Reduced intercellular communication and altered morphology of bovine corneal endothelial cells with prolonged time in cell culture

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**Reduced intercellular communication and altered morphology
of bovine corneal endothelial cells with prolonged time in cell culture**

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PURPOSE

Mechanical stimulation induces intercellular Ca^{2+} waves in the corneal endothelium. The extent of the wave propagation is dependent on the activity of gap junctions, hemichannels, and ectonucleotidases. To further establish the use of a cell culture model to investigate intercellular communication, in this study, we have characterized the changes in the Ca^{2+} wave propagation in bovine corneal endothelial cells with prolonged time in culture.

MATERIALS AND METHODS

Freshly isolated BCEC were cultured for a short term (8 to 14 days; referred to as “short term”) and a long term (21 to 30 days; referred to as “long term”). Cell surface area and size were measured by confocal microscopy and flow cytometry, respectively. Calcium wave propagation was assayed by imaging spread of the Ca^{2+} waves elicited by mechanical stimulation. ATP release was assayed using Luciferin-Luciferase bioluminescence technique.

RESULTS

Cells cultured for a long term showed larger surface area and size compared to those cultured for a short term, but a reduced spread of the Ca^{2+} wave. Exposure to exogenous apyrases, which can rapidly hydrolyze extracellular ATP, reduced the spread of the Ca^{2+} wave in both groups. The fractional decrease, however, was smaller in cells cultured for a long term. Exposure to ARL-67156 to inhibit the ectonucleotidases led to a larger enhancement of the active area in cells cultured for a long term. However, the active areas of the two groups were not significantly different in the presence of the drug. Furthermore,

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ATP release in response to mechanical stimulation was lower in cells cultured for a long term in the absence of ARL-67156 but not in its presence.

CONCLUSIONS

BCEC cultured for a long term show an increase in cell surface area and cell size similar to the effect of ageing in human corneas. Moreover, the cells cultured for a long term showed a reduced ATP-dependent paracrine intercellular communication largely due to an increase in the activity of the ectonucleotidases.

INTRODUCTION

The corneal endothelium forms a monolayer of hexagonal cells on the inner surface of the cornea. Its main physiological role is to maintain deturgescence of the stroma, which is essential for corneal transparency.^{1, 2} A challenge to this function arises with ageing because of a continuous decrease in endothelial density, which is reflective of continuous cell loss and the non-regenerative nature of the monolayer in humans. Loss of endothelial cells is especially accelerated in a number of pathological conditions (e.g., Fuchs's dystrophy) and by iatrogenic trauma (e.g., phacoemulsification). When cell density is reduced below a critical level (~ 500 cells/mm²), the functionality of the endothelial barrier is compromised, resulting in corneal edema.¹⁻⁵ In the absence of endothelial cell proliferation,^{6, 7} the denuded Descemet's membrane is covered by cell enlargement (polymegathism) and migration, resulting in a loss of cell shape (polymorphism).⁸⁻¹² The altered morphology may also reflect impaired cellular functions of the monolayer, since the cortical actin cytoskeleton, a determinant of cell shape, regulates cytokinesis,¹³ migration,¹⁴ trafficking,¹⁵ barrier integrity,¹⁶ ion transport mechanisms¹⁷ and intercellular communication.¹⁸⁻²⁰

Intercellular communication, which has been a focus of our studies, is critical to establishing a coordinated response against extracellular stresses, and therefore, would be essential for the resilience of epithelial and endothelial monolayers. In our recent studies,¹⁸⁻²³ we have investigated the mechanisms of intercellular communication in cultured bovine corneal endothelial cells (BCEC) using the paradigm of intercellular Ca²⁺ wave propagation. In this approach, a single cell of a monolayer is subjected to a mechanical stimulus. The subsequent onset and spread of Ca²⁺ rise in the mechanical stimulated cell and neighboring cells is followed by confocal microscopy.¹⁸⁻²³

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Like many other cell types, the spread of an intercellular Ca^{2+} wave in corneal endothelial cells involves two major modes of intercellular communication: gap junctional intercellular communication (GJIC) and paracrine intercellular communication (PIC). While the spread of the Ca^{2+} wave through GJIC may involve a direct exchange of signaling molecules via gap junctions, the PIC pathway is dependent on the release of ATP and its subsequent paracrine action, involving P2 subtype of purinergic receptors. Accordingly, we have shown that in corneal endothelial cells (specifically in bovine corneal endothelial cells) ATP is released through hemichannels and that its subsequent action on P2Y1 and P2Y2 receptors underlies Ca^{2+} wave propagation,²¹ as has also been shown in a number of other cell types.

Primary cultures of BCEC are frequently used as models to study functional properties of the corneal endothelium such as barrier integrity, ion transport, wound repair, and intercellular communication.^{16, 18-29} While a number of *in vivo* studies have investigated the age-related increases in cell size as noted above,³⁰⁻³² there are no similar reports on the effects of duration of culture. In this study, we have investigated the morphological changes and intercellular communication in BCEC as a function of duration of cell culture. The results show that, in addition to a significant increase in cell area, a marked reduction in paracrine and gap junctional intercellular communication is noted in BCEC cultured for a long term. Since primary cultures of BCEC are frequently used as endothelial cell models,^{16, 18-29} our results implicate the importance of duration of culture on the functional properties of the cells.

MATERIALS AND METHODS

Chemicals

Fluo-4 AM (F14217), Dulbecco's PBS, anti-fade agent (P7481; Prolong Antifade kit), anti-bovine α -tubulin mouse monoclonal antibody (A-11126), Alexa Fluor® 488 labeled F(ab')₂ goat anti-mouse IgG1 fragments (A-11017), Alexa Fluor® 546 labeled phalloidin (A-22283) and Alexa Fluor® 488 labeled isotype specific secondary goat anti-mouse IgG antibody (A-21121) were obtained from Invitrogen-Gibco (Karlsruhe, Germany). Apyrase VI (A6410), apyrase VII (A6535), ARL-67156, Triton X-100, and goat serum were obtained from Sigma-Aldrich (Deisenhofen, Germany). Paraformaldehyde was obtained from Merck (Darmstadt, Germany). BSA was obtained from Roche (Vilvoorde, Belgium).

Gap27 (SRPTEKTIFII), Gap26 (VCYDKSFPISHVR) and control peptide (SRGGEKNVFIV) were synthesized at the Laboratory of Biochemistry, KU Leuven. The peptides were analyzed by reverse-phase HPLC (high-performance liquid chromatography, Waters Corporation) on a C18-column (Phenomenex Luna 5u, 250x4.60 mm) using a linear gradient of acetonitrile–water containing 0.06% TFA (trifluoroacetic acid). The exact sequence of the peptide was confirmed by ESI-triple quadrupole mass spectrometry on an API-3000 mass spectrometer (PE-SCIEX, Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). The purity of the peptide was greater than 95%.

Cell Culture

Primary cultures of BCEC were established as described previously from fresh eyes (animal age < 18 months) obtained from a local slaughter house.^{18-23, 28, 29, 33,34} The growth medium consisted of Dulbecco's Modified Eagle's Medium and 10% fetal bovine serum, 6.6% L-glutamine and 1% antibiotic-antimycotic mixture. Cells were grown at 37°C in a

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humidified atmosphere containing 5% CO₂. Cells of the first, second, and third passages were harvested and seeded into two chambered glass slides (Laboratory-Tek; Nunc, Roskilde, Denmark) at a density of 165,000 cells per chamber (4.2 cm²). Cells were grown to confluence for three to four days before use.

Measurement of Cell Area

Cells were loaded with the Ca²⁺-sensitive dye Fluo-4 AM (10 µM) for 30 minutes at 37° C. The dye was excited at 488 nm, and its fluorescence emission was collected at 530 nm. Cells were then imaged with the confocal microscope (LSM510; 40x objective; Oil, 1.3 N.A.). Polygonal regions of interest were drawn on the images to define the borders of each cell and the area of each polygon was calculated with LSM510 software. The longest axis of actin cytoskeleton in BCEC was measured with Carnoy Software to carry out measurements on digital images.³⁵

Flow Cytometry

Cells were harvested by trypsinization and then washed with PBS. After centrifugation, the cell pellet was dispersed in PBS. Forward (FSC) and side scatter (SSC) plots were generated after 100,000 events using CellQuest™ software on a FACSort™ flow cytometer (Beckton-Dickinson; laser beam with excitation wavelength of 488 nm). After acquisition of data under physiological conditions (314.0 mOsm/(kg H₂O)), the same cells were analyzed after 5 minutes of hypotonic stress (192.3 mOsm/(kg H₂O)) to induce an increase in cell volume. The reliability of the FSC signal as a parameter for cell size was checked by determination of the light-scattering properties of unstained polystyrene microspheres, calibrated for size (Flow Cytometry Size Calibration kit; Molecular Probes, F-13838).

Visualization of F-actin and α -tubulin

Cultured cells grown to confluence were washed with Dulbecco's PBS, fixed at 37° C with 4% paraformaldehyde for 20 minutes, and permeabilized for 10 minutes with a 0.5% Triton-X100 solution. The cells were washed three times with PBS and then blocked with 3% BSA and 10% goat serum for 60 min. Cells were next incubated for 60 min. at room temperature with an anti-bovine α -tubulin mouse monoclonal antibody (1 μ g/ml) solution in PBS. Unbound antibody was then washed away before incubating the cells with Alexa Fluor® 488 labeled F(ab')₂ goat anti-mouse IgG1 fragments (dilution 1/200) at room temperature. F-actin was stained with Alexa Fluor® 546 labeled phalloidin (1/40 dilution) for 20 minutes at room temperature. Finally, cells were washed with PBS and mounted with an anti-fade agent. Confocal images were obtained with 40x oil objective using argon (488 nm) and helium-neon (543 nm) lasers for excitation.

Measurement of $[Ca^{2+}]_i$ and Propagation of the Ca^{2+} Wave

The Ca^{2+} wave propagation was assayed by imaging $[Ca^{2+}]_i$ in the mechanical stimulated cells and the neighboring cells using the LSM510 confocal microscope. Mechanical stimulation of a single cell consisted of an acute, short-lasting deformation of the cell by briefly touching less than 1% of the cell membrane with a glass micropipette (tip diameter < 1 μ m) coupled to a piezoelectric crystal nanopositioner (Piezo Flexure NanoPositioner P-280, operated through E463 amplifier/controller, PI Polytech, Karlsruhe, Germany) mounted on a micro-manipulator.

For imaging $[Ca^{2+}]_i$, cells were loaded with the Fluo-4 AM, as noted above, and the emission collected at 530 nm caused by excitation at 488 nm was captured using a 40x objective (Oil, 1.3 NA). In experiments involving ARL-67156, a 10x objective (Air, 0.3 NA)

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was used. Once the images were acquired, polygonal regions of interest (ROIs) were drawn on the images to quantify the $[Ca^{2+}]_i$ increase in the mechanically stimulated cell and the neighboring cells (NB cells). Cells immediately around the mechanically stimulated cell are defined as neighboring cell layer 1 (NB1), the ones immediately surrounding the NB1 cells are defined as neighboring cell layer 2 (NB2), and so on. Normalized fluorescence (NF) was then obtained by dividing the fluorescence by the average fluorescence before mechanical stimulation. Intercellular propagation of the Ca^{2+} wave was characterized by maximum NF and percentage of responsive cells (%RC), as well as by the total surface area of responsive cells (active area, AA) with $NF \geq 1.1$.

Measurement of ATP Release

After the mechanical stimulation, the accumulation of the released ATP in a solution bathing a monolayer was followed with the luciferin-luciferase bioluminescence protocol. Five microliters of the ATP assay mix-solution (FL-AAM, containing luciferin and luciferase), added to 100 μ l out of the 500 μ l bathing solution covering the cells, were taken to a custom-built photon-counting setup to measure the luminescence. Photons emitted as a result of the oxidation of luciferin in the presence of ATP and O_2 , a reaction that is catalyzed by luciferase, were detected by a photon-counting photomultiplier tube (H7360-01, Hamamatsu Photonics, Hamamatsu, Japan) that has a sensitive area of 25 mm diameter and is positioned 20 mm above the cells. Voltage pulses from the photomultiplier module were counted with a high-speed counter (PCI-6602, National Instruments, Austin, Texas, USA). The dark count of the photomultiplier tube was < 80 counts/second.

Lucifer Yellow Uptake Assay

Cells grown to $\pm 90\%$ confluence in chambered slides were incubated in Ca^{2+} -rich PBS containing the drug of interest for 30 min. Cells were then exposed to PBS containing the 2 mM EGTA and 2.5% LY for 5 min. in the continued presence of the drug. Following a wash with Ca^{2+} -containing PBS, LY fluorescence was recorded using the laser-scanning confocal microscope (LSM 510). The excitation was at 458 nm with emission recorded at 530 nm. Images with a frame size of $106,080 \mu\text{m}^2$ were acquired at a resolution of 1024×1024 pixels and 256 grey levels.

Data Analysis

Unpaired t-tests with Bonferroni correction were used to compare the cell area of cells of different ages. "N" indicates the number of experiments and "n" indicates the number of cells. Unpaired t-tests with Bonferroni correction were also used to compare results of the Ca^{2+} wave experiments for treatment vs. control (Prism 4.0 for Windows, GraphPad Software Inc., San Diego, California, USA). For all tests, a P-value of < 0.05 is considered statistically significant. Histograms are expressed as mean $\pm$ standard error of the mean (SEM). In the Ca^{2+} wave experiments, "N" indicates the number of independent experiments (the number of mechanically stimulated cells), while "n" represents the total number of responsive cells.

RESULTS

Changes in Morphology

We first examined the morphology of cells at different time points in culture. Figure 1 shows histograms and box plots of the distribution of the area of cells cultured for periods between 8 to 30 days after isolation. The average area increased from $831 \pm 2 \mu\text{m}^2$ ($n = 42,049$ and $N = 500$) to $1,650 \pm 8 \mu\text{m}^2$ ($n = 23,428$ and $N = 500$) in cells cultured for a

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short term (8 to 14 days) and a long term (21 to 30 days) (also shown in Table 1). The cells, which are usually dome-shaped, exhibited a decrease in height from $4.4 \pm 0.5 \mu\text{m}$ ($N = 6$) to $3.14 \pm 0.22 \mu\text{m}$ ($N = 6$) in short and long term cultures. Consistent with increase in cell area, the flow cytometry showed an increase in forward light scatter ($578 \pm 16 \text{ ALU}$, $N = 7$ for cells in the short term culture vs. $683 \pm 12 \text{ ALU}$, $N = 7$ for cells in the long term culture; $P < 0.001$; ALU: arbitrary light units), indicating an increase in cell size as well. The differences in area were not due to the variability in corneas from different animals, since they were found in cells isolated from the same cornea. This is illustrated in Figure 2 A-C, which shows confocal images of cells cultured for 10, 20 and 30 days after isolation from the same cornea. Another parameter that could have influenced the area is the number of passages during cell culture. In cells cultured for 8 to 14 days, we found no significant difference of cell area between passages 1 and 2 ($P = 0.83$), but there was a significant decrease of cell areas between passage 2 and 3 ($P = 1.93 \cdot 10^{-6}$). In cells cultured for 21 to 30 days, on the other hand, we noted a significant increase in cell area between passages 2 and 3 (Fig. 3). To corroborate these findings, we examined the organization of the cytoskeleton. Figure 4 shows co-staining of F-actin and α -tubulin on day 12 (Fig. 4A) and on day 26 (Fig. 4B) ($N = 7$). F-actin at the periphery showed an increase in the perimeter with prolonged time in cell culture (Fig. 4). The length of the longest axis of the F-actin band increased from $17.8 \pm 0.9 \mu\text{m}$ ($n = 80$) on day 12, to $30.9 \pm 0.6 \mu\text{m}$ ($n = 100$) on day 26.

Changes in Intercellular Communication

Figure 5 shows typical Ca^{2+} waves in monolayers obtained from short and long term cell cultures. The mechanically stimulated cells in both the groups show a transient $[\text{Ca}^{2+}]_i$ rise, which then spreads out to the neighboring (NB) cells as a Ca^{2+} transient (Fig. 5). The line graphs to the right show the time course of such Ca^{2+} transients (represented as

normalized fluorescence (NF) values) in the mechanically stimulated cell and in the neighboring cell layers one to five (NB1, NB2, NB3, NB4 and NB5). As can be seen from the figure, the normalized fluorescence decreases, while the delay for the onset of $[Ca^{2+}]_i$ rise increases with increasing distance from the mechanically stimulated cell. In cells cultured for a short term, Ca^{2+} transients were observed up to approximately 4 to 8 cell layers away from the mechanically stimulated cell (Fig. 5A), while in cells cultured for a long term, the Ca^{2+} wave reached cell layers 2 to 4 only (Fig. 5B). For cells cultured for a short term, the maximal normalized fluorescence in the mechanically stimulated cell was reached in about 0.95 ± 0.04 s ($N = 175$). Thereafter, the normalized fluorescence showed a very gradual and slow decline, returning to the basal value after 152 ± 6 seconds after application of the stimulus. For cells grown for a long term, the maximal normalized fluorescence in the mechanically stimulated cell was reached in about 1.6 ± 0.1 seconds. Thereafter, the normalized fluorescence returned to the basal value 142 ± 5 seconds after application of the stimulus ($N = 118$).

A quantitative summary of the effect of prolonged time in culture on Ca^{2+} wave propagation is given in Figure 6 and Table 2. In both groups, the normalized fluorescence (Fig. 6A and C) and the percentage of responsive cells (%RC), as shown in Fig. 6B and D, decrease as a function of the distance of the cell layer from the mechanically stimulated cell. The decrease of the responsive cells with distance from the mechanically stimulated is faster in cells cultured for long term. When comparing cells in corresponding cell layers, the delay is longer for cells cultured for a long term compared to those cultured for a short term. While in cells cultured for short term the Ca^{2+} wave propagates to cell layer 5 or further, the spread of the wave in cells cultured for a long term is limited to layer 4. In addition, the active area was significantly lower for cells grown for a long term than for cells grown for a short term, as shown in Table 2. In summary, the results in Figures 5 and

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6 show that the propagation of Ca^{2+} waves decreases significantly upon prolonged time in culture.

Changes in Paracrine Intercellular Communication

Our previous studies with BCEC cells have shown that paracrine intercellular communication (PIC) is the predominant mechanism underlying the Ca^{2+} wave propagation and that it is mediated through ATP release via hemichannels. Therefore, we investigated the reduction in the active area of Ca^{2+} wave in cells after the long term culture to see if it is associated with mechanisms underlying PIC. However, we first examined changes in the gap junctional intercellular communication (GJIC). Thus, Gap27 (30 min pre-treatment at 300 μM), which is known to reduce the Ca^{2+} wave by inhibition of GJIC,^{21, 22} reduced the Ca^{2+} wave in cells cultured for a short term (from $51,600 \pm 2,800 \mu\text{m}^2$ to $28,100 \pm 2,700 \mu\text{m}^2$; N = 70; Fig. 7A) but not in those cultured for a long term ($20,700 \pm 2,000 \mu\text{m}^2$ vs $19,400 \pm 1,500 \mu\text{m}^2$ in control conditions; N = 45; Fig. 7B). This result suggests that GJIC is reduced after a long term culture. We next examined modulation of PIC upon a long term culture by using Gap26, which is known to reduce PIC through inhibition of connexin hemichannels.^{18, 21} Gap26 (30 min. pre-treatment at 300 μM) reduced the active area of Ca^{2+} wave from $51,600 \pm 2,800 \text{ mm}^2$ to $30,300 \pm 2,600 \text{ mm}^2$ (N = 70) (Fig. 7A) in cells cultured for a short term. In contrast, Gap26 did not influence the Ca^{2+} wave in cells cultured for a long term ($21,600 \pm 2,400 \text{ mm}^2$ vs $19,400 \pm 1,500 \text{ mm}^2$ in control conditions; N = 45) (Fig. 7B). This finding provides striking evidence that PIC is significantly reduced in cells cultured for long term.

Effect of Ectonucleotidases

Since Ca^{2+} wave propagation, which is largely through ATP release,^{21, 22} is influenced to a very large extent by ectonucleotidases (CD73 and CD39 are present in BCEC²²), we

attempted to determine whether the reduction in active area in cells cultured for a long term is due to increased activity of the ectonucleotidases. In our first series of experiments, we exposed cells to exogenous apyrases known to rapidly hydrolyze ATP and ADP and thereby abolish PIC. In cells cultured for a short term, apyrase VI (known to preferentially hydrolyse ATP³⁶; 5 U/ml for 30 min) and apyrase VII (known to hydrolyse preferentially ADP³⁶; 5 U/ml for 30 min) in combination caused a five-fold decrease of the active area (from $67,500 \pm 3,700 \mu\text{m}^2$ to $12,900 \pm 1,600 \mu\text{m}^2$; N = 25) (Fig. 8A). In cells cultured for a long term, exposure to apyrases VI+VII caused only a two-fold decrease (from $22,400 \pm 3,200 \mu\text{m}^2$; N = 37 to $10,200 \pm 900 \mu\text{m}^2$; N = 40) (Fig. 8B). Thus, the inhibition of the Ca^{2+} wave by apyrases in cells cultured for a long term is smaller compared to those cultured for a short term ($P < 0.001$). This suggests that in cells cultured for a long term, PIC is significantly reduced.

To confirm the above findings, we also examined the effect of inhibition of the ectonucleotidases using ARL-67156 (ARL) on the active area of the Ca^{2+} wave. In our previous studies, we have reported that ARL (100 μM for 30 min) increases the active area significantly.^{18, 21, 22} Exposure to ARL led to a five-fold increase in the active area (from $55,500 \pm 3,700 \mu\text{m}^2$ to $258,300 \pm 23,600 \mu\text{m}^2$; $P < 0.001$; N = 20; Fig. 9A) in cells cultured for a short term compared to an 8-fold increase in cells cultured for long term (from $31,000 \pm 2,600 \mu\text{m}^2$ to $257,400 \pm 30,700 \mu\text{m}^2$; $P < 0.001$; N = 52; Fig. 9B). However, in the presence of ARL, the two cell groups did not show significantly different active areas ($P < 0.001$), indicating that the amount of released ATP and the expression level of purinergic receptors are not different between the two cell groups. Therefore, our findings with ARL suggest that the activity of the ectonucleotidases is higher in cells cultured for a long term compared to those cultured for a short term. Consistent with this finding, the ATP release following mechanical stimulation in the presence of ARL was not different in cells cultured

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for a long term compared to those cultured for a short term (N = 5). However, in the absence of ARL, the amount ATP measured upon mechanical stimulation was markedly reduced (median reduction of 75%; N = 5) in cells cultured for a long term when compared to those cultured for a short term, indicating that in cells cultured for a long term the released ATP is hydrolyzed at a higher rate compared to the released ATP of cells cultured for a short term.

The sensitivity of the Ca^{2+} wave to Gap26, demonstrated in cells cultured for a short term, in addition to similar findings reported previously,^{18,21,37} indicates an involvement of hemichannels in ATP release.²¹ To investigate whether the hemichannel-mediated PIC is inhibited in cells cultured for a long term, we examined LY uptake. As shown in Figure 10, LY uptake was noted in cells cultured for long term similar to in those cultured for short term (N = 6). Thus, cells cultured for a long term clearly possess functional hemichannels.

DISCUSSION

A number of *in vivo* studies have demonstrated age-related increase in cell size in corneal endothelium.³⁰⁻³² However, little attention has been given to altered morphology and function *vis-a-vis* duration of cells in primary culture. As noted earlier, BCEC are routinely used as models to study functional properties of the corneal endothelium such as regulation of its fluid pump, barrier integrity and intercellular communication. These properties are all critical for maintenance of stromal deturgescence, which is required for corneal transparency. In this study, we have investigated altered morphology and intercellular communication in BCEC upon prolonged time in cell culture. Our major findings are that cells cultured for long term show an increase in cell size as well as significantly increased activity of the ectonucleotidases.

Altered Morphology

We used confocal microscopy to assess changes in cell morphology in response to prolonged time in culture. Since BCEC isolated from animals of different ages showed age-related morphological differences, we employed only corneas of young animals (< 18 months) for isolation of cells. Our data show a significant increase in cell area with prolonged time in culture (Figs. 1-2, Table 1). In consistence with this finding, the perimeter of the peri-junctional actomyosin ring (PAMR) is larger on day 26 after isolation compared to cells on day 12 after isolation (Fig. 4).

In order to investigate whether the increase in cell area with time in culture, as visualized by confocal microscopy, is associated with an increase in cell volume or merely due to cell spreading, we performed flow cytometry studies to investigate differences in cell volume. We acquired data of forward-light scattering (FSC) as a measure of the cell size. In line with our finding of increased in cell area, FSC in cells cultured for 24 to 26 days was significantly higher by ~ 18% than those cultured for 10 to 12 days. Since the reliability of FSC as a measure for cell volume is still debated,^{38,39} we also measured increase in FSC by subjecting the cells to hyposmotic solution (192.3 mOsm/(kg H₂O)). According to van 't Hoff's law ($\pi = nRT/V$), changes in volume (V) in ideal osmometers are inversely related to changes in osmotic pressure (π) for constant number of particles (n) and temperature. Our flow cytometry data showed a 4.4 ± 0.7 % increase in FSC in the hyposmotic solution versus isosmotic (314 mOsm/(kg H₂O)) solution. Srinivas et al.⁴⁰ have shown a ~4% increase in forward scattering in hyposmotic (245 mOsm/(kg H₂O)) versus isosmotic (295 mOsm/(kg H₂O)) conditions. Finally, as in human corneal endothelial cells, which show polymegathism concomitant with pleomorphism, BCEC cells lost their shape and showed increased polygonality.

Altered Intercellular Communication and Enhanced Ectonucleotidase Activity

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Our data indicated that intercellular propagation of Ca^{2+} waves in BCEC decreases significantly upon prolonged time in culture (Figs. 5 and 6). We employed a number of different measures of intercellular communication, such as number of neighboring cell layers reached by the Ca^{2+} wave, the percentage of responsive cells (%RC) in each neighboring cell layers, and the normalized fluorescence (NF), as well as the active area. Each of these measures consistently indicated that intercellular communication is reduced in cells cultured for a long term. The active area is decreased by ~ 26% upon long term cell culture compared to those cultured for a short term. While in cells cultured for a short term the Ca^{2+} wave covers 4 to 8 cell layers (Fig. 5A), in cells cultured for a long term the Ca^{2+} wave reaches only cell layers 2 to 4 (Fig. 5B). The normalized fluorescence (Figs. 6A and C) and the percentage of responsive cells (Figs. 6B and D) in both groups decrease as a function of the distance of the cell layer from the mechanically stimulated cell. The decrease of the percentage of responsive cells with distance from the mechanically stimulated cell is faster in cells cultured for a long term.

In our previous studies^{18, 21-23} we showed that in BCEC two forms of intercellular Ca^{2+} communication pathways, namely paracrine (PIC) and gap junctional intercellular communication (GJIC), contribute to propagation of the Ca^{2+} wave in response to a mechanical stimulation. We also showed that PIC contributes more to the wave propagation than GJIC. In this study we have investigated the influence of prolonged time in culture on PIC and GJIC. Our experiments with Gap27 provided evidence for a reduced GJIC (Fig. 7). This was further confirmed by experiments based on fluorescence recovery after photobleaching (data not shown). However, ATP-release experiments and experiments with apyrases, ARL and Gap26 demonstrated that the decrease in the active area of the wave in cells cultured for a long term is mainly due to a decrease in ATP-mediated PIC (Figs. 7-9). The reduction of active area by apyrases was ~ 50% in cells

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3 cultured for a long term compared to ~ 80% in cells cultured for a short term (Fig. 8).
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5 Inhibition of ectonucleotidase activity by ARL-67156, which is known to induce an
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7 enhancement of the active area in BCEC,^{18,21-23} caused a larger enhancement of the
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9 active area in cells cultured for a long term (Fig. 9B) compared to the enhancement of the
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11 active area in cells cultured for a short term (Fig. 9A). In fact, the active area in the
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13 presence of ARL-67156 was not significantly different. These experiments, therefore,
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15 indicate that the amount of released ATP and the response of the purinergic receptors are
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17 not different between the two groups. The findings with ARL-67156 (Fig. 9) suggest that
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19 the reduced propagation of the Ca^{2+} wave in cells cultured for a long term is due to a
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21 higher rate of extracellular hydrolysis of ATP, presumably because of higher activity of the
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23 ectonucleotidases. Experiments with Gap26 are in agreement with such a conclusion,
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25 since Gap26 produced a strong decrease of the active area in cells cultured for a short
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27 term (Fig. 7A), while the peptide had no significant effect on cells cultured for a long term
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29 (Fig. 7B).
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38 In summary, BCEC in culture show an increase in cell surface area and cell size, similar to
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40 the effect of aging in human eyes. Moreover, cells cultured for a long term show reduced
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42 PIC, presumably due to an increase in the activity of the ectonucleotidases. This finding is
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44 relevant to cell culture studies of corneal endothelium, since the BCEC culture model is
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46 frequently used to study ion transport, cell proliferation, wound healing, intercellular
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48 communication and barrier integrity.^{16, 18-20, 24, 41} As far as the changes in the morphology
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50 are concerned, the pattern of changes in the BCEC cell culture model found in this study is
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52 similar to what is known in human corneal endothelial cells *in vivo* in response to ageing or
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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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FIGURE LEGENDS

Figure 1. Changes in cell size of cultured BCEC with prolonged time in culture

(A). Distribution of cell area of cultured BCEC for different periods after cell isolation. **(B).** Scatter and boxplots of area of cultured cells for different periods in culture after cell isolation ($N = 500$ for each period in culture after cell isolation). The box represents the 25th and 75th percentile, the line in the box represents the median value, the small rectangle represents the mean value.

Figure 2. Confocal images of a monolayer of bovine corneal endothelial cells at different times in culture

BCEC, cultured from the same cornea, 10 (left), 20 (middle) and 30 (right) days after isolation.

Figure 3. Changes in cell size of BCEC of different passages with time in culture

Cell area of BCEC cultured for 8 to 14 days or for 21 to 30 days after cell isolation ($N = 15$). In cells cultured for 8 to 14 days, the average area of cells of passage 1 and 2 is not significantly different ($n = 1,367$ and $n = 1,114$ respectively), but the cell area of cells of passage 3 is significantly decreased ($n = 1,614$). In cells cultured for 21 to 30 days the area of cells of passage 3 is significantly increased. ($n = 785$ for passage 2 and $n = 618$ for passage 3).

* signifies $P < 0.001$ between passage 3 vs passage 1 or 2 in cells cultured for 8 to 14 days and in cells cultured for 21 to 30 days.

Figure 4. Immunofluorescence images showing F-actin and α -tubulin staining

(A). Immunocytochemistry images from BCEC on day 12 after isolation.

(B). Immunocytochemistry images from BCEC on day 26 after isolation. First row shows F-actin staining, second row shows α -tubulin staining, third row shows colabeling of F-actin and α -tubulin and fourth row is a detail from the images in the third row.

Figure 5. Ca^{2+} wave propagation in control conditions in BCEC cultured for 10 days (top) and for 21 days (bottom)

Representative pseudocolored fluorescence images showing Ca^{2+} transients at different times after mechanical stimulation in BCEC. The line graphs at the right show the time course of the normalized fluorescence value (NF) in the mechanically stimulated cell (MS) and the average value of NF in the neighboring cell (NB) layers 1 to 5 (NB1 to NB5). The first image shows the fluorescence intensities before stimulation. The white arrow in the second image identifies the MS cell. **(A).** Control conditions in BCEC cultured for 10 days: the Ca^{2+} wave propagates to six neighboring cell layers. The total area of cells reached by the wave (active area: AA) is $62,870 \mu\text{m}^2$. **(B).** Control conditions in BCEC cultured for 21 days: the Ca^{2+} wave propagates to three neighboring cell layers. The total area of cells reached by the wave (active area: AA) is $22,030 \mu\text{m}^2$.

Figure 6. Quantification of the spread of the Ca^{2+} wave in control conditions in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)

(A). Average value of normalized fluorescence (NF) in the mechanically stimulated (MS) cell and in neighboring cell layers NB1 to NB5 in control conditions in BCEC cultured for 8 to 14 days ($N = 175$). **(B).** Percentage of responsive cells (%RC) in MS and NB1 to NB5 in control conditions in BCEC cultured for 8 to 14 days ($N = 175$). **(C).** Average value of normalized fluorescence (NF) in the mechanically stimulated (MS) cell and in neighboring cell layers NB1 to NB5 in control conditions in BCEC cultured for 21 to 30 days ($N = 118$). **(D).** Percentage of responsive cells (%RC) in MS and NB1 to NB5 in control conditions in BCEC cultured for 21 to 30 days. ^ signifies $P < 0.001$ between control condition in cells cultured for 21 to 30 days vs control condition in cells cultured for 8 to 14 days ($N = 118$).

Figure 7. Effect of Gap27 and Gap26 on the active area in control conditions in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)

Active area (AA) in BCEC after incubation with connexin mimetic peptides, namely control peptide (300 μ M), Gap27 (300 μ M) or Gap26 (300 μ M) for 30 min. **(A).** In BCEC cultured for 8 to 14 days, the AA is reduced in the presence of Gap26 (N = 70) or in the presence of Gap27 (N = 70).

* signifies $P < 0.001$ between each condition in cells cultured for 8 to 14 days vs control in cells cultured for 8 to 14 days (i.e., comparison of white bars). **(B).** In BCEC cultured for 21 to 30 days, the AA is not reduced in the presence of Gap26 (N = 45) or in the presence of Gap27 (N = 45).

^ signifies $P < 0.001$ between each condition in cells cultured for 21 to 30 days vs each condition in cells cultured for 8 to 14 days (i.e., comparison of identically colored bars).

Figure 8. Effect of exposure to exogenous nucleotidases on the active area (AA) in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)

(A). Effect on AA in the presence of exogenous apyrases in BCEC cultured for 8 to 14 days. Treatment of the cells with apyrase VI (5 U/ml) and apyrase VII (5 U/ml) for 30 min decreased AA ($N = 25$).

(B). AA in the presence of the combination of apyrase VI and apyrase VII in BCEC cultured for 21 to 30 days is decreased ($N = 37$).

* signifies $P < 0.001$ in the presence vs absence of apyrase. ^ signifies $P < 0.001$ between control in cells cultured for 21 to 30 days vs control in cells cultured for 8 to 14 days (i.e., comparison of white bars). In the presence of apyrase the difference between cells cultured for 21 to 30 days and cells cultured for 8 to 14 days was not statistically significant (i.e., comparison of black bars).

Figure 9. Effect of inhibition of ectonucleotidase activity on the active area (AA) in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)

(A). Effect on AA in BCEC cultured for 8 to 14 days in the presence of a selective ectonucleotidase inhibitor ARL-67156 (ARL; 100 μ M for 30 min). In cells, cultured for 8 to 14 days, treated with ARL, the AA is significantly increased ($N = 20$). **(B).** In cells, cultured for 21 to 30 days, treated with ARL, the AA is also significantly increased ($N = 52$).

* signifies $P < 0.001$ in the presence vs absence of ARL. ^ signifies $P < 0.001$ between control in cells cultured for 21 to 30 days vs control in cells cultured for 8 to 14 days (i.e., comparison of white bars). There is no significant difference in the presence of ARL between cells cultured for 21 to 30 days and cells cultured for 8 to 14 days (i.e., comparison of black bars).

Figure 10. Effect of time in culture on Lucifer Yellow uptake in Ca^{2+} -free solutions

Cells were exposed to the fluorescent dye Lucifer Yellow (2.5 % for 5 minutes) in Ca^{2+} -free solution containing 2 mM EGTA. **(A).** Uptake of Lucifer Yellow in control condition in BCEC cultured for 8 to 14 days. **(B).** Uptake of Lucifer Yellow in control condition in BCEC cultured for 21 to 30 days.

Figure 1. Changes in cell size of cultured BCEC with prolonged time in culture (A). Distribution of cell area of cultured BCEC for different periods after cell isolation. (B). Scatter and boxplots of area of cultured cells for different periods in culture after cell isolation (N = 500 for each period in culture after cell isolation). The box represents the 25th and 75th percentile, the line in the box represents the median value, the small rectangle represents the mean value.

215x324mm (150 x 150 DPI)

Figure 2. Confocal images of a monolayer of bovine corneal endothelial cells at different times in culture BCEC, cultured from the same cornea, 10 (left), 20 (middle) and 30 (right) days after isolation.

215x82mm (150 x 150 DPI)

Figure 3. Changes in cell size of BCEC of different passages with time in culture
 Cell area of BCEC cultured for 8 to 14 days or for 21 to 30 days after cell isolation (N = 15). In cells cultured for 8 to 14 days, the average area of cells of passage 1 and 2 is not significantly different (n = 1,367 and n = 1,114 respectively), but the cell area of cells of passage 3 is significantly decreased (n = 1,614). In cells cultured for 21 to 30 days the area of cells of passage 3 is significantly increased. (n = 785 for passage 2 and n = 618 for passage 3).
 * signifies P < 0.001 between passage 3 vs passage 1 or 2 in cells cultured for 8 to 14 days and in cells cultured for 21 to 30 days.

186x117mm (150 x 150 DPI)

Figure 4. Immunofluorescence images showing F-actin and tubulin staining (A). Immunocytochemistry images from BCEC on day 12 after isolation. (B). Immunocytochemistry images from BCEC on day 26 after isolation. First row shows F-actin staining, second row shows tubulin staining, third row shows colabeling of F-actin and tubulin and fourth row is a detail from the images in the third row.

108x197mm (150 x 150 DPI)

Figure 5. Ca²⁺ wave propagation in control conditions in BCEC cultured for 10 days (top) and for 21 days (bottom)

Representative pseudocolored fluorescence images showing Ca²⁺ transients at different times after mechanical stimulation in BCEC. The line graphs at the right show the time course of the normalized fluorescence value (NF) in the mechanically stimulated cell (MS) and the average value of NF in the neighboring cell (NB) layers 1 to 5 (NB1 to NB5). The first image shows the fluorescence intensities before stimulation. The white arrow in the second image identifies the MS cell. (A). Control conditions in BCEC cultured for 10 days: the Ca²⁺ wave propagates to six neighboring cell layers. The total area of cells reached by the wave (active area: AA) is 62,870 μm². (B). Control conditions in BCEC cultured for 21 days: the Ca²⁺ wave propagates to three neighboring cell layers. The total area of cells reached by the wave (active area: AA) is 22,030 μm².

215x184mm (150 x 150 DPI)

Figure 6. Quantification of the spread of the Ca²⁺ wave in control conditions in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right) (A). Average value of normalized fluorescence (NF) in the mechanically stimulated (MS) cell and in neighboring cell layers NB1 to NB5 in control conditions in BCEC cultured for 8 to 14 days (N = 175). (B). Percentage of responsive cells (%RC) in MS and NB1 to NB5 in control conditions in BCEC cultured for 8 to 14 days (N = 175). (C). Average value of normalized fluorescence (NF) in the mechanically stimulated (MS) cell and in neighboring cell layers NB1 to NB5 in control conditions in BCEC cultured for 21 to 30 days (N = 118). (D). Percentage of responsive cells (%RC) in MS and NB1 to NB5 in control conditions in BCEC cultured for 21 to 30 days. ^ signifies P < 0.001 between control condition in cells cultured for 21 to 30 days vs control condition in cells cultured for 8 to 14 days (N = 118).

215x145mm (150 x 150 DPI)

Figure 7. Effect of Gap27 and Gap26 on the active area in control conditions in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)
Active area (AA) in BCEC after incubation with connexin mimetic peptides, namely control peptide (300 μM), Gap27 (300 μM) or Gap26 (300 μM) for 30 min. (A). In BCEC cultured for 8 to 14 days, the AA is reduced in the presence of Gap26 (N = 70) or in the presence of Gap27 (N = 70). * signifies P < 0.001 between each condition in cells cultured for 8 to 14 days vs control in cells cultured for 8 to 14 days (i.e., comparison of white bars). (B). In BCEC cultured for 21 to 30 days, the AA is not reduced in the presence of Gap26 (N = 45) or in the presence of Gap27 (N = 45). ^ signifies P < 0.001 between each condition in cells cultured for 21 to 30 days vs each condition in cells cultured for 8 to 14 days (i.e., comparison of identically colored bars).

215x71mm (150 x 150 DPI)

Figure 8. Effect of exposure to exogenous nucleotidases on the active area (AA) in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)

(A). Effect on AA in the presence of exogenous apyrases in BCEC cultured for 8 to 14 days. Treatment of the cells with apyrase VI (5 U/ml) and apyrase VII (5 U/ml) for 30 min decreased AA (N = 25).

(B). AA in the presence of the combination of apyrase VI and apyrase VII in BCEC cultured for 21 to 30 days is decreased (N = 37).

* signifies P < 0.001 in the presence vs absence of apyrase. ^ signifies P < 0.001 between control in cells cultured for 21 to 30 days vs control in cells cultured for 8 to 14 days (i.e., comparison of white bars). In the presence of apyrase the difference between cells cultured for 21 to 30 days and cells cultured for 8 to 14 days was not statistically significant (i.e., comparison of black bars).

215x75mm (150 x 150 DPI)

Figure 9. Effect of inhibition of ectonucleotidase activity on the active area (AA) in BCEC cultured for 8 to 14 days (left) and for 21 to 30 days (right)

(A). Effect on AA in BCEC cultured for 8 to 14 days in the presence of a selective ectonucleotidase inhibitor ARL-67156 (ARL; 100 μM for 30 min). In cells, cultured for 8 to 14 days, treated with ARL, the AA is significantly increased (N = 20). (B). In cells, cultured for 21 to 30 days, treated with ARL, the AA is also significantly increased (N = 52).

* signifies $P < 0.001$ in the presence vs absence of ARL. ^ signifies $P < 0.001$ between control in cells cultured for 21 to 30 days vs control in cells cultured for 8 to 14 days (i.e., comparison of white bars). There is no significant difference in the presence of ARL between cells cultured for 21 to 30 days and cells cultured for 8 to 14 days (i.e., comparison of black bars).

215x75mm (150 x 150 DPI)

Figure 10. Effect of time in culture on Lucifer Yellow uptake in Ca^{2+} -free solutions
Cells were exposed to the fluorescent dye Lucifer Yellow (2.5 % for 5 minutes) in Ca^{2+} -free solution containing 2 mM EGTA. (A). Uptake of Lucifer Yellow in control condition in BCEC cultured for 8 to 14 days. (B). Uptake of Lucifer Yellow in control condition in BCEC cultured for 21 to 30 days.

187x81mm (150 x 150 DPI)

Table 1. Cell area of bovine corneal endothelial cells on different days after cell isolation.

# days	Endothelial cells				
	Area $\pm$ SEM (μm^2)	# Cells (n)	# Eyes	# Experiments (N)	
8	711 $\pm$ 8	3349	3	40	500
10	730 $\pm$ 3	9640	4	100	
11	789 $\pm$ 4	8985	3	90	
12	910 $\pm$ 6	6939	3	90	
13	920 $\pm$ 6	7115	3	90	
14	926 $\pm$ 7	6021	4	90	
15	962 $\pm$ 7	5771	3	130	500
16	1019 $\pm$ 8	4456	8	100	
19	1140 $\pm$ 12	5005	4	120	
20	1312 $\pm$ 9	6276	4	150	
21	1375 $\pm$ 14	4584	5	70	500
22	1400 $\pm$ 24	967	2	20	
23	1547 $\pm$ 15	7387	9	130	
24	1681 $\pm$ 18	4995	6	140	
25	1746 $\pm$ 28	1470	3	30	
26	1753 $\pm$ 29	1536	3	30	
27	2156 $\pm$ 49	798	5	20	
28	2350 $\pm$ 54	1110	4	40	
30	2517 $\pm$ 67	581	3	20	

Table 2. Average maximum Normalized Fluorescence (NF), Percentage Responsive cells (%RC), Delay and Active Area (AA) in the MS and NB layers during mechanical stimulation in control conditions in BCEC cultured for 8 to 14 days and for 21 to 30 days.

	MS	NB1	NB2	NB3	NB4	NB5	AA (μm^2)
Control: BCEC cultured for 8 to 14 days							
NF ± SEM	2.70 ± 0.08	2.90 ± 0.04	2.50 ± 0.03	2.10 ± 0.02	1.80 ± 0.05	1.60 ± 0.06	54,600 ± 1,000
% RC	100	99	94	77	51	40	
Delay ± SEM (s)	0.00 ± 0.00	0.90 ± 0.04	3.50 ± 0.07	6.3 ± 0.1	8.9 ± 0.2	12.8 ± 0.5	
n	175	1154	2158	2660	2096	1264	484
Control: BCEC cultured for 21 to 30 days							
NF ± SEM	2.50 ± 0.06	2.40 ± 0.03 [^]	2.10 ± 0.02 [^]	1.80 ± 0.02 [^]	1.70 ± 0.03	0.00 ± 0.00 [^]	43,300 ± 1,800*
% RC	100	89	68	44	26	0	
Delay ± SEM (s)	0.00 ± 0.00	1.6 ± 0.1 [^]	4.5 ± 0.2 [^]	7.5 ± 0.3 [^]	9.9 ± 0.5 [^]	0.00 ± 0.00 [^]	
n	118	672	1015	817	380	0	260

Data were collected during mechanical stimulation in control conditions.
[^] P < 0.05 control in BCEC cultured for 8 to 14 days vs control in BCEC cultured for 21 to 30 days.