Surgical stress and cytoskeletal changes in lens epithelial cells following manual and femtosecond laser-assisted capsulotomy

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Abstract
● AIM: To study the effect of mechanical stress on the cytoskeleton in lens epithelial cells following conventional phacoemulsification surgery (CPS) and femtosecond laser-assisted cataract surgery (FLACS).
● METHODS: The cytoskeleton of the epithelial cells of the anterior lens capsules (ALC) removed by CPS and FLACS was examined by immunohistochemistry. Expression of the intermediate filament, glial fibrillary acidic protein (GFAP), and glutamine synthetase (GS) immunoreactivity were detected. In order to map the actin network of cells, fluorescently labeled phalloidin was used. The samples were examined using confocal laser scanning microscopy.
● RESULTS: GFAP expression was visible in a larger number of the epithelial cells after CPS compared to FLACS. In CPS sample’s epithelial cells, GFAP immunoreactivity indicated robust morphological change. Regarding the actin filaments, the presence of tubular elements connecting epithelial cells, regular actin pattern and marked cortical network after CPS were found. Following FLACS, the actin cytoskeleton of the epithelial cells remained densely structured, and the tubular elements were undetectable, however, the above-mentioned regular actin pattern and the marked cortical network were visible.
● CONCLUSION: The conventional removal of the ALC induces more robust changes of the cytoskeleton of the lens epithelial cells.
● KEYWORDS: lens epithelial cell; cytoskeleton; capsulotomy; glial fibrillary acidic protein; actin
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INTRODUCTION
Cataract surgery underwent rapid and significant development in the past decades[1]. In addition to the conventional phacoemulsification surgery (CPS), the use of femtosecond laser-assisted cataract surgery (FLACS) gained acceptance and has become widespread. Based on previous studies, FLACS has several advantages over conventional surgical techniques, including the more accurate capsulorhexis, the reduced phacoemulsification time and the rapid visual recovery of the patients[2-3]. Moreover, FLACS may reduce the corneal endothelial cell damage and the postoperative thickening of the cornea[4-5]. However in contrast to the above-mentioned advantages, a constant debate exists on the benefit of FLACS[6].

It is well known that every surgical intervention induces certain changes at cellular level. Following mechanical stress, various molecular pathways are activated, and consequently, changes are induced in the cells including alteration of structure of the
cytoskeleton. According to the study of Mayer et al., apoptosis in cells death is induced in both CPS and FLACS. According to our previous results, the rate of anterior lens capsules (ALC) degeneration is higher following CPS than after FLACS. Compared to CPS, FLACS induced lower rate of cell death, and lower expression of the pro-apoptotic p53 gene. In contrast, anti-apoptotic bel-2 gene expression level is increased after the FLACS. Studies indicate correlation between the expression of proteins important in the epithelial cell death and survival, and the changes of actin pattern. According to Weber and Menko, the changes of cortical actin network results in decreased anti-apoptotic Bel-2 protein level. These results are in harmony with that of Zhang, who has shown that alterations of actin cytoskeleton are associated with pro-apoptotic transcription factor p53 protein level. Thus, we can hypothesize that CPS and FLACS induce different cytoskeletal changes. In this study, we compared the effect of the two surgical methods used for the removal of ALC (CPS and FLACS) on the epithelial cells. Our main focus was to reveal cytoskeletal changes due to mechanical stress induced by capsulotomy. In the epithelial cells of the removed ALC, we examined the actin pattern and the intermediate filament glial fibrillary acidic protein (GFAP), which is characteristically expressed in the immature epithelial cells. It is well known, that due to deleterious effects (mechanical damage, injury, etc.), proteins which characteristically appear in the cells throughout embryonic development show an increased expression level in various organs including the eye. In astrocytes as well as in retinal Müller glia, GFAP is colocalized with glutamine synthetase (GS). In the epithelial cells of the lens, a GS-like protein, lengsin is present that can be detected with antibodies against GS, although, lengsin has no enzymatic activity characteristic to GS. While lengsin is highly expressed in normal lens epithelial cells, expression is decreased in age-related cataract. In order to get information about lengsin expression, antibodies against GS were used. We show that the different surgical techniques affect lens epithelial cells differently. The goal of this study is to examine the cytoskeletal changes in the epithelial cells of the ALC due to capsulotomy.

SUBJECTS AND METHODS

Ethical Approval Investigations were carried out according to procedures approved by the Institutional Ethics Committee (University of Pécs 7624, Hungary). In all cases, we obtained written consent of the patients for further examination of the lens capsule.

Subjects Samples of ALC were taken during CPS (n=32) and FLACS (n=18; VICTUS Femtosecond Laser, Bausch+Lomb’s, USA) with the following parameters: energy, 7.2 µJ; spot spacing, 6 µm; path spacing, 4 µm; time, 400-550fs. CPS removed ALC were assorted in group 1, while FLACS samples were classified in group 2. Our inclusion criteria were the following: all patients with cataract, independent of gender and age.

Light Microscopy and Immunohistochemistry For routine light microscopy, ALC (n=8 with laser-assisted, n=17 with manual removal) were fixed in a buffered solution (phosphate buffer 0.1 mol/L, pH=7.4) of 2% formaldehyde and 2.5% glutaraldehyde for 24h at 4°C. Specimens were post-fixed with 1% osmium tetroxide diluted in phosphate buffer for 30 min at room temperature. Following dehydration with ethyl-alcohol, samples were washed and placed in propylene oxide and then embedded in Durcupan resin (Sigma, Budapest, Hungary). Semithin sections or whole mount preparations were stained with toluidine-blue and studied with Olympus BX50 light microscopy.

Extracted tissue samples (n=5 ALC in each group) were immediately fixed in 4% paraformaldehyde (PFA; Sigma, Budapest, Hungary) diluted in phosphate buffered saline (PBS, 0.1 mol/L, pH 7.4) for two hours at room temperature. The fixed samples were washed in PBS and Triton-X (1:1000, Sigma, Budapest, Hungary), and incubated in 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) for one hour at room temperature. Anti-GFAP (rabbit polyclonal antibody, 1:1000, Sigma, Budapest, Hungary) and anti-GS antibodies (GS; mouse monoclonal antibody, 1:1000, Transduction Laboratories, USA) diluted in PBS were applied to the samples and incubated for 72h at 4°C. The bound antibodies were visualized following incubation with Alexa Fluor® 568-conjugated anti-rabbit and Alexa Fluor® 488-conjugated anti-mouse (1:1000, Life Technologies, Budapest, Hungary) fluorescent secondary antibodies, respectively, for 24h. Finally, nuclear labeling was performed using 4’,6-diamidino-2-phenylindol (DAPI, 1:10 000, Sigma, Budapest, Hungary) and preparations were coverslipped using fluoromount-G (Southern Biotech, USA).

In addition, GFAP immunoreaction was also examined with another, anti-human GFAP antibody (mouse monoclonal antibody, 1:750, DAKO, Glostrup, Denmark) on whole-mount preparations of ALC (n=5). Following binding of the primary antibody overnight at room temperature, anti-mouse biotinylated secondary antibody (1:100), then the avidin-biotin peroxidase detection system (Vector, Burlingame, CA) were used for 2h each. Binding sites were visualized with the chromogene 3-3’diamino-benzidine (DAB, Sigma, Budapest, Hungary) and preparations were coverslipped using fluoromount-G (Southern Biotech, USA).

Examination of the Actin Pattern Samples (n=5 ALC in each group) were washed in PBS (0.1 mol/L, pH 7.4),
followed by fixation in 4% PFA (Sigma, Budapest, Hungary) for 10 min at room temperature. Fixed tissues were incubated in permeabilization solution (diluted in PBS containing 0.1% Triton X-100, 0.1% Na-azide, 5% BSA) for 20 min at room temperature. Then capsules were stained with Alexa Fluor® 488-conjugated fluorescent phalloidin (Life Technologies, Budapest, Hungary) for 45 min, light protected at room temperature. Samples were washed in PBS and nuclear labeling was performed by propidium iodide (1:1000, Life Technologies, Budapest, Hungary), followed by coverslipping using VectaShield medium (Vector Laboratories, Burlingame, CA, USA).

**Statistical Analysis** To determine the percentage of immunoreactive cells, anti-GFAP and anti-GS immunopositive cells as well as the immunonegative cells were counted on the full thickness of the whole-mount preparations (*n*=5 ALC in each group, 10 images were taken from each sample) using a Fluoview FV-1000 Laser Confocal Scanning Microscope (40× magnification). Examination of the actin pattern was performed by analyzing 5 ALC samples from each group, taking 10 images from every sample in a Zeiss LSM 710 microscope (63× magnification) and the number of cells was determined. Data were presented as mean±SEM and statistical significance (*P*<0.05) was set with Student’s *t*-test analysis, using GraphPad Prism 5.03 program. Images were processed with Adobe Photoshop CS6.

**RESULTS**

Routine light microscopy revealed remarkable morphological changes in toluidine blue-stained ALC epithelial cells following CPS capsulotomy (Figure 1A), while morphology of epithelial cells remained relatively normal following laser-assisted removal (Figure 1B). The alteration of cells’ shape indicated robust cytoskeletal changes especially following CPS capsulotomy (Figure 1C and 1D), therefore, we further examined components of the cytoskeleton.

Immunohistochemical examinations performed in tissue samples obtained by different capsulotomy methods showed intense GFAP and GS immunopositivity of the epithelial cells. Immunoreactivity, especially following CPS capsulotomy, was stronger in the peripheral area of the ALC, and the immunoreactivity became patchy and gradually weaker towards the central area (Figure 2). The nuclei of the epithelial cells remained unstained, but the cytoplasm showed GFAP and GS immunoreactivity (Figure 2 and Figure 3A-3D).

In contrast, samples obtained from FLACS showed only 26.38% immunoreactivity to GFAP, while the GS immunoreactivity
Cytoskeletal changes in lens epithelial cells following capsulotomy

was observed in 46.78% of the cells. Colocalization of GFAP and GS was found in 19.07% of epithelial cells. Apparently, no meaningful difference could be detected in the GS-immunoreactive cell density between FLACS and CPS capsulotomy.

Comparing the results of the quantification, significant differences were found between the two groups (CPS and FLACS) in the expression of GFAP (\(P = 2.24 \times 10^{-6}\)), as well as GFAP and GS coreactivity (\(P = 0.003\)). In conclusion, GFAP expression can be an indicator of mechanical damage in case of CPS and FLACS capsulotomy.

Examination of the actin pattern revealed a marked cortical network (Figure 5A, 5B) as well as regular actin filaments (Figure 5C, 5D) both in CPS and FLACS capsulotomy samples. However, a difference was found between the two groups when the compactness of the actin pattern was studied (Figure 5E, 5F). In case of FLACS capsulotomy, that supported robust change of their cytoskeleton proposed on the base of toluidine blue staining. Therefore, another, a more dynamic component of the cytoskeleton, microfilaments were also studied.

Figure 3 GFAP and GS detected in lens epithelial cells using immunohistochemistry, following CPS (A, C, E) and FLACS capsulotomy (B, D, F) Representative fluorescence microphotographs taken from whole ALC, processed for GFAP, GS immunohistochemistry. A, B: GFAP positivity in the epithelial cells; C, D: Positive staining of epithelial cells for GS immunohistochemistry; E, F: Colocalization of GFAP and GS (arrows) in the epithelial cells. G, H: The morphology of GFAP-immunoreactive cells following CPS. Scale bar: 50 μm for A-F, 40 μm in G and 15 μm in H.

Figure 4 Quantification of immunohistochemical data In CPS removed ALC on the whole mount preparations, GFAP positive cell density was significantly higher than in FLACS capsulotomy specimens (\(P = 2.24 \times 10^{-6}\)).

Figure 5 The organization of actin filaments visualized by phalloidin labeling following CPS (A, C, E) and FLACS capsulotomy (B, D, F) Representative fluorescence microphotographs taken from ALC. Marked cortical network (A) and regular actin pattern (C) could be observed after CPS removal, like in case of FLACS capsulotomy (B, D). Remarkable difference was visible regarding the compactness of the actin pattern after CPS removal of ALC (E). In contrast, the actin pattern remained compact after FLACS capsulotomy (F). Scale bar: 10 μm.
the compactness of the actin pattern was similar that seen in control epithelial cells. In contrast, the epithelial cells after CPS capsulotomy showed a reorganization of the actin pattern. Instead of compact actin filaments, actin islands appeared and gap formation was observed between them (Figure 6A). In these gaps, tubular elements connecting epithelial cells were visible (Figure 6B). The tubular elements appeared only after CPS of the ALC.

Quantitative measurements using analysis of variance from whole ALC resulted in a significant difference ($P<0.0001$) between the two capsulotomy methods when the presence of tubular elements was examined (Figure 7).

**DISCUSSION**

Due to the introduction of femtosecond laser technology, the critical points of cataract surgery have become automated. Macroscopic changes, and postoperative refractive consequences are in the center of interest of the studies on femtosecond laser-assisted and manually performed capsulotomy [19-20]. The goal of our study was to compare cytoskeletal changes associated with CPS and FLACS capsulotomy.

Several mild injuries including mechanical damage cause stress to the cells. We suppose that all surgical interventions have an influence on the affected cells, and induce stress signaling pathways which results in the change of cytoskeleton. GFAP is one component of intermediate filaments that is found in astrocytes of the central nervous system. Due to various damages including stress, GFAP expression is increased [21-22]. In addition to astrocytes, GFAP can also be detected in many non-glial cells under normal conditions such as renal capsule, chondrocytes of epiglottic cartilage as well as in various types of tumors [23-25]. Moreover, GFAP can be observed during the embryonic development of the lens. Following differentiation it cannot be detected in lens epithelial cells anymore [13,26-28], and cytokeratin characteristic for epithelial cells is present instead. It is known, that due to damage or stress on the cells, proteins being characteristically present during embryonic development show an increased expression in adults in many organs including the eye [14-15]. Our results revealed that after ALC removal, the intermediate filament GFAP can be detected in epithelial cells. We used two different antibodies to verify the presence of GFAP and both indicated the expression of it. Boyer et al [13] described the presence of GFAP-immunoreactive fibers only during development. Based on our knowledge, there is no data in the literature about GFAP expression in the adult’s ALC epithelial cells, and we suggest that re-expression of GFAP occurred in our samples may be due to the stress caused by mechanical damage.

Our immunohistochemical and light microscopic qualitative and quantitative examinations of GFAP-immunoreactivity revealed that the rate of GFAP-immunoreactive epithelial cells were significantly higher after CPS removal of the ALC compared to FLACS capsulotomy. Repair mechanism following cellular damage involve recapitulation of developmental processes in various organs including the eye and induce elevated expression of those gene that are expressed during embryonic development [14-15]. Some of these proteins such as growth factors and their receptors can promote recovery processes [29-30]. Others, like cytoskeletal proteins (such as GFAP, vimentin or nestin) indicate the presence of cell damage [31-32]. Part of the GFAP-positive cells revealed GS-immunoreactivity indicating the presence of lengsin in those cells [16-18]. Apparently, no meaningful difference could be detected in the GS-immunoreactive cell density between FLACS and
CPS capsulotomy. Since expression of lenssin decreases in age-related cataract, the similarity in the rate of GS-immunoreactivity of lens epithelial cells indicates similar lenssin expression, and consequently, similar severity of cataract. Thus, it signifies the homogeneity of our cataract patients’ groups treated by the two different surgical methods and even highlights the difference found in GFAP expression. In harmony with the strongly significant difference in GFAP-immunoreactive cell density, the GFAP and GS double-labelled cell density revealed significant differences between the groups, although only part of GS-immunoreactive cells co-expressed GFAP. The higher density of GFAP positive cells observed after CPS capsulotomy than following FLACS ALC removal may suggest that CPS capsulotomy exerts stronger mechanical stress on the epithelial cells than the laser-assisted method.

This is important because during capsulotomy a part of the anterior capsule is not removed, and wound healing process will begin on the residual epithelial cells. We suggest that similar alterations can occur in the residual lens epithelial cells following the surgical removal of ALC and the damage caused by surgery may induce those pathways that can trigger the epithelial-mesenchymal transformation of residual capsular epithelial cells that eventually results in fibrosis and posterior capsule opacification[33]. By the migration and proliferation of residual lens epithelial cells after extracapsular cataract extraction, secondary cataract can appear due to surgical trauma[34]. The alteration of the cytoskeleton of epithelial cells around capsulorhexis showed not only changed morphology, but the alteration observed, especially in actin pattern may indicate a change in contractility and in ability to migrate.

In addition, due to stress, direct cell-cell communication can also be changed. Until recently, direct cell-cell communication was supposed to happen via gap junctions and different paracrine mechanisms. In 2004 Rustom and co-worker’s[35] described a new-actin containing-tubular structure of intercellular communication that provides continuity between distantly positioned cells. Since then similar tubular structures have been reported in many cell types (astrocytes, various tumour cells, immune cells, etc.)[36-37]. By comparing the two types of capsulotomy, we have identified cytoskeletal changes that may indicate different rates of mechanical stress on ALC epithelial cells, therefore we focused onto the examination of the actin pattern in our investigations.

It is known that stress leads to the reorganization of the actin pattern with the appearance of stress fibers[38-39]. According to Weber and Menko[11], stress fibers convert to normal actin pattern in epithelial cell culture. The change in actin cytoskeleton can trigger the expression of different signaling proteins playing a role in the epithelial cells’ response and survival[11]. Examination of the actin pattern revealed similar cortical networks in cell after CPS and FLACS as well. This is important, because changes in the cortical actin network can influence different molecular biological signaling pathways. By contrast we have found a marked difference in the compactness of the actin pattern.

In CPS capsulotomy, remodeling of the actin cytoskeleton of epithelial cells was observed. Instead of compact actin filaments, actin “islands” appeared. Between these actin “islands” gap formation was found. These gaps were not visible after FLACS capsulotomy. Gap formation can presumably be explained with the higher mechanical effect of CPS capsulotomy, which is supported with our previous light and electron microscopic results and the increased GFAP expression found in this study[10]. Between the gaps, actin-containing tubular elements could be seen. These structures were only observed after CPS removal of the ALC. Both after CPS and FLACS capsulotomy, regular actin filaments were visible.

Our results suggest that FLACS capsulotomy causes milder mechanical stress on epithelial cells, than CPS. The robust cytoskeletal changes detected following CPS indicate the possibility of altered cell-cell communication and increased ability to migration that may result in epithelial-mesenchymal transformation and consequently, development of secondary cataract. The milder cytoskeletal changes following FLACS allows us to suggest that following laser-assisted method fewer residual epithelial cells undergo epithelial-mesenchymal transformation, which may decrease the risk for the formation of posterior capsule opacification.

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Cytoskeletal changes in lens epithelial cells following capsulotomy