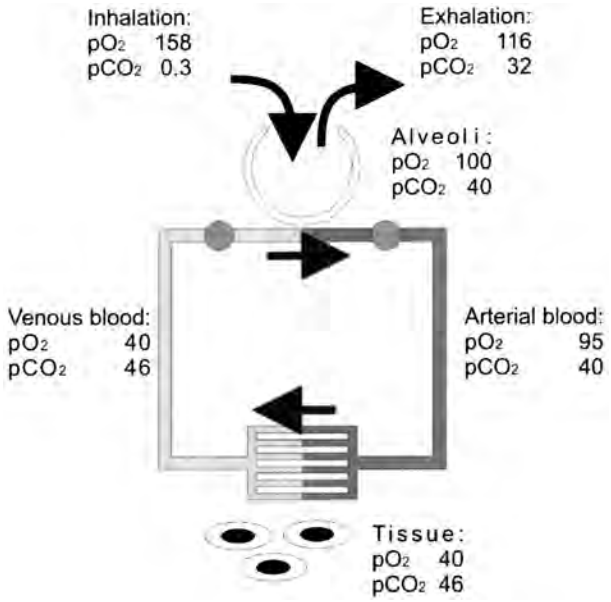

FIG. 1. Schematic representation of the availability of respiratory gases *in vivo*. Inhaled atmosphere contains approximately 158 mmHg O_2 and 0.3 mmHg CO_2. Arterial blood contains approximately 95 mmHg O_2 and 40 mmHg CO_2. Tissue that is supplied by capillaries contains approximately 40 mmHg O_2 and 46 mmHg CO_2.

ing with the abundance of oxygen during *in vitro* culture.[8,16] To overcome the resulting problem of tissue damage caused by lack of oxygen, the constructs should be gradually adapted to a hypoxic atmosphere.

The present series of experiments was performed to answer the question concerning whether varying concentrations of oxygen promote or inhibit the functional development of embryonic renal collecting duct epithelia. Besides serving as an experimental model the suggested setup could be employed to routinely adapt tissue constructs to a low-oxygen environment before implantation, thus avoiding cellular shock. Embryonic renal tissue was isolated from developing rabbit kidneys and cultured under permanent perfusion of medium equilibrated to individually defined gas atmospheres. Using cell biological and morphological analysis, we could demonstrate oxygen-dependent changes in the differentiation profile of the embryonic collecting duct ampulla-derived epithelium for the first time.

MATERIALS AND METHODS

Tissue preparation

Generation of embryonic renal collecting duct epithelia was performed by isolating cortical explants from the kidneys of newborn New Zealand rabbits according to methods described earlier.[17] The explants consisted of capsula fibrosa with adherent collecting duct ampullae containing epithelial cells, S-shaped bodies, and nephrogenic blastema, which were mounted on tissue carriers. For multiplication of cells the explants were cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL Life Technologies, Eggenstein, Germany) supplemented with 10% fetal bovine serum (Roche, Mannheim, Germany) in a CO_2 incubator (5% CO_2/95% air). Within 24 h the entire surface of the explant (6 mm in diameter) was covered with a monolayer of polarized renal epithelium derived from the collecting duct ampullae.

Tissue culture

The tissue carriers were placed in perfusion culture containers (Minucells and Minutissue, Bad Abbach, Germany). The perfusion container was placed within a gas-exchange container that was flooded with a continuous flow of a defined gas mixture from a pressurized bottle (Fig. 2). Fresh culture medium was continuously supplied to the perfusion culture container from a storage bottle by an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany) at a rate of 1 mL/h. The medium was transported through thin, gas-permeable silicone tubing. A length of 1 m of this tubing was curled up within the gas-exchange container just before the inlet of the perfusion culture container to allow equilibration of the culture medium to the surrounding gas atmosphere. The atmosphere within the gas exchanger was produced by a constant flow of 20 mL/min of a defined

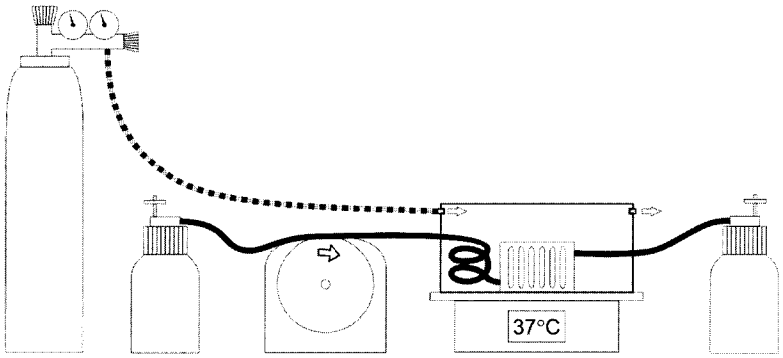

FIG. 2. Schematic representation of the culture setup. The perfusion culture setup consisted of a storage bottle, a culture container, and a waste bottle connected by thin gas-permeable silicone tubing. A peristaltic pump transported the medium through the culture container at a rate of 1 mL/h and a thermo plate with a lid maintained at a constant temperature of 37°C. The perfusion container and a 1-m length of curled-up silicone tubing just before the inlet of the perfusion culture container were contained within a gas exchanger to allow equilibration of the culture medium with the surrounding gas atmosphere. The gas atmosphere within the gas exchanger was produced by a constant flow of 20 mL/min of a defined mixture from a gas cylinder.

mixture from a gas cylinder. A temperature of 37°C within the culture setup was maintained by a thermoplate (Medax, Kiel, Germany). All used culture medium was collected in a waste bottle.

The culture medium used for the test groups (0–12% O_2/5% CO_2) was IMDM (GIBCO-BRL Life Technologies). The culture medium used for the room atmosphere control (21% O_2/0.3% CO_2) was IMDM (GIBCO-BRL Life Technologies) supplemented with 25 mM HEPES (GIBCO-BRL Life Technologies) to maintain a pH of 7.4 at atmospheric CO_2 levels.

O_2/CO_2 measurement

To determine the respiratory gas partial pressures in the culture medium, 500-μL samples were collected from within the perfusion culture container. The undiluted samples were analyzed immediately in a Stat Profile 9 Plus analyzer according to the manufacturer's instructions (Nova Biomedical, Rödermark, Germany). Solutions with defined electrolyte concentrations served as controls.

Light microscopy

Small pieces of freshly prepared tissue were immediately fixed in 2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate buffer (12 h, 4°C), postfixed in 1% osmium tetroxide in 0.1 M cadodylate buffer, and block contrasted in 1% uranyl acetate in maleate buffer. The pieces of tissue were then dehydrated in a graded series of ethanols and embedded in Epon, which was polymerized at 60°C for 48 h. Semithin sections were cut with a glass knife on an OmU3 ultramicrotome (Reichert, Vienna, Austria), stained with Richardson solution, and analyzed in a Zeiss Axiovert 35 (Zeiss, Oberkochen, Germany) in bright-field mode. For documentation, Agfa Pan 25 film (Agfa, Leverkusen, Germany) was used.

Immunohistochemistry

Monoclonal antibodies and lectins were used to determine the degree of differentiation within the collecting duct epithelia. A monoclonal antibody (MAb) against Na^+, K^+-ATPase, developed by D.M. Fambrough, was obtained from the Development Studies Hybridoma Bank (Department of Biological Sciences, University of Iowa, Iowa City, IA) (maintained under contract NO1-HD-7-3263 from the NICHD). MAb anti-cytokeratin 19 was produced in the laboratory of R. Moll (Marburg, Germany). Monoclonal antibodies directed against cyclooxygenase 2 (COX-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). *Dolichos biflorus* lectin was purchased from Vector Laboratories (Burlingame, CA).

For the immunohistochemical detection of proteins 7-μm cryosections of the tissue were prepared with an HM 500 cryostat (Microm, Walldorf, Germany). Immunolabeling was initiated by fixing the cryosections for 10 min in ice-cold ethanol. After several washing steps with phosphate-buffered saline (PBS, pH 7.2) the sections were incubated in a blocking solution (PBS) containing 10% horse serum and 1% bovine serum albumin (BSA) for 30 min. The primary antibodies were diluted in blocking buffer and were incubated for 1.5 h at room temperature. After several washes with PBS containing 1% BSA the sections were incubated for 45 min with a donkey anti-IgG fluorescein isothiocyanate-conjugated

secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in blocking buffer. After several washes in PBS the sections were embedded in photobleaching-resistant mounting medium (SlowFade Light Antifade kit; Molecular Probes, Eugene, OR) and examined with an Axiovert 35 fluorescence microscope (Zeiss, Oberkochen, Germany).

Scanning electron microscopy

For scanning electron microscopy (SEM) exactly oriented pieces of tissue were prepared, fixed in 2% glutaraldehyde in PBS under isotonic conditions (24 h, 4°C), dehydrated in a graded series of ethanols, transferred to acetone, and critical point dried in CO_2. Finally they were

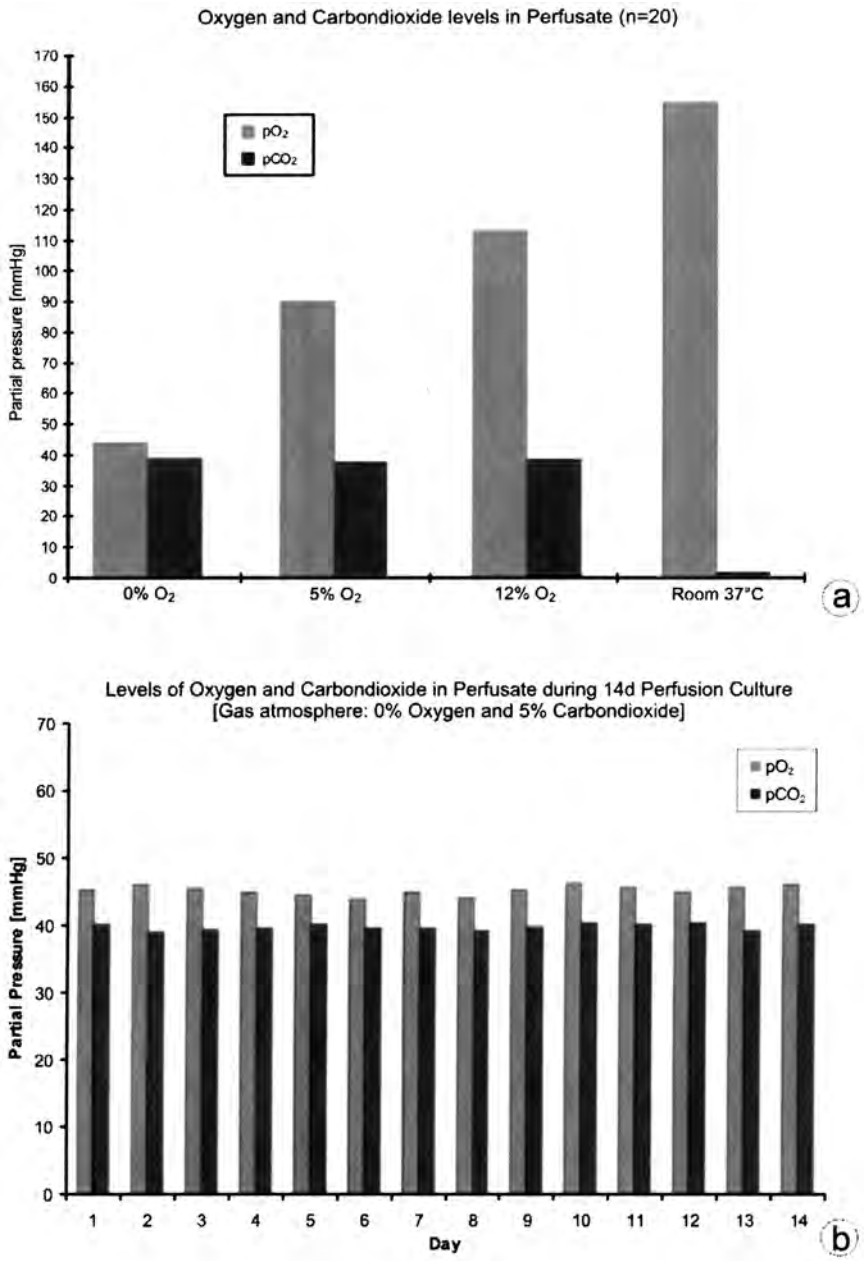

FIG. 3. Evaluation of the culture system. (**a**) Partial pressures for O_2 and CO_2 in perfusion culture. No O_2 in the gas mix resulted in 45 mmHg O_2 in the culture medium, 5% O_2 in the gas mixture resulted in 90 mmHg in the culture medium, 12% O_2 in the gas mixture resulted in 115 mmHg O_2 in the culture medium, and room atmosphere (21% O_2) produced a partial pressure of 160 mmHg O_2 in the culture medium. All gas mixes also contained 5% CO_2 to maintain a pH of 7.4 in the culture medium. (**b**) Levels of O_2 and CO_2 during 14 days of perfusion culture. Respiratory gas partial pressures in the culture medium could be maintained at a constant level over a period of 14 days.

sputter coated with gold (Polaron, Watford, UK). The specimens were examined in a DSM 940 A scanning electron microscope (Zeiss). For documentation Agfa Pan 100 film was used.

RESULTS

The experimental setup consisted of a perfusion system with additional modules to allow control over the respiratory gas concentrations in the culture medium.

Using this setup, the respiratory gas partial pressures within the culture medium could be adjusted to defined values (Fig. 3a). A 0% O_2 level in the gas mix resulted in 45 mmHg O_2 in the culture medium, 5% O_2 in the gas mixture resulted in 90 mmHg in the culture medium, 12% O_2 in the gas mixture resulted in 115 mmHg O_2 in the culture medium, and room atmosphere (21% O_2) produced a partial pressure of 160 mmHg O_2 in the culture medium. All gas mixtures also contained 5% CO_2, which led to a constant pH of 7.4 in the culture media through the bicarbonate–CO_2 buffer system.

The defined respiratory gas partial pressures in the culture medium could be maintained at a constant level over a period of 14 days (Fig. 3b). Using a gas atmosphere of 0% O_2 and 5% CO_2, the O_2 partial pressure ranged from 43 to 47 mmHg while the CO_2 partial pressure varied between 38 and 40 mmHg.

To investigate the effect of reduced O_2 partial pressure in this system, renal epithelial tissue explants from neonatal rabbit were cultured in serum-free IMDM at 45, 90, 115, and 160 mmHg O_2 partial pressure for 14 days. After the culture period the tissue was processed for histology, immunohistochemistry, and scanning electron microscopy. Semithin sections of the renal epithelial explants showed that the tissue survived at all oxygen partial pressures tested. The explants cultured at 21% O_2 in the control (Fig. 4a), at 5% O_2 (Fig. 4b), and at 0% O_2 (Fig. 4c) displayed similar morphology with an intact collecting duct epithelium covering the whole surface of the explant. No morphological signs of apoptosis of necrosis could be found.

The effect of lowered oxygen partial pressures on the differentiation of cultured epithelia was investigated with a panel of monoclonal antibodies and a lectin on cryosections prepared from the cultured tissue (Fig. 5). Expression of epithelial cytoskeleton protein cytokeratin 19 within the epithelium was not affected by the lowered O_2 partial pressures, which indicates a structurally intact epithelium. COX-2 expression within the epithelium increased drastically, suggesting a cellular stress response to the low O_2 availability. Na^+,K^+-ATPase, however, was clearly downregulated under low O_2 conditions, which indicates a loss of functional differentiation. In contrast, an increase in glycoprotein expression due to lowered O_2 concentrations could be demonstrated with the lectin *Dolichos Biflorus* agglutinin.

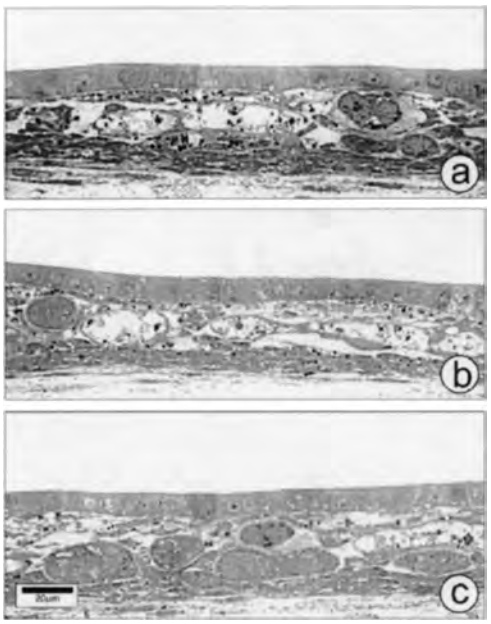

FIG. 4. Semithin sections of renal explants after 14 days in perfusion culture. (**a**) Control; (**b**) 5% O_2; (**c**) 0% O_2. Explants cultured at 21% O_2 (control), at 5% O_2, and at 0% O_2 displayed intact morphology with no signs of apoptosis or necrosis.

The apical differentiation of cells within cultured epithelia was studied by scanning electron microscopy of the explant surface. In explants cultured under room atmosphere (control) conditions for 14 days six distinct surface differentiations could be found: type a with a cilium and dense microvilli (Fig. 6a), type b with a cilium and sparse microvilli (Fig. 6b), type c with a cilium but no microvilli (Fig. 6c), type d without a cilium but with dense microvilli (Fig. 6d), type e without a cilium and only sparse microvilli (Fig. 6e), and type f without a cilium and without microvilli (Fig. 6f). These different surface differentiations can also be found under lowered oxygen conditions, but the ratios of the individual surface differentiations change with the amount of oxygen present in the culture medium. Most strikingly, the total percentage of all ciliated cells, which is more than 90% under room atmosphere conditions in the control, drops to less than 60% in a 0% O_2 atmosphere. The total percentage of all nonciliated cells, on the other hand, increases from less than 10% under room atmosphere conditions to more than 40% in a 0% O_2 atmosphere.

DISCUSSION

Oxygen is one of the essential elements necessary for survival. Depending on location within individual organs

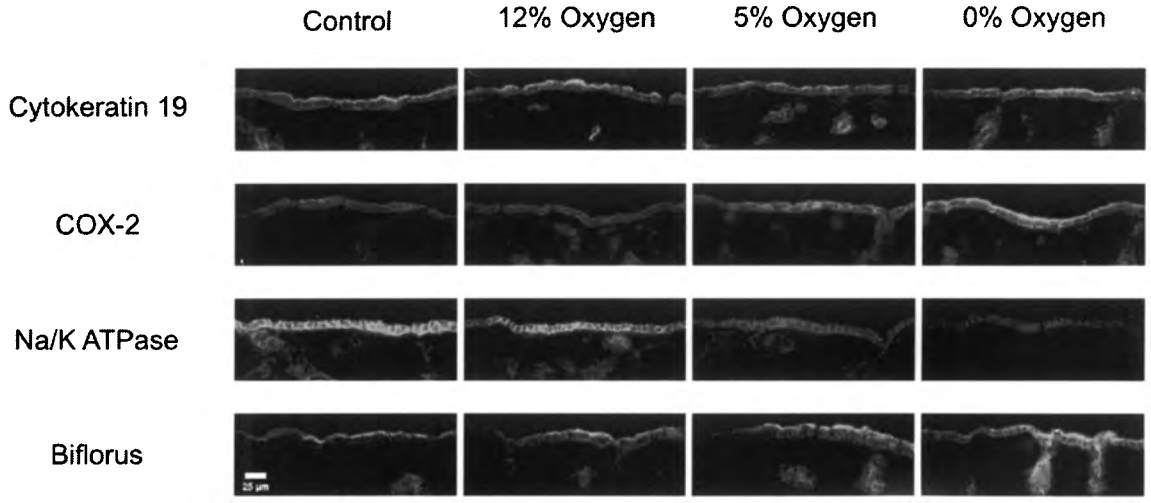

FIG. 5. Immunoincubation with a panel of monoclonal antibodies against renal differentiation markers and a lectin that specifically binds to glycoproteins. Expression of cytokeratin 19 within the established epithelium remains unchanged. Na^+, K^+-ATPase is clearly downregulated under low O_2 conditions, whereas COX-2 expression increases drastically. In contrast, low oxygen tension in the medium increases glycoprotein expression, which can be demonstrated with *Dolichos biflorus* agglutinin.

and tissues the availability of oxygen differs considerably. The oxygen partial pressure found in tissue depends on the blood pressure, the caliber of the vessels, and the amount of capillaries.[18] All tissues except cartilage and epithelia are vascularized. Epithelial and cartilage tissues are supplied with oxygen only by diffusion across more or less long distances.

When artificial tissues are generated for regenerative medicine under *in vitro* conditions oxygen usually is present in high concentrations, comparable to the dissolved oxygen concentrations within arteries[19] unless large constructs bring about long diffusion distances. However, when transferring the construct from the culture environment into the recipient's tissue, it faces a harmful atmosphere because initially there are no intact capillaries within the construct or in the surrounding tissue. For a certain period the implant must survive in an atmosphere with an extremely low oxygen supply. Normally it takes days until functional capillaries are developed. During that time the implant is provided with oxygen only by diffusion over a long distance. In each case the change from a more or less optimal culture atmosphere to the implantation site is connected with abrupt and extreme physiological changes that can result in a shock situation with a stress response or even severe damage to the generated implant.[16,20–22]

Our aim is to develop a strategy to minimize the physiological changes experienced by the developing tissue during transition from the culture atmosphere to the environment of freshly implanted tissue. In the present article we show data from experiments performed with a culture system that allows us to adapt cultured tissue to chronic deprivation of oxygen. By this treatment it is possible to determine the degree of oxygen deprivation a developing tissue construct is able to tolerate and which level will lead to irreversible damage.

We show data from culture experiments using embryonic renal tissue as model system, which is microsurgically isolated from the outer cortex of neonatal rabbit kidney. The explant consists of the fibrous capsule with adherent collecting duct (CD) ampullae. Some cells inside the CD ampullae represent renal stems cells growing in a tissue layer that lacks functional blood vessels.[23] In perfusion culture the cells of the CD ampulla have been shown to develop into a functional epithelium showing features of adult principal and intercalated cells.[17]

It has been shown earlier that the development of embryonic collecting duct cells can be influenced by the application of aldosterone[24–26] as well as by urea[27] and increased electrolyte concentrations.[28] Astonishingly, the present experiments show that chronically decreasing oxygen partial pressure in a defined manner during culture can trigger similar changes in the differentiation profile of the embryonic epithelia. It is, however, unknown whether different stimuli such as steroid hormones, electrolytes, urea, and oxygen partial pressure affect the same pathway or different pathways that lead to similar results.

The effect of chronic application of lowered oxygen partial pressures on the cultured epithelia was unexpected. The fact that the tissue showed no morphological signs of necrosis or apoptosis after 2 weeks of culture under low oxygen conditions (Fig. 4) was most

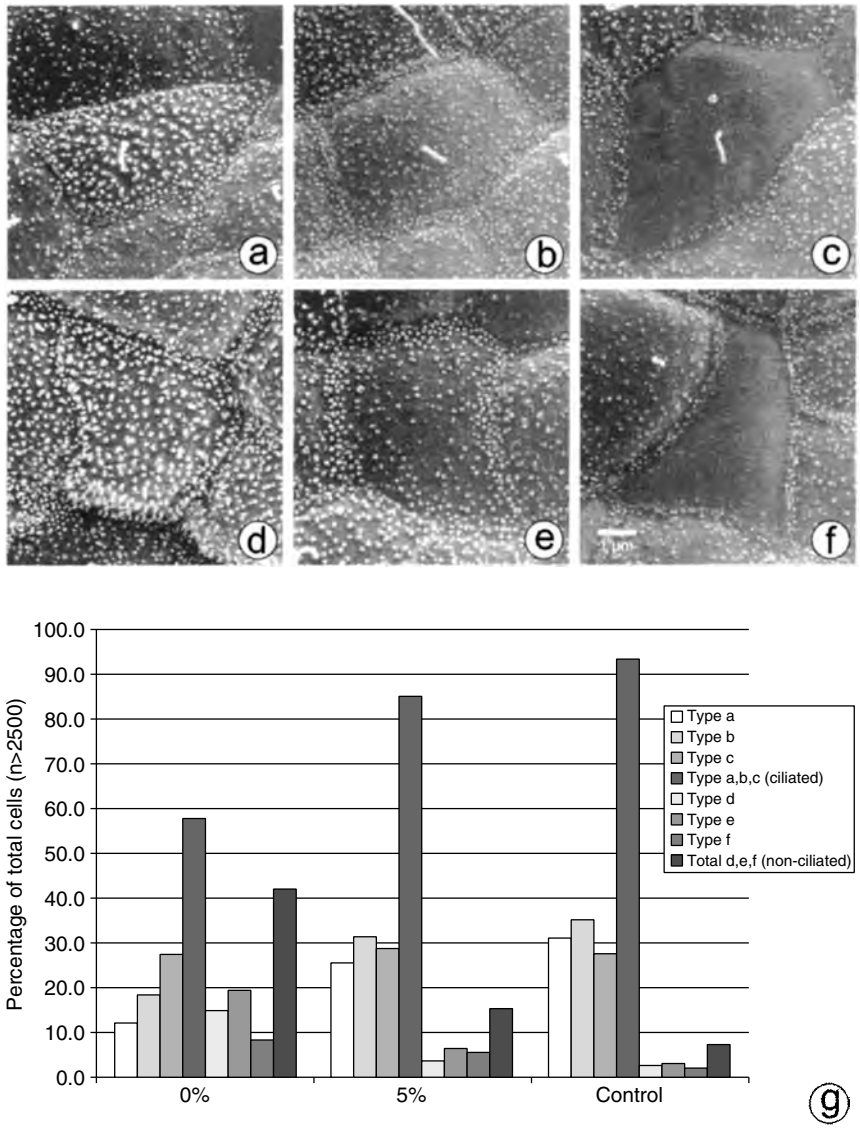

FIG. 6. Analysis of apical cell differentiation depending on decreased oxygen availability. (**a**–**f**) Scanning electron micrographs of the apical surface of cell types found within the epithelium of renal explants after 14 days in perfusion culture. (**a**) One cilium, dense microvilli; (**b**) one cilium, sparse microvilli; (**c**) one cilium, no microvilli; (**d**) no cilium, dense microvilli; (**e**) no cilium, sparse microvilli; (**f**) no cilium, no microvilli. (**g**) Percent occurrence of the various cell types in the collecting duct epithelium, depending on oxygen levels.

striking. This can in part be explained by the fact that the explants were derived from an embryonic zone of the kidney, which lacks functional blood vessels. The upregulation of COX-2, on the other hand, proves that the lack of oxygen indeed results in a cellular stress response within the epithelium. At the same time the development of characteristic features such as Na^+, K^+-ATPase expression within the collecting duct epithelium is suppressed under low oxygen conditions compared with high oxygen conditions (Fig. 5). This means that the development of blood vessel in the maturing organ is a prerequisite for the functional development of the epithelia.

The implications of transferring a partially matured tissue construct from the high oxygen environment of the culture dish to the low oxygen environment of the implantation site are illuminated by these experiments. Even if the implanted tissue survives the sudden transition, quite severe changes in differentiation along with changes in functionality must be expected. It is essential to be aware of these phenomena when attempting to per-

form functional tissue repair. A gradual adaptation to the target environment in combination with monitoring the functionality of the tissue construct could be an initial attempt to control and counteract these changes.

REFERENCES

1. Ganong, W.F. Review of Medical Physiology. Los Altos, CA; Lange Medical, 1979.
2. Martin, H., *et al.* Morphological and biochemical integrity of human liver slices in long-term culture: Effects of oxygen tension. Cell Biol. Toxicol. **18,** 73, 2002.
3. Suleiman, S.A., and Stevens, J.B. The effect of oxygen tension on rat hepatocytes in short-term culture. In Vitro Cell. Dev. Biol. **23,** 332, 1987.
4. Soller, B.R., *et al.* Simultaneous measurement of hepatic tissue pH, venous oxygen saturation and hemoglobin by near infrared spectroscopy. Shock **15,** 106, 2001.
5. Gonsalves, M., *et al.* Scanning electrochemical microscopy as a local probe of oxygen permeability in cartilage. Biophys. J. **78,** 1578, 2000.
6. O'Driscoll, S.W., Fitzsimmons, J.S., and Commisso, C.N. Role of oxygen tension during cartilage formation by periosteum. J. Orthop. Res. **15,** 682, 1997.
7. Ye, G.F., and Silverton, S.F. Computer-modeling of oxygen supply to cartilage: Addition of a compartmental model. Adv. Exp. Med. Biol. **361,** 31, 1994.
8. Carlsson, P.O., and Mattsson, G. Oxygen tension and blood flow in relation to revascularization in transplanted adult and fetal rat pancreatic islets. Cell Transplant. **11,** 813, 2002.
9. Erecinska, M., and Silver, I.A. Tissue oxygen tension and brain sensitivity to hypoxia. Respir. Physiol. **128,** 263, 2001.
10. Landgraf, H. Correlation between plasma viscosity and tissue oxygen tension. Clin. Hemorrheol. Microcirc. **20,** 37, 1999.
11. Niinikoski, J. Oxygen tension and tissue perfusion. Ann. Chir. Gynaecol. **80,** 7, 1991.
12. Davidson, J.D., and Mustoe, T.A. Oxygen in wound healing: More than a nutrient. Wound Repair Regen. **9,** 175, 2001.
13. LaVan, F.B., and Hunt, T.K. Oxygen and wound healing. Clin. Plast. Surg. **17,** 463, 1990.
14. Mano, T., *et al.* Comparison of oxygen supply methods for cultures of shear-stress sensitive organisms including animal cell culture. J. Chem. Technol. Biotechnol. **47,** 259, 1990.
15. Wang, S., *et al.* Studies on oxygen transfer process in animal cell culture bioreactor. Chin. J. Biotechnol. **9,** 19, 1993.
16. Hyder, A., Laue, C., and Schrezenmeir, J. Variable responses of islet cells of different ages and species to hypoxia. Transplant. Proc. **30,** 578, 1998.
17. Minuth, W.W. Neonatal rabbit kidney cortex in culture as tool for the study of collecting duct formation and nephron differentiation. Differentiation **36,** 12, 1987.
18. Vovenko, E. Distribution of oxygen tension on the surface of arterioles, capillaries and venules of brain cortex and in tissue in normoxia: An experimental study on rats. Pflugers Arch. **437,** 617, 1999.
19. Minuth, W.W., *et al.* Physiological and cell biological aspects of perfusion culture technique employed to generate differentiated tissues for long term biomaterial testing and tissue engineering. J. Biomater. Sci. Polym. Ed. **11,** 495, 2000.
20. Helfman, T., and Falanga, V. Gene expression in low oxygen tension. Am. J. Med. Sci. **306,** 37, 1993.
21. Seta, K.A., *et al.* Responding to hypoxia: Lessons from a model cell line. Sci. STKE **2002,** RE11, 2002.
22. Yun, J.K., *et al.* Cellular adaptive responses to low oxygen tension: Apoptosis and resistance. Neurochem. Res. **22,** 517, 1997.
23. Kloth, S., *et al.* Three-dimensional organization of the developing vasculature of the kidney. Cell Tissue Res. **287,** 193, 1997.
24. Minuth, W.W., *et al.* Aldosterone modulates PNA binding cell isoforms with renal collecting duct epithelium. Kidney Int. **44,** 537, 1993.
25. Minuth, W.W., Zwanzig, M., and Gross, P. Action of aldosterone on protein expression in cultured collecting duct cells from neonatal rabbit kidney. Ren. Physiol. Biochem. **12,** 213, 1989.
26. Schumacher, K., *et al.* Cyclooxygenases in the collecting duct of neontal rabbit kidney. Cell. Physiol. Biochem. **12,** 63, 2002.
27. Schumacher, K., Strehl, R., and Minuth, W.W. Urea restrains aldosterone-induced development of PNA-binding on embryonic renal collecting duct. J. Am. Soc. Nephrol. **14,** 2758, 2003.
28. Minuth, W.W., *et al.* Electrolyte environment modulates differentiation in embryonic renal collecting duct epithelia. Exp. Nephrol. **5,** 414, 1997.

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