Original Article

Effect of Culture Substrate and Culture Conditions on Lens Epithelial Cell Proliferation and α-smooth Muscle Actin Expression

(α-smooth muscle actin / cell proliferation / culture substrates / lens epithelial cells)

G. MAHELKOVÁ[1,2], L. BAČÁKOVÁ[5], J. KORYNTA[6], L. VAJNER[3], R. VYTÁSEK[4]

Charles University in Prague, 2nd Faculty of Medicine: [1]Institute of Physiology, [2]Clinic of Ophthalmology, [3] Institute of Histology and Embryology, [4]Institute of Medical Chemistry and Biochemistry, Prague, Czech Republic
[5]Academy of Sciences, Institute of Physiology, Department of Growth and Differentiation of Cell Populations, Prague, Czech Republic
[6] University of Edinburgh, Princess Alexandra Eye Pavillion, Edinburgh, Scottland, United Kingdom

Abstract. The most common complication following cataract surgery is posterior capsule opacification. This results from migration, proliferation and transdifferentiation of residual lens epithelial cells (LECs). We studied the effect of several culture substrates and culture conditions on LEC proliferation and α-smooth muscle actin (α-SMA) expression. We used primary and secondary cultures of porcine LECs cultivated on collagen I, collagen IV, microscopic glass slides, and uncoated plastic dishes. We studied the cell proliferation and expression of α-SMA and α-, β-, and γ-crystallins. The effect of the medium exchange protocol was studied using the TOTL-86 rabbit epithelial lens cell line. There was no difference in growth characteristics of primary cultures on different substrates. In secondary cultures, LECs adhered better to collagen-coated surfaces. The culture substrate influenced LEC proliferation and α-SMA expression. The proliferation was greater when the medium was changed than when extra medium was added on the 4th day. The cells did not synthesize α-, β- or γ-crystallin. The culture substrate influences the adhesion ability, proliferation and α-SMA expression in lens epithelial cells. In addition, it is necessary to consider the effects of the medium exchange protocol, serum supplementation, cell density and other cell culture conditions in lens epithelial cell experiments.

Received September 12, 2008. Accepted February 28, 2009.

This work was supported by grant No. 51/2001/C/2.LF of Charles University in Prague, Czech Republic, grant No. 305/08/0108 of the Grant Agency of the Czech Republic and grant No. 1QS500110564 of the Academy of Sciences of the Czech Republic.

Corresponding author: Gabriela Mahelková, Charles University in Prague, 2nd Faculty of Medicine, Institute of Physiology, Plzeňská 221, 150 00 Prague 5, Czech Republic. Phone: (+420) 257 296 201, Fax: (+420) 257 210 995; e-mail: gabriela.czakoova@post.cz

Abbreviations: α-SMA – α-smooth muscle actin, D-MEM – Dulbecco's modified Eagle's Minimum Essential Medium, DT – doubling time, EDTA – ethylene diamine tetra-acetic acid, ELISA – enzyme-linked immunosorbent assay, FBS - foetal bovine serum, LEC – lens epithelial cell, N – number of cells, PBS – phosphate buffered-saline solution, PCO – posterior capsule opacification, SD – standard deviation, SEM – standard errors of means, TGF-β – transforming growth factor β.

Introduction

The most common cause of reduced visual acuity following cataract surgery is posterior capsule opacification (PCO). This is a consequence of migration and proliferation of lens epithelial cells (LECs) onto the central region of the posterior capsule. These cells can be stimulated to differentiate into various cell types. They can undergo fibromuscular transdifferentiation, which is manifested by the expression of α-smooth muscle actin (α-SMA), i.e. an isoform of actin normally restricted to smooth muscle cells. Because of their contractile elements, metaplastic LECs can cause wrinkling and haze of the adjacent capsule (Kurosaka et al., 1996; Marcantonio and Vrensen, 1999; Nagamoto et al., 2000; Bertelmann and Kojetinsky, 2001; Aslam et al., 2003a, b; Marcantonio et al., 2003; Mahoney and Schwartz, 2005).

In order to prevent PCO, it is necessary to understand the regulatory mechanisms of proliferation and transdifferentiation of LECs. Many studies have been carried out to determine the effects of additional external growth factors on lens cell proliferation, fibrous metaplasia and α-SMA expression (Hales et al., 1994; Liu et al., 1994; Kurosaka et al., 1995; Nishi et al, 1996; Lee and Joo, 1999; Meacock et al., 2000; Wilhelm et al., 2007). However, the α-SMA expression in LECs is affected by the

culture substrate itself (Kurosaka et al., 1999; de Jong-Hesse et al., 2005). The effect of extracellular matrix composition on the growth and phenotype characteristics of different types of cells is well known (Greenburg and Hay, 1986; Iwig et al., 1989; Iwig and Glaesser, 1991; Wu et al., 1995; Oharazawa et al., 1999; Engler et al., 2004a). It has been reported that various intraocular lens biomaterials cause different degrees of reactive fibrous metaplasia (Ursell et al., 1998), and the lens capsule composition after cataract surgery plays an important role in the regulation of LEC proliferation and differentiation (Saika et al., 1998).

Various LEC culture models have been developed to elucidate the reasons for postoperative LEC proliferation (Nishi, 1999). The viability and growth characteristics of the human LECs depend markedly on the age of the donor and the type of cataract. An animal model is therefore considered more suitable and more homogeneous for evaluating the effects of various external factors on LEC growth. Therefore, we decided to use porcine lenses in our experiments.

The goal of our experiments was to study different effects of several commonly used cell culture substrates on α-SMA expression. As the results of previous studies in this field (Kurosaka et al., 1999; de Jong-Hesse et al., 2005) have been heterogeneous, we also focused on other aspects of cell culture conditions, such as the protocol of medium exchange, addition of extra medium, serum supplementation, cell seeding density, and also the differences in behaviour of primary and secondary cultures.

Material and Methods

Cell culture techniques, testing primary culture

Fresh porcine lens were obtained from the local slaughterhouse and processed within four hours. The whole eyes were rinsed in 96% ethanol for 30 s to minimize a possible bacterial contamination of the lenses from the eye surface during lens preparation and were then washed in phosphate buffered-saline solution (PBS). The cornea was dissected aseptically and the lens was released using a sterile plastic tube. Any remainders of zonular fibres were cut off. The whole lenses (40 lenses) were incubated in 30 ml of 0.03% collagenase solution (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's Minimum Essential Medium (D-MEM, Sigma) supplemented with gentamicin (80 μg/ml, LEK, Ljubljana, Slovenia) for 4–5 hours at 37 °C. The released cells were centrifuged (10 min, 900 *g*), rinsed in D-MEM in order to eliminate collagenase, and resuspended in 10 ml of the culture medium consisting of D-MEM supplemented with 10% foetal bovine serum (FBS, Pan-systems, Bochum, Germany) and gentamicin (40 μg/ml). Cultivation wells were prepared by sticking FlexiPERM micro 12 cell culture chamber system (growth area 0.2 cm^2, Greiner-Bio-One, Frickenhausen, Germany) to polystyrene Petri dishes (NUNC, Roskilde, Denmark, diameter 9 cm) and microscopic glass slides (KnittelGläser, Braunchweig, Germany). Seventy-two wells were prepared on polystyrene dishes: 24 of them were coated with collagen type I (isolated from rat tails, 10 μg/ml, 40 μl per well), 24 of them were coated with collagen type IV (Sigma, 10 μg/ml, 40 μl per well), and 24 were left uncoated. Forty-eight wells were prepared on microscopic glass slides: 24 of them were coated with collagen type I (isolated from rat tails, 1 mg/ml, 40 μl per well), and 24 of them were left uncoated. The coating of the wells had been prepared two days earlier (40 μl of the specific collagen solution were injected in each well and then dried in the biohazard box overnight). One hundred μl of the cell suspension were inoculated into each well. On the 3rd and 10th days, 100 μl of fresh medium were added, and all the medium was changed on the 7th day. The cells were cultured for 1, 3, 7 and 12 days, then rinsed with PBS and fixed by methanol at 4 °C. The entire experiment was performed twice. The fixed cells were analysed for expression of α-SMA and for expression of α-, β- and γ-crystallins (see later).

Cell culture techniques, testing secondary cultures

The eyes were processed and the lenses were isolated as described above. After incubation in the collagenase solution, the cells were centrifuged and seeded in 24-well culture plates (NUNC). The cells obtained from 40 lenses were inoculated in ten wells of a 24-well culture plate. The cells were grown in D-MEM supplemented with 10% foetal bovine serum (Pan-systems) and gentamicin 40 μg/ml (LEK). In each well, 0.5 ml of the cell suspension was inoculated, and on the next day 0.5 ml of the medium was added. All the medium was changed once a week.

Cell confluence was reached after 10 days. The cells were trypsinized (0.1% trypsin in PBS) and newly inoculated in wells created by the FlexiPERM micro 12 system (Greiner-Bio-One) on plastic dishes (NUNC) either uncoated or coated with collagen type I (isolated from rat tails, 10 μg/ml, 40 μl/well), collagen type IV (Sigma, 10 μg/ml, 40 μl/well), and on uncoated microscopic glass slides or slides coated with collagen type I (isolated from rat tails, 1 mg/ml, 40 μl/well). The coatings of the wells were prepared as described above. For each experimental group, 18 wells were prepared. Each well contained 4800 cells and 100 μl of the culture medium. One hundred μl of D-MEM were added to the wells on the 4th day of the 8-day culture period. After cultivation for 1, 4 and 8 days, the cells were rinsed in PBS and fixed in methanol at 4 °C. The experiment was performed twice. The cells were analysed for expression of α-SMA and for expression of α-, β- and γ-crystallins. A cell proliferation analysis was also performed.

Immunocytochemical and cell proliferation analysis

The fixed cells were incubated with 3% solution of porcine blood plasma in PBS with 0.05 % of Tween 20 (PBS-Tween, Sigma) for 30 min to block non-specific

binding. Next, the cells were rinsed twice with PBS and immunostained for α-SMA according to the manufacturer's instructions. The primary antibody was mouse monoclonal antibody against human α-SMA (Sigma, 1 : 200). The secondary antibody was porcine anti-mouse immunoglobulin labelled with peroxidase (SwAM, Sevapharma, Prague, Czech Republic, 1 : 40). Peroxidase visualization was accomplished by adding a solution containing 3-amino-9-ethyl-carbazol and hydrogen peroxide. Finally, the cell nuclei were counterstained with haematoxylin. Control staining without a primary or secondary antibody (replaced by PBS) was performed to exclude non-specific staining.

The total number of cells was counted in each well of the secondary cultures using computer image analysis software (LUCIA G, Laboratory Imaging, Prague, Czech Republic). The doubling time (DT) was estimated from the *k* parameters calculated from the regression line after logarithmic transformation of the number of cells (N), according to the formula $\log N = \log N_0 + kt$. The times *t* selected for cell counting were 24 and 96 hours, because logarithmic growth of cells occurred during this interval. DT is calculated from the parameter *k* of a regression line $DT = \log 2/k$.

The number of α-SMA-positive cells was counted in each well of the secondary cultures using computer image analysis software (LUCIA G) and is expressed as a percentage of all counted cells.

Preparation of antigens (porcine lens crystallins)

Porcine eyes were collected from animals (age 6–8 months) from a local slaughterhouse and were brought to the laboratory on ice. The lenses were excised and homogenized in PBS with 2 mM EDTA (gel filtration buffer). α- and β-crystallins were purified by chromatography on a Sepharose 6B-CL column (2.5 × 88 cm) and the γ-crystallins were isolated on a Sephacryl-200 column (2.5 × 90 cm).

Monoclonal antibody production

Six-week-old female BALB/c mice were immunized intraperitoneally with porcine lens crystallin (α, β or γ), isolated as described above, in complete Freund's adjuvant. The second immunization of the animals was performed in a similar manner with the same antigen in incomplete Freund's adjuvant after four weeks of resting. The animals were boosted with an intrasplenic injection of the antigen in PBS, pH 7.2, 8 weeks later. Splenocytes of the immunized mice were isolated after 4 days and fused with cells of myeloma line Sp2/0 by electrofusion in an isotonic medium (Neil and Zimmermann, 1993). Hybridoma supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) for the presence of immunoglobulins against the original immunizing antigen. Briefly, mouse immunoglobulins in the culture media were detected on microtitre plates coated with 5 μg/ml of the appropriate porcine crystallin (α, β or γ) in a bicarbonate buffer at pH 9.2. Positive colonies of hybridomas were expanded and subcloned at least twice by the method of limiting dilution. Three hybridomas, α-8-D5 (α-crystallin-positive) β-9-F10 (β-crystallin-positive) and γ-4-A4 (γ-crystallin-positive) were used to produce ascitic fluids by an intraperitoneal injection of 10^6 cells into male BALB/c mice previously primed with an injection of Freund's incomplete adjuvant. Ascitic fluids were harvested 10–14 days later and stored with the addition of 0.05% NaN_3 at 4 °C. The specificity of the prepared monoclonal antibodies was verified by immunoblotting with individual fractions of crystallins (α, β or γ) and also by staining of histology sections through porcine lenses.

α-, β- and γ-crystallin production testing

The cells were grown on collagen type I, type IV or uncoated plastic dishes. The secondary culture was tested for α-, β- and γ-crystallin expression after 4, 8 and 22 days of culture. Fixed cells were incubated with a 3% solution of porcine blood plasma in PBS-Tween for 30 min to eliminate non-specific antibody affinity. Next, the cells were rinsed twice with PBS and immunostained for α-, β- or γ-crystallin. The primary antibodies were represented by mouse monoclonal antibodies directed against porcine α-, β- or γ-crystallin (α-8-D5, β-9-F10, and γ-4-A4, respectively, see above). The secondary antibody was porcine anti-mouse immunoglobulin conjugated with peroxidase (SwAM, dilution 1 : 40). Peroxidase visualization was accomplished by adding a solution containing 3-amino-9-ethyl carbazol and hydrogen peroxide. Finally, the cell nuclei were counterstained with haematoxylin. Control staining without a primary or secondary antibody (replaced with PBS) was performed to exclude non-specific staining.

The effect of the culture medium exchange schedule

TOTL-86 cells (rabbit lens epithelial cell line, a gift from Dr. Noboyuki Ohguro, Osaka University Medical School, Japan) were used in this experiment (Sasabe et al., 1986). The cells were seeded in 96-well culture plates (NUNC). The cells were grown for 1, 4 and 8 days in 100 μl of D-MEM with 10% FBS. On the 4th day, 100 μl of the medium were added to one half of the wells, whereas the total volume of the medium was changed carefully in the other half of the cultures. The cells were then grown for further 4 days. At the end of each culture period, the cell samples were immersed in methanol at 4 ˚C for fixation, stained with haematoxylin, and the total number of cells was counted in six wells of each group. The doubling time (DT) was estimated as described above. The times *t* selected for counting the cells were 24 and 96 hours.

Statistical analyses

The values are expressed as mean ± standard deviation (SD). The results were statistically evaluated by ANOVA, Fisher's protected least significant difference test. The differences were considered significant at $P \leq 0.05$. The statistical analyses were performed using

StatView 5.0 statistical software (SAS Institute Inc, Cary, NC).

The significances of the measured differences in DT between cells grown on various surfaces were computed from the estimated *k* parameters and their standard errors of means (SEM) by Student's *t*-test for unpaired data.

Results

Cell behaviour in primary cultures

After 24 hours of cultivation, no adhering cells were found on any of the surface coatings in the primary cultures. However, as we found proliferating cells on the samples after four and more days of cultivation, these cells might be washed up during the fixative procedure, or required more than 24 hours for their adhesion. After four days of cultivation, small clusters of cells appeared, but no cell was α-SMA positive. According to our assumption of a low ability of lens cells to adhere within 24 hours of cultivation, it is still possible that part of the cells were washed up even after three days of culture. After seven days of cultivation, the cells were almost confluent. They displayed epithelial morphology and there were still no α-SMA-positive cells. However, after 12 days of cultivation, some of the cells became α-SMA positive. The confluent culture became disorganized and areas free of cells appeared again. The α-SMA-positive cells became larger and spindle-shaped and they tended to create multilayered regions in some areas (Fig. 1).

The cell proliferation was less satisfactory on the microscopic glass slides. The cells did not reach confluence after 12 days; nevertheless, some of them became α-SMA positive during cultivation and acquired mesenchymal phenotype characterized by a spindle-like shape and multilayered growth. There were no differences in growth characteristics in the primary cultures grown on collagen type I (10 µg/ml), collagen type IV and uncoated plastic dishes. The collagen sheet (i.e. coating with collagen type I, 1 mg/ml) was contracted after four days of cultivation, and cells continued to proliferate on the uncoated glass. Therefore, we did not evaluate further proliferation of the cells on this sample.

In view of these growth characteristics, we decided to cultivate the primary cultures on uncoated plastic dishes for ten days and to perform all experiments with secondary cultures.

Cell behaviour in secondary cultures from day 1 to 4

In the secondary cultures, significantly more cells adhered to the collagen surfaces, i.e. collagen type I (1192 ± 119 cells/well) and collagen type IV (1160 ± 159 cells/well) than to uncoated plastic dishes (781 ± 152 cells/

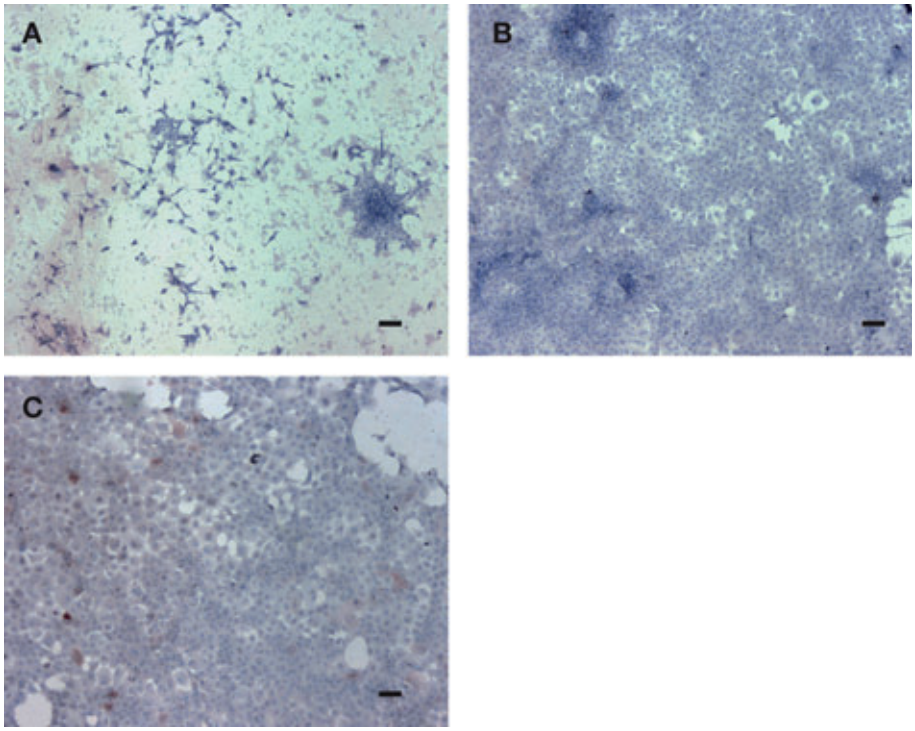

Fig. 1. Primary culture of porcine LECs on plastic dishes. Immunoperoxidase reaction of α-SMA after 3 (a), 7 (b), and 12 days (c) of culture was visualized with 3-amino-9-ethyl carbazole, and the cells were counterstained with haematoxylin. The cell debris is visible between the cells after 3 days of culture. After 3 days of cultivation, small clusters of cells appeared, but no cell was α-SMA positive. After 7 days of cultivation, the cells were almost confluent, displayed epithelial morphology and there were still no α-SMA-positive cells. After 12 days of cultivation, some of the cells became α-SMA positive. The confluent culture became disorganized and areas free of cells appeared again. The α-SMA-positive cells became larger and they tended to create multilayered regions in some areas. The bar represents 100 µm.

well; P = 0.0014 and P = 0.002, respectively) and microscopic glass slides (711 ± 155 cells/well; p = 0.0003 and 0.0004, respectively) after 24 hours of culture. There was no significant difference in the number of cells between collagen types I and IV (P = 0.7683). Also, there was no difference in the number of cells found on uncoated plastic dishes and on glass (P = 0.5359; Fig. 2). Generally, the cells on collagens were better spread than on plastic dishes and glass slides. On the latter substrates, there were a higher amount of small and condensed cells, and some of them probably lost viability. The percentage of α-SMA-positive cells on day 1 after seeding was significantly higher on the collagen type I coating (10 μg/ml, 9.3 ± 0.8 %, P = 0.0015), on glass (8.5 ± 2.7 %, P = 0.0109) and on uncoated plastic (8.7 ± 1.1 %, P = 0.0072) than on collagen type IV (5.9 ± 1.4 %, Figs. 3 and 4). The control staining showed no immunoreactivity.

Contraction of the collagen sheets was observed when there was a higher coating concentration of collagen I (1 mg/ml) after four days of cultivation. The cells on the sheets were small, round and had pycnotic nuclei, and they did not proliferate during the next four days. It seemed that the cells lost their viability in the absence of mechanical stability of their growth substrate. It is known that the anchorage-dependent cells grow and survive only on such substrates that are able to resist the tractional forces generated by the cell cytoskeleton (Engler et al., 2004a). Part of the cells started to proliferate on the exposed glass surface, and they acquired the characteristics of the cells grown on that type of surface. After collagen sheet contraction and partial detachement, we did not continue to evaluate the number of cells and proliferation characteristics on this type of coating.

On the other surface coatings, the number of cells increased significantly after four days of cultivation (P < 0.0001). In areas of confluence, the cells acquired epithelial morphology, but in low cell density areas, the cells were more spindle-shaped even if they did not express α-SMA. Generally, the α-SMA-positive cells were more spread and had larger cell-substrate contact areas (data not shown). On day 4 after seeding, the total number of cells did not differ significantly between collagen type I (5069 ± 434 cells/well) and collagen type IV (5866 ± 1044 cells/well; P = 0.0609), and between the uncoated plastic (3004 ± 194 cells/well) and glass (2390 ± 501 cells/well; P = 0.1398). The total number of cells was significantly higher on the collagen type I and type IV coating than on uncoated plastic (P < 0.0001 and P < 0.0001, respectively) and on glass (P < 0.0001 and P < 0.0001, respectively) (Fig. 2). The number of α-SMA-positive cells was significantly higher on glass surfaces (19.6 ± 3.2 %) than on collagen type I (9.5 ± 0.6 %, P < 0.0001), type IV (9.1 ± 1.7 %, P < 0.0001) and uncoated plastic dishes (10.32 ± 1.4 %, P < 0.0001). In addition, the number of α-SMA-positive cells on day 4 after seed-

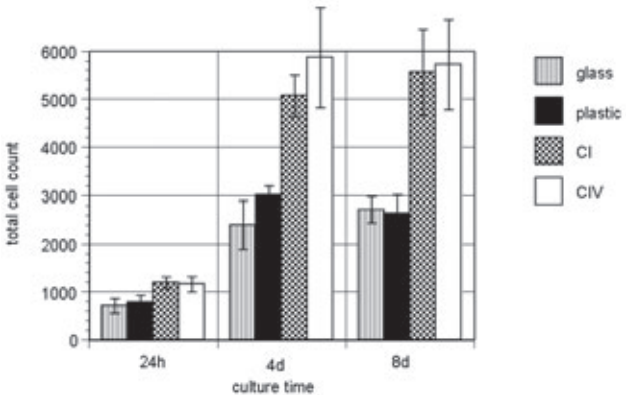

Fig. 2. The total cell count/well on the collagen type I (CI), collagen type IV (CIV), glass and uncoated plastic after 24 hours (24h), 4 days and 8 days (4d, 8d) of culture. After 24 hours, significantly more cells adhered to the collagen surfaces (1192 ± 119 cells/well on collagen I and 1160 ± 159 cells/well on collagen type IV) than to uncoated plastic (781 ± 152 cells/well) and glass (711 ± 155 cells/well). The number of cells increased significantly on all tested surfaces after four days of cultivation. The total number of cells was significantly higher on collagen type I (5069 ± 434 cells/well) and IV (5866 ± 1044 cells/well) than on glass (2390 ± 501 cells/well) and uncoated plastic (3004 ± 193 cells/well). From day 4 to 8, the proliferation rate decreased on all tested surfaces.

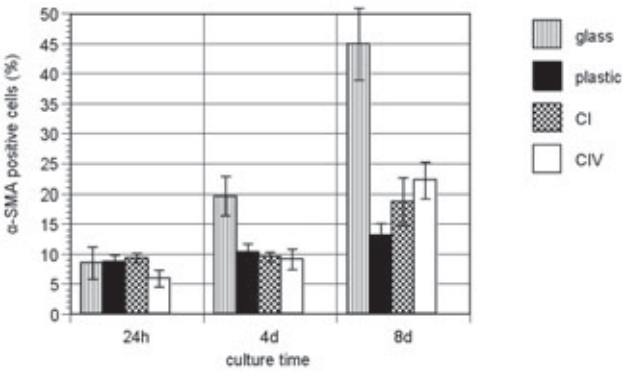

Fig. 3. The percentage of α-SMA-positive cells on the collagen type I (CI), collagen type IV (CIV), glass and uncoated plastic after 24 hours (24h), 4 days and 8 days (4d, 8d) of culture. The number of α-SMA-positive cells was significantly higher on collagen type I (10 μg/ml, 9.3 ± 0.8 %), glass (8.5 ± 2.7 %) and uncoated plastic (8.7 ± 1.1 %) than on collagen type IV (5.9 ± 1.4 %) after 24 hours of culture. The number of α-SMA-positive cells was significantly higher on glass surfaces (19.6 ± 3.2 %) than on collagen type I (9.5 ± 0.6 %), IV (9.1 ± 1.7 %) and uncoated plastic dishes (10.32 ± 1.4 %) after 4 days of culture. The number of α-SMA-positive cells increased significantly during the next 4 days on every surface coating that was used. The number of α-SMA-positive cells was significantly higher on collagen type I (18.7 ± 3.9 %) and IV (22.2 ± 3.0 %) than on uncoated plastic dishes (13.1 ± 2.0 %) after 8 days of culture. The number of α-SMA-positive cells was significantly higher on glass (44.9 ± 6.0 %) than on any other surface coating.

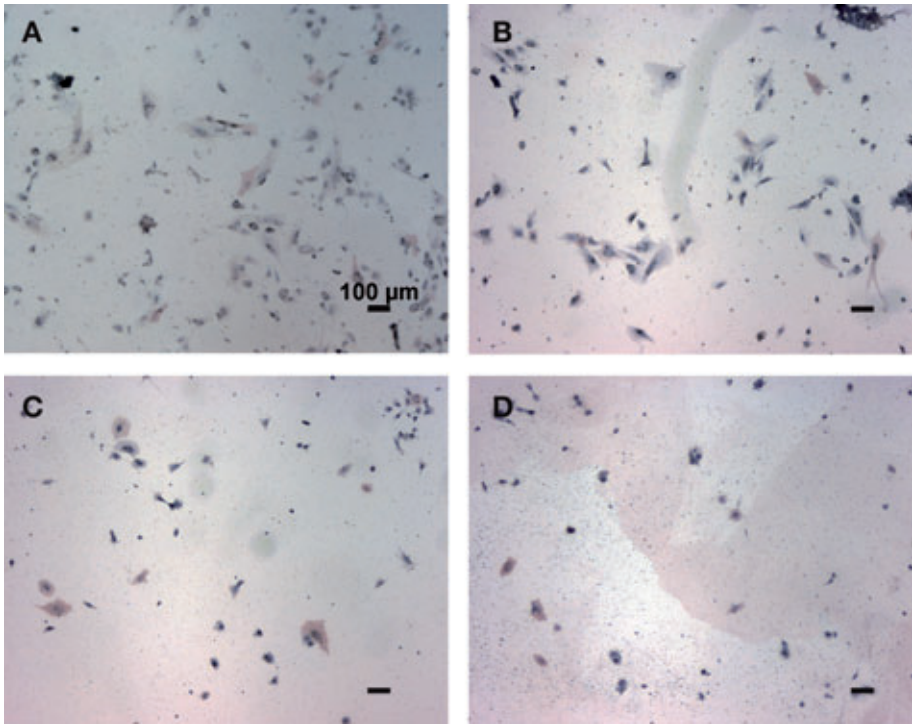

Fig. 4. Immunoperoxidase staining of porcine LECs with monoclonal antibody against α-SMA after 24 hours of culture. The cells were cultured on collagen type I (a), collagen type IV (b), uncoated plastic (c) and glass (d). The reaction was visualized with 3-amino-9-ethyl carbazole; the cells were counterstained with haematoxylin. Generally, the cells on collagens were better spread than on plastic dishes and glass slides. On the plastic dishes and glass slides, there were more small and condensed cells, and part of them probably lost viability. The bar represents 100 µm.

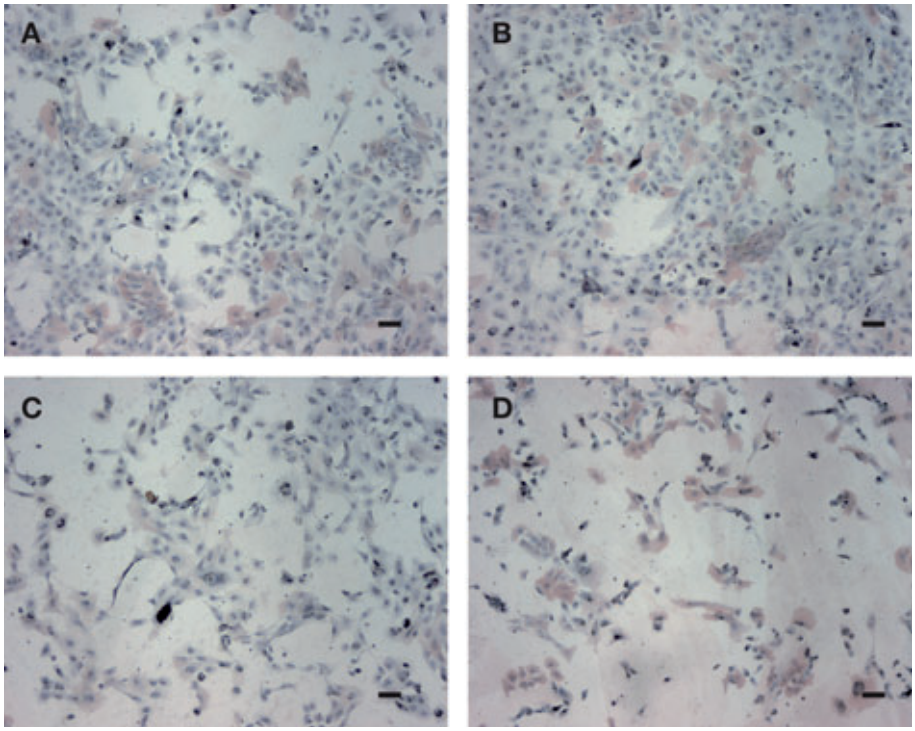

Fig. 5. Immunoperoxidase staining of porcine LECs with monoclonal antibody against α-SMA after 4 days of culture. The cells were cultured on collagen type I (a), collagen type IV (b), uncoated plastic (c) and glass (d). The reaction was visualized with 3-amino-9-ethyl carbazole; the cells were counterstained with haematoxylin. In areas of confluence the cells acquired epithelial morphology, but in low cell density areas the cells were more spindle-shaped even if they did not express α-SMA. Generally, the α-SMA-positive cells were more spread and had larger cell-material contact area. The bar represents 100 µm.

ing increased significantly on glass (P = 0.0013) and on collagen IV (P = 0.0431) in comparison with the corresponding values on day 1. At the same time, the number of α-SMA-positive cells did not change on plastic (P = 0.1262) and on collagen I (P = 0.9311, Figs. 3 and 5). The control staining showed no immunoreactivity.

The calculated DT was 41.1 h for the cells grown on glass, 36.7 h for the cells grown on uncoated plastic dishes, 34.5 h for the cells grown on collagen type I and 30.9 h for the cells grown on collagen type IV. The DT was significantly shorter on collagen type IV than on glass (P = 0.031). Although the differences between the DT of collagen I and glass and collagen IV and pure plastics were not significant (P = 0.136 and 0.075, respectively, according to the two-tail *t*-test), the increased rate of growth on both collagen types is remarkable.

Cell behaviour in secondary cultures from day 4 to 8

The proliferation rate measured from days 4 to 8 decreased in comparison with the values between days 1 and 4. The number of cells on collagen I, collagen IV and glass did not change, but the number of cells decreased significantly on uncoated plastic dishes, as revealed by a statistical evaluation (P = 0.0440). The total number of cells remained significantly higher on collagen type I (5558 ± 895 cells/well) and type IV (5719 ± 937 cells/well) than on uncoated plastic dishes (2629 ± 385 cells/well, P < 0.0001 and P < 0.0001, respectively) and on glass (2705 ± 270 cells/well, P < 0.0001 and P < 0.0001, respectively) after eight days of culture (Fig. 2). These differences were probably not due to the production of acid metabolites by the cells and by low pH of the culture medium, because the colour of the phenol red in the culture media indicated a physiological pH about 7.35. A possible reason was that the cells in later culture intervals detached spontaneously and more easily from the growth substrates that were not pre-coated with extracellular matrix proteins mediating cell adhesion, i.e. collagens in our experiments. Also, production of soluble factors by cells in post-confluent cultures, lowering the cell proliferation activity, cannot be excluded. As a result, the confluent cell layer became disorganized and areas free of cells appeared. In adddition, the cells often acquired a mesenchymal phenotype (i.e., they became larger and spindle-shaped), especially at the margins of the cell-free areas.

The number of α-SMA-positive cells increased significantly during the cultivation period on all tested surface coatings (collagen I P < 0.0001, collagen IV P < 0.0001, plastic P = 0.0161, glass P < 0.0001). The number of α-SMA-positive cells was significantly higher on collagen type I (18.7 ± 3.9 %, P = 0.0399) and type IV (22.2 ± 3.0 %, P = 0.0022) than on uncoated plastic dishes (13.1 ± 2.0 %) after eight days of culture. The number of α-SMA-positive cells was significantly higher on glass (44.9 ± 6.0%, P <0.0001) than on the other surface coatings (Figs. 3 and 6). The control samples showed no immunoreactivity.

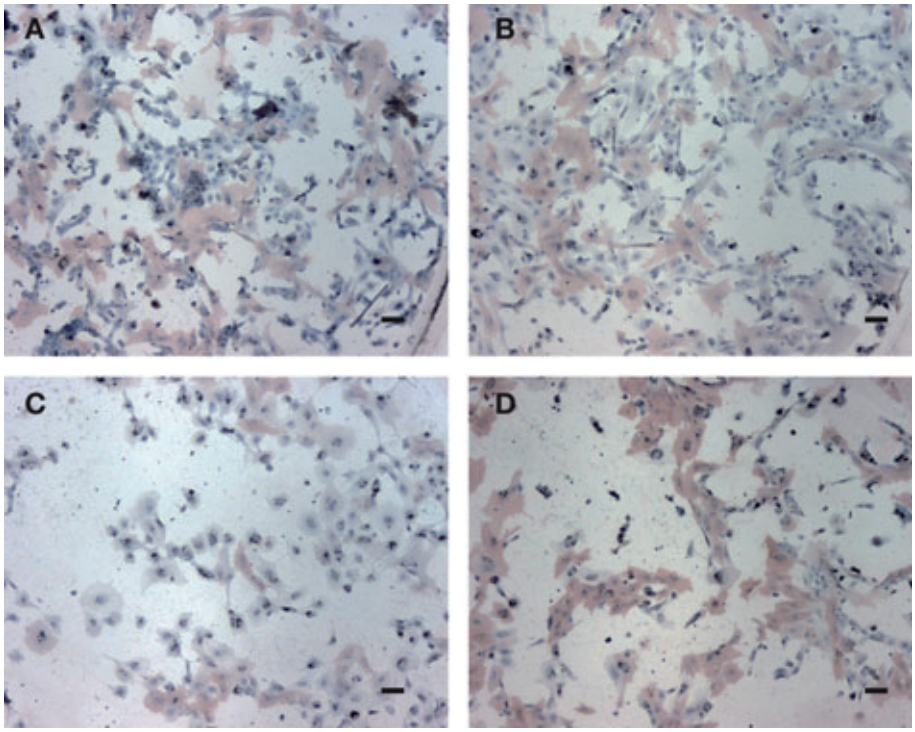

Fig. 6. Immunoperoxidase staining of porcine LECs with monoclonal antibody against α-SMA after 8 days of culture. The cells were cultured on collagen type I (a), collagen type IV (b), uncoated plastic (c) and glass (d). The reaction was visualized with 3-amino-9-ethyl carbazole; the cells were counterstained with haematoxylin. The cultures were over their confluency. The culture became disorganized and areas free of cells appeared. More cells acquired mesenchymal phenotype (became larger, some of them spindle-shaped), especially in the margin of the cell-free areas. The bar represents 100 μm.

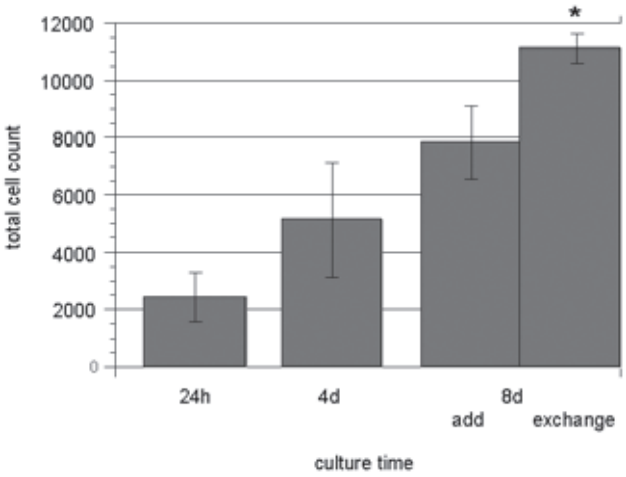

Fig. 7. Effect of the medium exchange protocol on the proliferation activity of the TOTL-86 cell line, apparent from the total cell count after 24 hours (24h), 4 days and 8 days (4d, 8d) of culture. There were significantly more cells in the wells on the 8th day if the medium was changed (11380 ± 517 cells/well, exchange) than if extra medium was added (7970 ± 1296 cells/well, add) on the 4th day.

The cultures were tested for production of specific lens proteins α-, β- or γ-crystallins, i.e. markers of lens cell differentiation. The cells did not start to differentiate into fibre cells. There were no α-, β- or γ-crystallin-positive cells on either of the cultivation substrates in any of the time intervals.

Effects of the medium exchange protocol on cell proliferation

The culture medium exchange schedule significantly influenced the proliferation rate of the cells. The number of cells increased significantly between days 1 (2420 ± 871 cells/well) and 4 (5233 ± 2056 cells/well, P = 0.0014), and also between days 4 and 8. However, on day 8, the cells reached a significantly higher number if the medium was changed (11380 ± 517 cells/well) than if only extra medium was added (7970 ± 1296 cells/well) on the 4th day (P = 0.0002, Fig. 7). During the first four days of growth, the cell population DT was 65.6 h. If the medium was changed on the 4th day, the DT was 79.0 h during the next four days of culture. If only extra medium was added, the DT was prolonged to 139.7 h during the next four days. In both cases the medium was not acidified on the fourth day, as indicated by a visual check of the colour of the phenol red in the medium.

Discussion

In the present study, essential differences were apparent in the growth characteristics of the porcine lens epithelial cells between the primary and secondary cultures. Mainly, the anchorage ability of the cells seemed to be lower in the primary culture. The cells probably required more than 24 hours to adhere and spread on the cultivation substrate. As indicated by the absence of cells on the samples after 24 hours of cultivation, the weakly adhering cells might be removed during the fixation procedure, and some of the cells were probably washed out even after four days of cultivation. When the cultivation medium was not changed for seven days, a sufficient number of cells were able to adhere and grow to form a confluent culture. In contrast to the secondary culture, no α-SMA-positive cells appeared within seven days on any of the tested growth surfaces, except glass. The morphology of the culture changed markedly after the cells reached confluence. The cell-free areas developed again, some of the cells became α-SMA positive and multilayered regions were formed in some places. All these features mimic the process observed during the formation of posterior capsular opacification after cataract surgery. First, a monolayer of cells appears. It continues to develop into structures that scatter the light and are responsible for loss of transparency. These developments include aggregation of cells into multilayered islets with regression of cells from some adjacent areas of the posterior capsule, increased deposition of extracellular matrix and wrinkle formation in the capsule (Marcantonio et al., 2000, Lois et al., 2003). A similar process was observed during the cultivation of bovine LECs in cultures established by an explant technique (Nagamoto et al., 2000). However, we did not study the primary LEC culture in more detail. Interestingly, the described arrangement of LECs *in vitro* as well as under pathological *in vivo* conditions resembles the organization of cultured vascular smooth muscle cells (VSMC) into characteristic “hills and valleys”. At the same time, the LECs acquire, at least partly, a contractile phenotype of VSMC, manifested by the presence of α-SMA, an important marker of VSMC differentiation (Bacakova et al., 1999).

The subcultured cells increased their adhesive ability to a remarkable extent. We found some cells on every tested surface coating, even after 24 hours of culture. The type of surface influenced the number of adhered cells.

The observed effect of various cell growth surfaces on the α-SMA expression has been variable in different studies. Kurosaka reported more α-SMA-positive cells on plastic, collagen type I and fibronectin than on laminin and collagen type IV after six days of cultivation (Kurosaka et al., 1999). On the contrary, De Jong-Hesse et al. described more α-SMA-positive cells on fibronectin and collagen type I than on plastic after four days of cultivation (de Jong-Hesse et al., 2005). Both studies used porcine LECs, as we did. We found significantly more α-SMA-positive cells on glass after four and eight days of cultivation. The number of α-SMA-positive cells did not differ on collagen type I, type IV and plastic after four days of growth. Surprisingly, the number of α-SMA-positive cells was higher on collagen type I and type IV than on plastic after eight days of cultivation, although the percentage of α-SMA-positive cells

was lowest on collagen type IV at the beginning (i.e., after 24 hours) of culture.

To summarize all these results, the growth surface affects the α-SMA expression in the LECs, but it is not the only factor that influences the α-SMA expression. It seems that another factor is cell density. On the 8th day of the culture the number of cells on collagen type I and especially on collagen type IV was significantly higher than that on the uncoated plastic surface. This situation may reflect a similar phenomenon observed in the case of prolonged cultivation of the primary culture (12 days). Therefore, the differences in the results among the studies mentioned here may reflect the different cell density at the time of culture evaluation.

Some studies have described the effect of serum on α-SMA expression in LECs (Liu et al., 1996; Ong et al., 2003; Kim et al., 2004). The lens epithelium and superficial cortical fibres *in vivo* are exposed to serum protein concentration less than 0.1 %. The markers of LEC differentiation were described in bovine lens cultures grown in a medium supplemented with low serum concentrations of 1, 3 or 4 % (Ong et al., 2003). In contrast, transdifferentiation into myofibroblast-like cells has been reported when the LECs were cultivated in a medium with 10 % or more serum (Kim et al., 2004). The cultivation of LECs in a serum-free medium or in a medium supplemented with 10% FBS had little effect on the rate or extent of α-SMA expression (Nagamoto et al., 2000). Kim et al. used a medium with 10% FBS and observed that the expression of α-SMA, determined by Western blotting, was enhanced, though the morphology of the LECs was similar to that of the epithelial cells. At the same time, the concentration of αA- and αB-crystallin decreased markedly (Kim et al., 2004). This is in agreement with our results, because a similar behaviour was observed in our experiments on porcine LECs in the presence of 10% FBS in the culture medium, where we did not find typical markers of differentiation in the LECs (i.e. morphology and production of lens-specific crystallins). Thus, serum supplementation is an important determining factor for the behaviour of LECs in the *in vitro* culture model.

The effect of the growth surface on proliferation has also been studied in LECs. Some authors have reported increased cell proliferation when the cell adhesion substrate was coated with collagen type I and type IV (de Jong-Hesse et al., 2005). It is not clear whether the different number of initially adhered cells was taken into account. Coating with collagen type IV, laminin or fibronectin did not affect the cell proliferation of human LEC line (SRA 01/04) in the cell culture system using a serum-free medium (Oharazawa et al., 1999). In contrast, as revealed by the DT calculated in the present study, the growth surface affected the porcine LEC proliferation. The DT was the shortest on collagen type IV, and the cell proliferation was the slowest on glass.

A decrease in the cell proliferation rate was apparent between the 4th and 8th day of culture, in comparison with the values between days 1 and 4. For a deeper analysis, we simulated this situation using rabbit LEC line TOTL-86. When we only added some fresh medium on the 4th day, the decrease in cell proliferation rate was more apparent than after exchanging the complete medium. This phenomenon may be due to a lower supply of fresh nutrients and growth factors in the first case. In addition, the cells may be exposed to some growth-attenuating factors produced by the LECs and accumulated in the culture media during eight days of cultivation. The effect of different growth-regulating factors on LEC proliferation and differentiation has been widely studied (Hales et al., 1994; Liu et al., 1994; Hales et al., 1995; Lee and Joo,1999; Meacock et al., 2000; de Iongh et al., 2005). One growth factor potentially produced by LEC is transforming growth factor β (TGF-β). TGF-β has been shown to inhibit proliferation of cultured LECs (Nishi et al., 1996) and to induce LEC transdifferentiation (i.e., epithelial-mesenchymal transition) (de Iongh et al., 2005). However, the level of TGF-β in our LEC cell culture system remains to be evaluated.

The rate of α-SMA expression was further modified by the composition of the growth surface, as documented by the highest percentage of α-SMA-containing cells on glass. Aside from the effect of cell density, the direct effect of the cell culture substrate may also be the reason for the lower rate of α-SMA-positive cells on uncoated plastic.

Our experiments on the collagen sheet (i.e., using a higher concentration of collagen type I for the culture surface coating) indicated that stability of the growth surface, i.e. its resistance to cell tractional forces, is necessary for the viability and proliferation of cells (Iwig and Glaesser, 1985; Iwig et al., 1989; Iwig and Glaesser, 1991; Engler et al., 2004a, b). Although it is generally believed that the α-SMA expression increased the LEC contractility, and that this is the reason for postoperative capsule wrinkling (Kurosaka et al., 1995), instability of the capsule may also participate in this process.

In the present study, the cultivation surface influenced the adhesive ability, proliferation and α-SMA expression in porcine LECs. The expression of the adhesion molecules may be changed by modifying the composition of the cell adhesion substrate. The changes in adhesion molecule expression as well as cytoskeletal components have been described during LEC transdifferentiation both *in vitro* and *in vivo* (Nishi et al., 1997; Kivela and Uusitalo, 1998; Saika et al., 1998; Kim et al., 2004). The cytoskeletal reorganization and actin remodeling reflect the adaptation of cells to injury and to changes in the external environment (Zelenka, 2004). More studies are needed in order to clarify the effect of the cell-extracellular matrix interaction on LEC transdifferentiation. It is also necessary to determine the effects of the medium exchange schedule, serum supplementation of the medium and cell density in more detail.

Acknowledgements

Mr. Robin Healey (Czech Technical University, Prague) is gratefully acknowledged for the language revision of the manuscript.

References

Aslam, T. M., Aspinall, P., Dhillon, B. (2003a) Posterior capsule morphology determinants of visual function. *Graefes Ach. Clin. Exp. Ophthalmol***. 241**, 208-212.

Aslam, T. M., Devlin, H., Dhillon, B. (2003b) Use of Nd:YAG laser capsulotomy. *Surv. Ophthalmol.* **48**, 594-612.

Bacakova, L., Mares, V., Lisa, V. (1999) Gender-related differences in adhesion, growth and differentiation of vascular smooth muscle cells are enhanced in serum-deprived cultures. *Cell Biol. Int.* **23,** 643-648.

Bertelmann, E., Kojetinsky, C. (2001) Posterior capsule opacification and anterior capsule opacification. *Curr. Opin. Ophthalmol.* **12,** 35-40.

de Iongh, R. U., Wederell, E., Lovicu, F. J., McAvoy, J. W. (2005) Transforming growth factor-β-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. *Cells Tissues Organs* **179,** 43-55.

de Jong-Hesse, Y., Kampmeier, J., Lang, G. K., Lang, G. E. (2005) Effect of extracellular matrix on proliferation and differentiation of porcine lens epithelial cells. *Graefes Arch. Clin. Exp. Ophthalmol.* **243,** 695-700.

Engler, A., Bacakova, L., Newman, C., Hategan, A., Griffin, M., Discher, D. (2004a) Substrate compliance versus ligand density in cell on gel responses. *Biophys. J.* **86**, 617-628.

Engler, A. J., Griffin, M. A., Sen, S., Bönnemann, C. G., Sweeney, H. L., Discher, D. E. (2004b) Myotubes differentiate optimally on substrate with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J. Cell. Biol.* **166**, 877-887.

Greenburg, G., Hay, E. D. (1986) Cytodifferentiation and tissue phenotype change during transformation of embryonic lens epithelium to mesenchyme-like cells in vitro. *Dev. Biol.* **115,** 363-379.

Hales, A. M., Schulz, M. W., Chamberlain, C. G., McAvoy, J. W. (1994) TGF-β 1 induces lens cells to accumulate α-smooth muscle actin, a marker for subcapsular cataracts. *Curr. Eye Res.* **13,** 885-890.

Hales, A. M., Chamberlain, C. G., McAvoy, J. W. (1995) Cataract induction in lenses cultured with transforming growth factor-β. *Invest. Ophthalmol. Vis. Sci.* **36,** 1709-1713.

Iwig, M., Glaesser, D. (1985) On the role of microfilaments in cell-shape-mediated growth control of lens epithelial cells. *Cell Tissue Kinet.* **18,** 169-182.

Iwig, M., Ngoli, D., Glaesser, D. (1989) Autoradiographic investigations on cell shape-mediated growth regulation of lens epithelial cells in culture. *Biomed. Biochim. Acta* **48,** 121-127.

Iwig, M., Glaesser, D. (1991) Cell-substratum interactions and the cytoskeleton in cell shape-mediated growth regulation of lens epithelial cells. *Lens Eye Toxic. Res.* **8,** 281-309.

Kim, J. T., Lee, E. H., Chung, K. H., Kang, I. C., Lee, D. H., Joo, C. K. (2004) Transdifferentiation of cultured bovine lens epithelial cells into myofibroblast-like cells by serum modulation. *Yonsei Med. J.* **45,** 380-391.

Kivela, T., Uusitalo, M. (1998) Structure, development and function of cytoskeletal elements in non-neuronal cells of the human eye. *Prog. Retin. Eye Res.* **17,** 385-428.

Kurosaka, D., Kato, K., Nagamoto, T., Negishi, K. (1995) Growth factors influence contractility and α-smooth muscle actin expression in bovine lens epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **36,** 1701-1708.

Kurosaka, D., Kato, K., Nagamoto, T. (1996) Presence of α smooth muscle actin in lens epithelial cells of aphakic rabbit eyes. *Br. J. Ophthalmol.* **80,** 906-910.

Kurosaka, D., Kato, K., Oshima, T., Kurosaka, H., Yoshino, M., Ogata, M. (1999) Extracellular matrixes influence α-smooth muscle actin expression in cultured porcine lens epithelial cells. *Curr. Eye Res.* **19,** 260-263.

Lee, E. H., Joo, C. K. (1999) Role of transforming growth factor-β in transdifferentiation and fibrosis of lens epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **40,** 2025-2032.

Liu, C. S., Wormstone, I. M., Duncan, G., Marcantonio, J. M., Webb, S. F., Davies, P. D. (1996) A study of human lens cell growth in vitro. A model for posterior capsule opacification. *Invest. Ophthalmol. Vis. Sci.* **37,** 906-914.

Liu, J., Hales, A. M., Chamberlain, C. G., McAvoy, J. W. (1994) Induction of cataract-like changes in rat lens epithelial explants by transforming growth factor β [see comments]. *Invest. Ophthalmol. Vis. Sci.* **35,** 388-401.

Lois, N., Dawson, R., McKinnon, A. D., Forrester, J. V. (2003) A new model of posterior capsule opacification in rodents. *Invest. Ophthalomol. Vis. Sci.* **44**, 3450-3457.

Mahoney, W. M., Schwartz, S. M. (2005) Defining smooth muscle cells and smooth muscle injury. *J. Clin. Invest.* **115**, 221-224.

Marcantonio, J. M., Vrensen, G. F. (1999) Cell biology of posterior capsular opacification. *Eye* **13 (Pt 3b),** 484-488.

Marcantonio, J. M., Rakic, J. M., Vrensen, G. F., Duncan, G. (2000) Lens cell populations studied in human donor capsular bags with implanted intraocular lenses. *Invest. Ophthalmol. Vis. Sci.* **41,** 1130-1141.

Marcantonio, J. M., Syam, P. P., Liu, C. S., Duncan, G. (2003) Epithelial transdifferentiation and cataract in the human lens. *Exp. Eye Res.* **77,** 339-346.

Meacock, W. R., Spalton, D. J., Stanford, M. R. (2000) Role of cytokines in the pathogenesis of posterior capsule opacification. *Br. J. Ophthalmol.* **84,** 332-336.

Nagamoto, T., Eguchi, G., Beebe, D. C. (2000) α-smooth muscle actin expression in cultured lens epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **41,** 1122-1129.

Neil, G. A., Zimmermann, U. (1993) Electrofusion. *Methods Enzymol.* **220,** 174-196.

Nishi, O., Nishi, K., Fujiwara, T., Shirasawa, E., Ohmoto, Y. (1996) Effects of the cytokines on the proliferation of and collagen synthesis by human cataract lens epithelial cells. *Br. J. Ophthalmol.* **80,** 63-68.

Nishi, O., Nishi, K., Akaishi, T., Shirasawa, E. (1997) Detection of cell adhesion molecules in lens epithelial cells of human cataracts. *Invest. Ophthalmol. Vis. Sci.* **38,** 579-585.

Nishi, O. (1999) Posterior capsule opacification. Part 1: Experimental investigations. *J. Cataract Refract. Surg.* **25,** 106-117.

Oharazawa, H., Ibaraki, N., Lin, L. R., Reddy, V. N. (1999) The effects of extracellular matrix on cell attachment, proliferation and migration in a human lens epithelial cell line. *Exp. Eye Res.* **69,** 603-610.

Ong, M. D., Payne, D. M., Garner, M. H. (2003) Differential protein expression in lens epithelial whole-mounts and lens epithelial cell cultures. *Exp. Eye Res.* **77,** 35-49.

Saika, S., Kawashima, Y., Miyamoto, T., Okada, Y., Tanaka, S. I., Ohmi, S., Minamide, A., Yamanaka, O., Ohnishi, Y., Ooshima, A., Yamanaka, A. (1998) Immunolocalization of prolyl 4-hydroxylase subunits, α-smooth muscle actin, and extracellular matrix components in human lens capsules with lens implants. *Exp. Eye Res.* **66,** 283-294.

Sasabe, T., Kishida, K., Kiritoshi, A., Uni, A., Manabe, R. (1986) A newly established cell line of rabbit lens epithelium. *Jpn. J. Ophthalmol.* **30,** 367-375.

Ursell, P. G., Spalton, D. J., Pande, M. V., Hollick, E. J., Barman, S., Boyce, J., Tilling, K. (1998) Relationship between intraocular lens biomaterials and posterior capsule opacification. *J. Cataract Refract. Surg.* **24,** 352-360.

Wilhelm, J., Smistik, Z., Mahelkova, G., Vytasek, R. (2007) Redox regulation of proliferation of lens epithelial cells in culture. *Cell Biochem. Funct.* **25**, 317-321.

Wu, X. Y., Svoboda, K. K., Trinkaus-Randall, V. (1995) Distribution of F-actin, vinculin and integrin subunits (α 6 and β 4) in response to corneal substrata. *Exp. Eye Res.* **60,** 445-458.

Zelenka, P. S. (2004) Regulation of cell adhesion and migration in lens development. *Int. J. Dev. Biol.* **48,** 857-865.