Toxic Effects of Recombinant Tissue Plasminogen Activator on Cultured Human Corneal Endothelial Cells

Efidal Yoeruek,1 Martin S. Spitzer,1 Olcay Tatar,1 Tilo Biedermann,2 Salvatore Grisanti,1 Matthias Lüke,1 Karl U. Bartz-Schmidt,1 and Peter Szurman1

PURPOSE. Human corneal endothelial cells (HCECs) are nonmitotic cells. Any intracameral application of a drug requires evaluation of the potential apoptotic and toxic effects. Intracameral recombinant tissue plasminogen activator (rtPA) is successfully used for the treatment of severe and prolonged postoperative fibrin reaction. This study was undertaken to investigate the toxicity of rtPA on cultured HCECs to determine its safety for clinical use.

METHODS. Cell cultures of HCECs were harvested from human donor eyes and exposed to various concentrations of rtPA (10–200 μg/mL). For cytotoxicity testing, the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and the live/dead viability/cytotoxicity assay were performed. Annexin V binding combined with propidium iodide (PI) co-staining was used for the distinction of viable, early, and late apoptotic cells. Odds ratios (ORs) and confidence intervals (CIs) were calculated for 50 μg/mL and 100 μg/mL, and 200 μg/mL. Cell morphology was studied after 24 hours of exposure to rtPA to identify cellular damage. Immunolocalization of zonula occludens 1 (ZO1) was performed to analyze intercellular barrier disturbance in the presence of rtPA.

RESULTS. Reduction of mitochondrial dehydrogenase activity after rtPA exposure was dose dependent and suggested comparable toxicity with the data obtained from the live/dead assay. The mean number of Annexin V+ cells was not significantly increased at concentrations of 50 μg/mL and 100 μg/mL. At 200 μg/mL, however, the ORs were 5.082 ± 0.213 (95% CI, 3.962–6.203; P < 0.001) for apoptosis and 6.154 ± 0.196 (95% CI, 5.123–7.181; P < 0.001) for necrosis. In addition, increasing concentrations of rtPA resulted in a fading immunopositive staining for ZO1.

CONCLUSIONS. These data suggest a dose-dependent toxic effect of rtPA on HCECs in vitro. Although intracameral rtPA concentrations up to 100 μg/mL seem to be clinically safe, use of concentrations higher than 125 μg/mL might induce irreversible cell death and should be restricted to selected cases.

Isolation of Human Corneal Cells and Culture Conditions

Human corneoscleral rims were obtained through the Tuebingen Eye Bank from six donors. Small explants from the endothelial layer, including Descemet membrane, were removed with sterile surgical forceps under a stereoscopic dissection microscope, trypsinized for 10 minutes, and seeded in 24-well plates. HCECs were maintained in Dulbecco modified Eagle medium (DMEM)/F12 containing 5% fetal calf serum (FCS), 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, 1.25 g/mL amphotericin B, 0.1 ng/mL epidermal growth factor (EGF), 1.0 ng/mL basic fibroblast growth factor (bFGF), and 1.0 μg/mL hydrocortisone in 5% carbon dioxide humidified environment. For all experiments, passages 1 to 3 were used.

MTT Stationary Toxicity Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed in serum-free medium to investigate the cytotoxicity of rtPA on HCECs. HCECs (5–8 × 104 in 100 μL/well) were grown in 96-well plates for 48 hours and treated with serially diluted rtPA in DMEM/F12 over a range of concentrations (10–200 μg/mL). After 24 hours, HCECs were washed, and the extent of cell growth was assessed using MTT assay (CellTiter-96 Non-Radioactive Cell Proliferation Assay; Promega Corporation, Madison, WI). A volume of 20 μL MTT was added to each well.
added to each well and mixed. Plates were incubated for 2 hours at 37°C in a humidified, 5% CO₂ atmosphere, after which 100 μL solubilization/stop solution were added to each well. Formazan levels, corresponding to the cellular mitochondrial dehydrogenase activity, were measured using a microplate reader (SLT Spectra 400 ATX, Salzburg, Austria) at a wavelength of 570 nm and a correction of interference at 690 nm.

**Live/Dead Viability/Cytotoxicity Kit**

To assess the cytotoxicity of rtPA on HCECs under nonstarving conditions, cell viability was investigated using a live/dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Staining was performed according to the manufacturer’s instructions. Adequate negative (cells without rtPA) and positive controls (cells treated with 0.3% Triton X-100 detergent; Serva, Heidelberg, Germany) for cell death were run with each set of experiments. Cell viability was analyzed by fluorescence microscopy after 24 hours of incubation. Green and red cells were counted per eight fields at 200-fold magnification. The percentage of cells with green fluorescence (interpreted as viable cells) was then calculated.

**Morphologic Changes in Corneal Endothelial Cells after Exposure to rtPA**

Cell morphology was assessed with a phase-contrast microscope 24 hours after incubation with respective concentrations of rtPA. Signs of cellular damage were sought, such as pleomorphism, disruption of the intercellular junctional complexes, prominent nuclei, shrunk cytosol, cytoplasmic vacuolization, nuclear swelling, rupture of nuclear and plasma membranes, or nuclear fragmentation in the rtPA-added cells and were compared with those in the control group.

**Flow Cytometric Assay for Apoptotic/Necrotic Cell Death**

Cellular responses after exposure to rtPA were investigated to identify “early” apoptotic cells and to discriminate from necrotic/late apoptotic and vital cells. For simultaneous detection of apoptotic and necrotic cell death, a costaining technique with fluorochrome-conjugated Annexin V (Merck Biosciences, Bad Soden, Germany), in tandem with the DNA-binding dye propidium iodide (PI), was used according to the method of Vermes et al. 17 Externalization of phosphatidylserine occurs in the earlier stages of apoptosis, and Annexin V-FITC staining precedes the loss of membrane integrity that accompanies the latest stages of cell death (PI labeled), thus permitting the discrimination of early apoptotic cells from necrotic/late apoptotic cells. 18 Briefly, HCECs were grown to confluence and incubated for 24 hours in 24-well plates using the same conditions as in the previous sets of experiments. After centrifugation and washing in cold PBS, HCECs for Annexin V-FITC and PI staining were resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, pH 7.4) at a concentration of 106 cells/mL. Five hundred microliters, containing 5 × 10⁵ cells, was transferred to a culture tube, and 1.25 μL FITC-conjugated Annexin V was added. After centrifugation at 1000 rpm for 5 minutes and removal of the supernatant, cells were gently resuspended in 500 μL cold binding buffer, and 10 μL PI was added. Positive controls were provided for both apoptotic and necrotic (10% ethanol) cell death. For simultaneous scoring of the differential cellular response, aliquots of 10⁵ cells each were immediately processed for fluorescence-activated cell sorting (FACS) on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). Excitation parameters were set at λex = 488 nm, and fluorescence emission was detected at λem = 518 nm for Annexin V-FITC and λem = 620 nm for PI. Data analysis was performed with appropriate software (CellQuest; BD Biosciences, Mountain View, CA).

**Immunohistochemistry**

After 24 hours of exposure to different concentrations of rtPA, the HCECs were fixed in 4% paraformaldehyde and 3% sucrose in PBS (pH 7.4) for 15 minutes at room temperature. As the primary antibody, rabbit polyclonal anti-ZO1 (1:100 dilution; Zymed Laboratories, South San Francisco, CA) was used. Cells were incubated with the antibody overnight at 4°C and washed three times in PBS. Monolayers were then incubated for 1 hour at room temperature with the secondary antibody alkaline phosphatase/RED, rabbit/mouse (Dako, Glostrup, Denmark). Slides were examined under a microscope (Axiovert 135; Zeiss, Oberkochen, Germany).

**Statistical Analysis**

All data were expressed as the mean ± SEM. For each rtPA concentration, mean values of the mean absorbance rates from eight wells have been calculated. These measurements were repeated four times. Flow cytometric data were plotted as mean number of the events of PI, Annexin V-positive cells in each quadrant, and the odds ratios (ORs) and 95% confidence intervals (CIs) were calculated in comparison with the control group. Student’s t-test was used to compare mean values from two groups, and P < 0.05 was considered statistically significant (marked with an asterisk). All analyses were performed with commercial software (SPSS version 12.0; SPSS, Inc., Chicago, IL).

**RESULTS**

**MTT Stationary Toxicity Assay and Live/Dead Viability/Cytotoxicity Kit**

A stationary confluent cell culture is better suited than a proliferating culture to detect a toxic drug effect, and it is more comparable to the natural state of the corneal endothelial cells in vivo. 19, 20 No cytotoxicity under starving conditions was observed up to a concentration of 125 μg/mL (P = 0.064). Only a moderate, insignificant decrease of cell number and an increase of dead cells were noted. However, at concentrations of 150 μg/mL (P < 0.001) or higher, rtPA caused a significant toxic effect on HCECs. At 125 μg/mL, the mean absorbance rate was 0.243 ± 0.014, which meant a reduction of the number of viable cells of 12.63% in comparison with the control; this represented an insignificant decrease. At 150 μg/mL, the mean absorbance rate was 0.195 ± 0.011 in comparison with the control, which presented an insignificant decrease. At 150 μg/mL, the mean absorbance rate was 0.278 ± 0.09, which meant a decrease of 29.86% (Fig. 1). To assess the viability of the HCECs in the presence of rtPA, intracellular esterase activity was used to identify the living cells. Alterations of cell membrane permeability were synchronized with loss of esterase activity. Percentages of cells with esterase activity (viable cells, green fluorescence) were as follows: 25 μg/mL, 98.40% ± 0.54%; 50 μg/mL, 95.90% ± 0.97%; 75 μg/mL, 94.30% ± 0.64%; and 150 μg/mL, 90.10% ± 0.72% (P < 0.001).
125 μg/mL, 86.80% ± 1.22%; 200 μg/mL, 25.50% ± 1.57%. The control group (without rtPA) showed 98.50% ± 0.67% viable cells. There was also no significant difference between the control group and 125 μg/mL (P = 0.064). With higher concentrations, however, rtPA showed a dose-dependent toxic effect with an increasing amount of dead cells (red fluorescence). At 200 μg/mL, the percentage of viable cells decreased by 73.9% in comparison with the control (P < 0.001). This dose-dependent reduction of esterase activity was in accordance with the results of the MTT-cytotoxicity test (Fig. 2).

Morphologic Changes in Human Corneal Endothelial Cells after Exposure to rtPA

Morphologic characteristics of HCECs in the presence of different rtPA concentrations changed in a dose-dependent manner. In the absence of rtPA, the HCEC monolayer consisted of small, polygonal cells. With increasing concentrations of rtPA, typical signs of cellular damage, such as pleomorphism, prominent nuclei, shrunken cytosol, and disruption of the intercellular junctional complexes, were observed (Fig. 3). Beginning at a concentration of 125 μg/mL, nuclear enlargement could be observed.

Apoptotic and Necrotic Effects of rtPA on HCECs

In an additional set of flow cytometry-based experiments, the mean number of cells that were actively undergoing apoptosis during rtPA exposure was determined to investigate the sensitivity of HCECs to rtPA-induced apoptosis. The number of Annexin V-FITC and PI-positive cells was not significantly increased at concentrations of 50 μg/mL and 100 μg/mL. ORs were 1.063 ± 0.018 (95% CI, 0.952–1.181; P = 0.192) and 1.124 ± 0.013 (95% CI, 1.021–1.213; P = 0.069) for apoptosis and 1.072 ± 0.017 (95% CI, 0.972–1.171; P = 0.214) and 1.114 ± 0.013 (95% CI, 1.034–1.202; P = 0.072) for necrosis at 50 and 100 μg/mL, respectively. With higher concentrations of rtPA, the number of Annexin V- and PI-positive cells increased, indicating an activation of the proapoptotic pathway with consecutive apoptotic cell death. After exposure to 200 μg/mL rtPA, however, the late apoptotic/necrotic cells predominated with higher ORs of 6.154 ± 0.196 (95% CI, 5.123–6.246) at a concentration of 200 μg/mL.
after exposure to 200 μg/mL rtPA, ZO1 immunopositive areas were only occasionally detected, indicating the loss of tight junctions. Scale bars, 200 μm.

**DISCUSSION**

The corneal endothelium plays a crucial role in maintaining corneal turgescence. Regarded as nonproliferative in vivo, HCECs decrease in number with age because of their limited regenerative capacity. If the endothelial cells are seriously damaged, for example, by Fuchs endothelial dystrophy, after penetrating keratoplasty, glaucoma surgery, or cataract surgery, decompensation of HCECs may occur and the cornea may become irreversibly edematous and opaque. Any physical or pharmacologic insult to this sensitive monolayer bears the potential risk of corneal decompensation.19,20

Many studies have shown that apoptosis is involved in this cell loss process. The reasons for triggering the apoptotic program in the human corneal endothelium are not well understood, but metabolic changes in the medium, mechanical stress, endotoxins, loss of survival factors, and nutrient deprivation may be involved.21 In this context, calcium ions especially seem to play an important role because one characteristic property of apoptosis is that it is associated with increased intracellular calcium levels and with various changed ion channel activities, such as L-type Ca2+ channels.22–24 Therefore, for analysis of the toxicity of a drug on corneal endothelial cells, the induction of apoptosis is crucial and thus should be evaluated. In the long term, the induction of apoptosis could lead to profound cell loss, especially after repetitive use of a drug. Since the first reports of successful fibrinolysis by intracameral rtPA injection, this effective and widespread treatment has been under debate for suspected cytotoxicity. Thus a safety evaluation is essential so that the substance may be used clinically with confidence.

Our results show a dose-dependent toxic effect of rtPA on HCECs, with significant cell death at concentrations higher than 125 μg/mL. The dose-dependent reduction of the esterase activity was confirmed by the results of the MTT-cytotoxicity test. An induction of the apoptotic cascade seems to be involved in rtPA cytotoxicity. Progressive disruption of typical monolayer architecture was accompanied by a rapid loss of tight junctions in a dose-dependent manner. Lower concentrations seem to be safe in view of the absence of direct cytotoxicity and the lack of induction of apoptosis. In general, the stationary confluent cell culture better represents the nonmitotic HCECs.

Previous cell culture experiments comparable to our study have not been reported, but clinical studies and animal models suggest the safe use of rtPA with regard to the corneal endothelium at concentrations of 100 μg/mL.15,16 However, the recommended concentrations for intracameral use vary widely in the literature, and the maximum safe concentration has not yet been determined. Our data are in accordance with those of several other clinical studies and ex vivo investigations. McDermott et al.15 performed corneal endothelial perfusion for 3 hours on paired human corneas with tissue plasminogen activator reconstituted at a concentration of 100 μg/mL. They observed no significant difference in the swelling rates between corneas perfused with plasminogen tissue activator or with balanced salt solution alone. They concluded that tissue plasminogen activator did not affect endothelial ultrastructure or function.15 A clinical study also reported rtPA to be safe for human use. Slit lamp examinations and endothelial cell counts revealed no visible toxic effects. Only clinical complications
such as recurrent fibrin, anterior chamber hemorrhage, and increased intraocular pressure were seen, and these were managed medically. The authors reported the results of slit lamp investigations, measurements of intraocular pressure, and corneal endothelial cell density, size, and morphology after the use of 25 μg rtPA (resulting in an anterior chamber concentration of 100 μg/mL).

Even lower rtPA concentrations might cause inadvertent effects, such as in a pathologic endothelial situation in which a lower rtPA concentration might be toxic enough to compromise the barrier function between endothelial cells and to induce paracellular fluid flow. Spitzer et al. reported the rapid onset of band keratopathy following intracameral injection of rtPA after penetrating keratoplasty. This shows that the pathologic endothelium may be more prone to rtPA-induced toxicity than the healthy endothelium.

It should be kept in mind that several previous reports suggest a lower dose of rtPA in the anterior chamber might also be effective. Ozveren et al. performed an intracameral application 3 μg, resulting in a final concentration of 15 μg/mL in the anterior chamber. Klais et al. described successful fibrinolysis after 10 μg/mL. Stark et al. achieved successful fibrinolysis with 4 to 6 μg/mL rtPA.

Comparable with our results, dose-dependent retinal toxicity of intravitreal rtPA has been described in rabbit and cat eyes with a dose equal to or greater than 50 μg/mL. Reduced scotopic and photopic ERG A- and B-waves have been reported in human eyes after two intravitreal injections (50 μg/mL each) of rtPA. In fact, most authors agree that intravitreal application of rtPA should be restricted to concentrations of 50 to 100 μg/mL to avoid toxic effects on the human retina. However, the concentrations for intravitreal or intracameral use are not easily comparable because the cornea and retina differ in function in the human eye. Moreover, the respective cells are of different origin and have different susceptibility to drugs.

To our knowledge, this is the first laboratory study that evaluates the toxic and apoptotic effects of rtPA on corneal endothelial cells of human origin. Our experimental series not only conveys new information about a safe dosage but also demonstrates for the first time apoptosis induction after exposure to rtPA. However, one has to bear in mind that cell culture experiments cannot be directly transferred to the in vivo situation. Through several passages, endothelial cells can lose their phenotypic properties with potentially lower sensitivity to toxic agents. Donor age and endothelial cell status are important for the phenotypic properties in a cell culture model. Another limitation of our cell culture study was that in vivo, in the anterior chamber of the eye, multiple mechanisms exist that protect the endothelium from damage. Growth factors, therefore, are important. Examples include epithelial growth factor, bFGF, and platelet-derived growth factor, all of which are present in the anterior chamber and are known to affect L-type Ca2+ channel activity in human endothelial cells and which can also protect the corneal endothelium. Thus, the “real” toxic dose is not evaluable with in vitro experiments, but it can nevertheless give important results for orientation.

In summary, our investigations demonstrate the dose-dependent toxicity of rtPA on cultured HCECs. Increasing concentrations of rtPA result in an accelerated loss of cell viability. In conclusion, we suggest the use of rtPA at concentrations less than 125 μg/mL in postoperative fibrin reaction in the anterior chamber in the clinical routine. Higher concentrations should be used with caution, especially if the endothelium is compromised. Further studies are required to elucidate the precise mechanisms leading to cell death of HCECs and intercellular tight junction breakdown after rtPA use.

References


