# Role of Insulin in Regulation of $Na^+$-/$K^+$-Dependent ATPase Activity and Pump Function in Corneal Endothelial Cells

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**Purpose.** The $Na^+$-/$K^+$-dependent ATPase (Na,K-ATPase) expressed in the basolateral membrane of corneal endothelial cells plays an important role in the pump function of the corneal endothelium. The role of insulin in the regulation of Na,K-ATPase activity and pump function in corneal endothelial cells was investigated.

**Methods.** Confluent monolayers of mouse corneal endothelial cells were exposed to insulin. ATPase activity was evaluated by spectrophotometric measurement of phosphate released from ATP with the use of ammonium molybdate; Na,K-ATPase activity was defined as the portion of total ATPase activity sensitive to ouabain. Pump function was measured with the use of a Ussing chamber; pump function attributable to Na,K-ATPase activity was defined as the portion of the total short-circuit current sensitive to ouabain. Western blot analysis and immunocytochemistry were performed to measure the expression of the Na,K-ATPase $\alpha_1$-subunit.

**Results.** Insulin increased the Na,K-ATPase activity and pump function of cultured corneal endothelial cells. These effects were blocked by protein kinase C (PKC) inhibitors and protein phosphatases 1 and 2A inhibitor. Western blot analysis indicated that insulin decreased the ratio of the inactive Na,K-ATPase $\alpha_1$-subunit. Immunocytochemistry indicated that insulin increased the cell surface expression of the Na,K-ATPase $\alpha_1$-subunit.

**Conclusions.** These results suggest that insulin increases the Na,K-ATPase activity and pump function of cultured corneal endothelial cells. The effect of insulin is mediated by PKC and presumably results in the activation of PP1, 2A, or both, which are essential for activating Na,K-ATPase by $\alpha_1$-subunit dephosphorylation. (*Invest Ophthalmol Vis Sci.* 2010;51: 3935–3942) DOI:10.1167/iovs.09-4027

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Clinical observations of a higher incidence of persistent corneal edema after vitrectomy and other surgical procedures for patients with diabetes mellitus have suggested that there is abnormal corneal endothelial function associated with diabetes mellitus.[1–6] Specular microscopic studies have shown morphologic abnormalities such as less endothelial cell density and increased endothelial pleomorphism in patients with type 1 and type 2 diabetes mellitus.[6–15] Some clinical studies have shown that patients with diabetes tend to have slightly thicker corneas and reduced recovery rates from hypoxia-induced corneal edema.[16–19]

The $Na^+$-/$K^+$-dependent ATPase (Na,K-ATPase), expressed in the basolateral membrane of corneal endothelial cells, is primarily responsible for the pump function of the corneal endothelium.[20] Herse and Adams[21,22] have shown that functional abnormalities, such as increased corneal thickness and decreased ability to recover from corneal edema in alloxan-induced diabetic rabbits, are associated with decreased Na,K-ATPase activity in the corneal endothelium. Whikehart et al.[23] have reported that elevated glucose levels reduce Na,K-ATPase activity in cultured bovine corneal endothelial cells. McNara et al.[24] have shown that acute hyperglycemia affects corneal hydration control in humans. These results suggest that a high glucose level itself is responsible for reduced Na,K-ATPase activity.

However, there might be another mechanism of reduced Na,K-ATPase activity in the corneal endothelia of persons with diabetes. It is well known that a lack of insulin plays a principal role in the pathogenesis of type 1 diabetes mellitus and that the intracellular insulin signal is reduced because of insulin resistance in type 2 diabetes mellitus.[25,26] Several studies have shown that insulin directly enhances Na,K-ATPase activity in skeletal muscle, liver, kidney, adipocytes, lymphocytes, avian salt glands, and many other cells and organs.[27–37] Insulin is present in the aqueous humor of rabbits at a concentration of approximately 3% of that in plasma, and the aqueous humor insulin concentration of alloxan-induced diabetic rabbits after feeding is lower than that of normal control animals.[38,39] Anderson and Fischberg[40] have reported that insulin has a significant effect on transendothelial fluid transport in rabbit cornea. Therefore, we hypothesized that a lack of insulin or a reduced level of intracellular insulin signaling may have a direct effect on the Na,K-ATPase activity of the corneal endothelial cells. Insulin and insulin-like growth factor-I (IGF-I) have been reported to stimulate DNA synthesis and cell proliferation in corneal endothelium by insulin receptor or IGF-I receptor,[41,42] whereas the effect of insulin on the Na,K-ATPase activity of corneal endothelial cells remains unknown.

Insulin and several other hormonal agents activate signaling pathways, including those mediated by protein kinases such as protein kinase C (PKC). A direct effect of PKC on Na,K-ATPase activity has been demonstrated in various tissues.[27,43] To date,

PKC is regarded to trigger the rapid action of insulin on the Na,K-ATPase and to be involved in the stimulation of the Na,K-ATPase by insulin in muscle cells.[27]

To investigate the role of insulin in the control of the Na,K-ATPase in corneal endothelial cells, we examined the effects of insulin activation on the enzymatic activity and pump function of Na,K-ATPase in cultured mouse corneal endothelial cells. We also examined whether PKC and its related enzymes, protein phosphatase 1 and 2A, might mediate the insulin activation of Na,K-ATPase.

## METHODS

### Chemicals

Ammonium molybdate reagent (Biomol Green) and phosphate standards were obtained from Biomol Research Laboratories (Plymouth, PA). Mammalian protein extraction reagent (M-PER) and BCA protein assay kit were obtained from Pierce Biotechnology (Rockford, IL). Anti-Na,K-ATPase $\alpha_1$ antibody was obtained from Cosmo Bio (Tokyo, Japan). Anti-phospho-Na,K-ATPase $\alpha_1$ (Ser18) antibody was obtained from Cell Signaling (Danvers, MA). Anti-$\beta$-actin antibody (AC-15) was obtained from Abcam (Cambridge, MA). ABC rabbit IgG kit (Vectastain Elite) was obtained from Funakoshi (Tokyo, Japan). Western blot analysis detection reagent (ECL Plus) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Reagent (ProLong Gold Antifade) with DAPI and signal enhancer (Image iT) were obtained from Life Technologies Japan (Tokyo, Japan). Insulin, ouabain, staurosporine, okadaic acid, and other chemicals were obtained from Sigma (St. Louis, MO). Water-insoluble compounds were dissolved in a minimal volume of methanol or dimethyl sulfoxide, with equal amounts of these solvents added to control solutions; the final concentration of methanol or dimethyl sulfoxide in incubations was <0.3% and was found to have no effect on adenosine triphosphate (ATP) hydrolysis or short-circuit current (data not shown).

### Cell Culture

A simian virus 40 (SV40)-transformed mouse corneal endothelial cell line (C3H derived) was the kind contribution of the late J. Wayne Streilein (Harvard Medical School). The cells were cultured under a humidified atmosphere of 5% $CO_2$ at 37°C in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 $\mu$g/mL). The cells were passaged at a split ratio of 1:2 to 1:4, at which they become confluent in 4 to 7 days.

Experiments examining PKC activity or Na,K-ATPase activity were performed with cells cultured in 24-well culture plates (Nunc, Roskilde, Denmark). Ussing chamber experiments were performed with confluent monolayers of cells cultured on six-well polycarbonate inserts (Snapwell; Corning, Acton, MA) with a membrane pore size of 0.4 $\mu$m. The insert membrane growth area was 4.67 $cm^2$. All experiments were performed with cell monolayers within 1 day of the cells reaching confluence and with the cells maintained in the culture incubator at 37°C.

### Measurement of Na,K-ATPase Activity

The culture medium was removed from cell monolayers, ultrapure distilled water (150 $\mu$L) was added to each well, and the culture plate was then placed in liquid nitrogen for 10 seconds before the addition to each well at room temperature of 150 $\mu$L solution containing 80 mM histidine, 20 mM KCl, 6 mM $MgCl_2$, 2 mM EGTA, alamethicin (2 $\mu$g/mL), 30 $\mu$M digitonin, and 200 mM NaCl at pH 7.4.[44] To duplicate wells, 10 $\mu$L of 30 mM ouabain (final concentration, 1 mM) or vehicle were added, and the plate was incubated for 30 minutes at 37°C. After the further addition of 10 $\mu$L of 300 mM ATP (final concentration, 10 mM), the reaction mixtures were incubated for an additional 30 minutes at 37°C. The ATP hydrolysis reaction was terminated by the addition of 75 $\mu$L of 50% trichloroacetic acid to each well. The contents of each well were then centrifuged at 3000 rpm for 10 minutes at room temperature.

The resultant supernatants were diluted 50-fold with ultrapure distilled water, and portions (50 $\mu$L) of the diluted samples were added to tubes containing 100 $\mu$L ammonium molybdate reagent (Biomol Green; Biomol Research Laboratories) for determination of phosphate content by measurement of absorbance at 640 nm. Phosphate solutions of 0 to 40 $\mu$M were used as standards. The Na,K-ATPase activity was calculated as the difference in ATPase activity between cells exposed to ouabain and those not exposed and was expressed as millimoles of ATP hydrolyzed per milligram of protein per hour. The protein concentration of the reaction mixtures was determined by the method of Lowry et al.,[45] with bovine serum albumin as the standard.

### Measurement of Pump Function

The pump function of confluent monolayers of corneal endothelial cells was measured with the use of a Ussing chamber basically as described previously.[46,47] The cells cultured on inserts (Snapwell; Corning) were placed in a Ussing chamber (EM-CSYS-2; Physiologic Instruments, San Diego, CA). The endothelial cell surface side was in contact with one chamber, and the insert membrane side was in contact with another chamber. The chambers were carefully filled with Krebs-Ringer bicarbonate (120.7 mM NaCl, 24 mM $NaHCO_3$, 4.6 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM $Na_2HPO_4$, 1.5 mM $NaH_2PO_4$, and 10 mM glucose bubbled with a mixture of 5% $CO_2$, 7% $O_2$, and 88% $N_2$ to pH 7.4). The chambers were maintained at 37°C by an attached heater. The short-circuit current was sensed by narrow polyethylene tubes positioned close to either side of the insert, filled with 3 M KCl and 4% agar gel, and connected to silver electrodes. These electrodes were connected to a computer through a Ussing system (VCC-MC2; Physiologic Instruments) and research grade recorder (iWorx 118; iWorx Systems, Dover, NH), and the short-circuit current was recorded (Labscribe 2 Software for Research; iWorx Systems). After the short-circuit current had reached steady state, ouabain (final concentration, 1 mM) was added to the chamber, and the short-circuit current was measured again. The pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit current measured before and after the addition of ouabain.

### Measurement of PKC Activity

After removal of the culture medium, cells were washed with phosphate-buffered saline and lysed in 1 mL solution containing 20 mM MOPS (pH 7.4), 50 mM $\beta$-glycerophosphate, 50 mM NaF, 1 mM sodium vanadate, 5 mM EGTA, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 $\mu$g/mL), and aprotinin (10 $\mu$g/mL). The lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C, and the resultant supernatants were assayed for PKC kinase activity with the use of a PKC kinase activity assay kit (Assay Designs, Ann Arbor, MI) based on a solid-phase enzyme-linked immunosorbent assay that recognizes the phosphorylated form of the substrate. PKC activity was expressed as nanograms of phosphorylated substrate formed per milligram of protein. The protein concentration of lysate supernatants was assayed by the method of Lowry et al.,[45] with bovine serum albumin as the standard.

### Western Blot Analysis of Na,K-ATPase $\alpha_1$-Subunit

The culture medium was removed from cell monolayers and then lysed with M-PER. The supernatant was collected, and total protein in each sample was measured with the use of a protein assay reagent (Advanced; Cytoskeleton, Inc., Denver, CO) BCA protein assay kit. Approximately 1 $\mu$g of each sample was separated on a 7.5% polyacrylamide gel containing sodium dodecyl sulfate and then was transferred to polyvinylidene membranes. After blocking with 1% normal goat or bovine serum and 0.1% Tween-20 in TBS, the membrane was incubated with anti-Na,K-ATPase $\alpha_1$ antibody (1:5000 dilution with TBS), anti-phospho-Na,K-ATPase $\alpha_1$ (Ser18) antibody (1:2000 dilution with TBS), or anti $\beta$-actin (AC-15) antibody (1:2000 dilution with TBS)

overnight at 4°C. Incubation with biotinylated secondary antibodies was followed with the use of elite reagent (Vectastain ABC; Vector Laboratories) for 30 minutes at room temperature. Positive immunoreactions were made visible by an enhanced chemiluminescence Western blot analysis reagent detection system (ECL Plus; Amersham Pharmacia Biotech). A densitometer (ChemiDoc XRS; Bio-Rad, Hercules, CA) was used for quantization of band intensities.

### Immunocytochemistry

The Na,K-ATPase $\alpha_1$-subunit was studied by indirect immunocytochemistry. Corneal endothelial cells cultured on four-well chamber slides were fixed at room temperature for 15 minutes in 4% formaldehyde in PBS. After three PBS washes, the specimens were incubated for 30 minutes in signal enhancer (Image iT; Life Technologies Japan). After two PBS washes, the specimens were incubated for 30 minutes in 10% normal goat serum to block nonspecific binding. This was followed by overnight incubation at 4°C with 1:500-diluted rabbit anti-Na,K-ATPase $\alpha_1$ antibody (final concentration, 2 $\mu$g/mL) and three washes in PBS. Corneal endothelial cells were then incubated for 1 hour in a 1:500 dilution of FITC-conjugated goat anti-rabbit IgG antibody and again washed three times in the dark. Specimens were mounted on glass slides with anti-fading mounting medium containing 4′,6-diamino-2-phenylindole (ProLong Gold Antifade Reagent with DAPI; Life Technologies Japan), and the slides were inspected with a confocal microscope (Radiance 2100; Bio-Rad).

### Statistical Analysis

Data are presented as mean ± SD and were compared by Student's *t*-test with the use of spreadsheet software (Excel 2003; Microsoft, Redmond, WA). $P < 0.05$ was considered statistically significant.

## Results

### Effect of Insulin on Na,K-ATPase Activity

To determine whether insulin affects Na,K-ATPase activity in corneal endothelial cells, we exposed the cells to 0.1 $\mu$M insulin for various times, and ouabain-sensitive ATP hydrolysis was measured. Insulin had a transient, stimulatory effect on Na,K-ATPase activity that was significant at 6 hours and 12 hours; after that, Na,K-ATPase activity returned to the baseline (Fig. 1A). The stimulatory effect of insulin on Na,K-ATPase activity was also concentration dependent and was apparent at 0.01 to 10 $\mu$M (Fig. 1B).

### Effect of Insulin on Pump Function

We next examined whether insulin affects the pump function of corneal endothelial cells. Tracings of short-circuit current obtained with a Ussing chamber revealed that insulin at 0.1 $\mu$M increased the ouabain-sensitive pump function of the cells compared with that observed for control cells (Fig. 2A). This effect of insulin was statistically significant at 6 hours, similar to the results obtained from Na,K-ATPase activity measurements (Fig. 2B). The stimulatory effect of insulin on pump function was concentration dependent and was apparent at 0.01 to 10 $\mu$M (Fig. 2C).

### Activation of PKC by Insulin in Corneal Endothelial Cells

To examine whether insulin activates PKC in cultured mouse corneal endothelial cells, we measured PKC activity in cell extracts after treatment of the cells with various concentrations of insulin for 30 minutes Insulin indeed increased PKC activity in a concentration-dependent manner; this effect was significant at concentrations of 0.1 to 10 $\mu$M (Fig. 3).

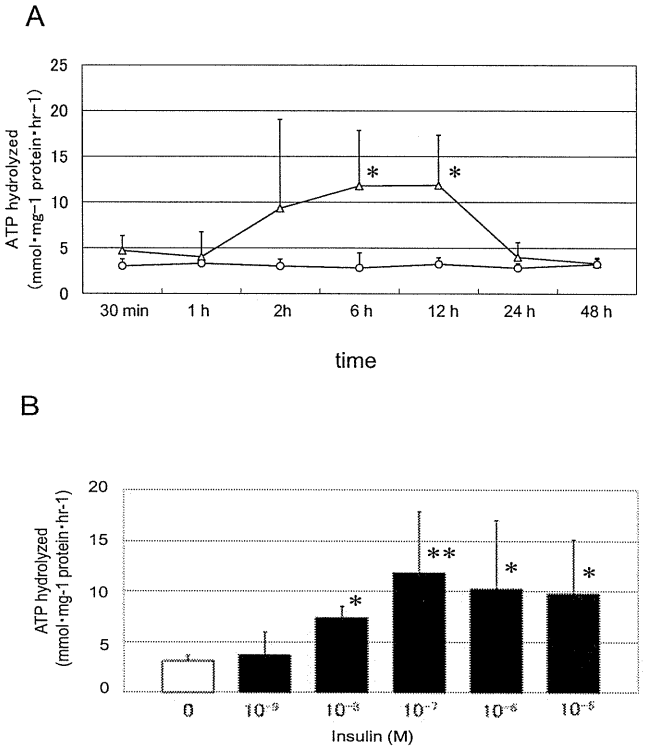


**FIGURE 1.** Effect of insulin on Na,K-ATPase activity in cultured mouse corneal endothelial cells. (**A**) Cells were incubated in the absence (*open circles*) or presence (*open triangles*) of 0.1 $\mu$M insulin for the indicated times and then assayed for Na,K-ATPase activity. Data are mean ± SD of values of four replicates from a representative experiment. $^*P < 0.05$ compared with the corresponding value for cells incubated without insulin (Student's *t*-test). (**B**) Cells were incubated with the indicated concentrations of insulin for 6 hours and then assayed for Na,K-ATPase activity. Data are mean ± SD of values of four replicates from four representative experiments. $^*P < 0.05$, $^{**}P < 0.01$ for the indicated comparisons (Student's *t*-test).

### Effect of Insulin on Na,K-ATPase $\alpha_1$-Subunit Phosphorylation

To determine whether insulin affects Na,K-ATPase expression in corneal endothelial cells, we exposed the cells to 0.1 $\mu$M insulin for 6 hours and then measured expression of the total Na,K-ATPase $\alpha_1$-subunit and the phospho-Na,K-ATPase $\alpha_1$-subunit by Western blot analysis (Fig. 4A). We used 0.1 $\mu$M phorbol 12,13-dibutyrate (PDBu), which is an established specific and strong activator for PKC and immediately phosphorylates Ser18 of Na,K-ATPase $\alpha_1$-subunit, for 30 minutes as a positive control to confirm that the phospho-Ser18 antibody works. The phosphorylation of Na,K-ATPase $\alpha_1$-subunit by PDBu was decreased at 6 hours. Ser18 phosphorylation triggers the endocytosis of the Na,K-ATPase $\alpha_1$-subunit and results in the inhibition of Na,K-ATPase activity.[48,49] Thus, the phospho-Na,K-ATPase $\alpha_1$-subunit is considered to be the inactive state of the Na,K-ATPase $\alpha_1$-subunit. Expression of the Na,K-ATPase $\alpha_1$-subunit and phospho-Na,K-ATPase $\alpha_1$-subunit were measured as the ratio of the signal intensity to $\beta$-actin. Although there were no statistically significant differences in the expression of total Na,K-ATPase $\alpha_1$-subunit (Fig. 4B), insulin significantly decreased the ratio of phospho-Na,K-ATPase $\alpha_1$-subunit expression to the total Na,K-ATPase $\alpha_1$-subunit (Fig. 4C). These results indicate that insulin decreases the ratio of the inactive state (i.e., increases the ratio of the active state of the Na,K-ATPase $\alpha_1$-subunit). In the presence of staurosporine and GF109203X, an established PKC inhibitor, and okadaic acid, an

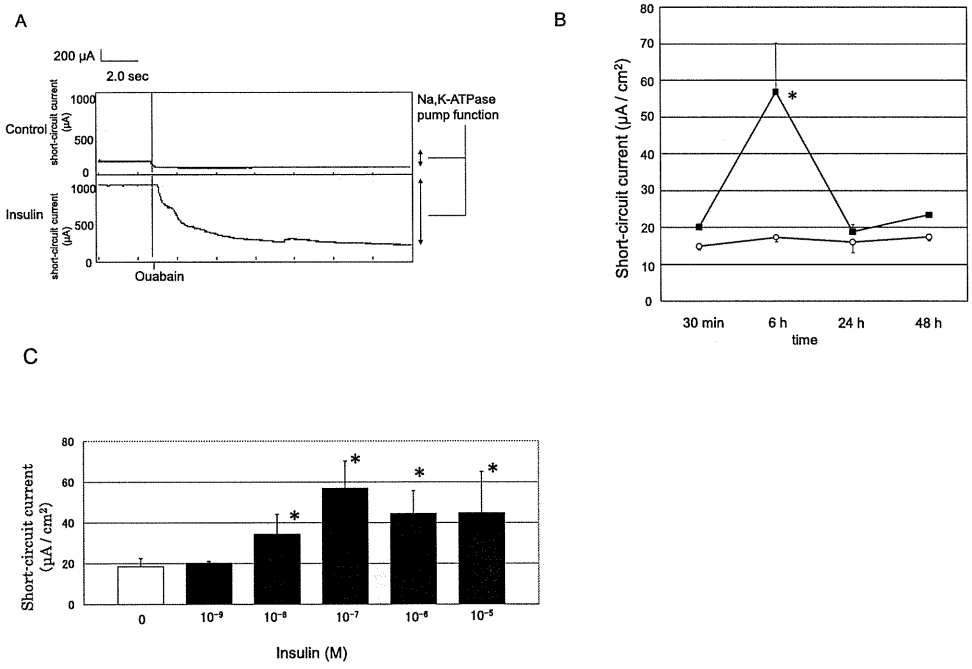


FIGURE 2. Effect of insulin on the pump function of cultured mouse corneal endothelial cells. (**A**) Representative tracings of short-circuit current (μA/well) obtained with cell monolayers in a Ussing chamber. The insert well membrane growth area was 4.67 $cm^2$. The cells were incubated in the absence (*upper*) or presence (*lower*) of 0.1 μM insulin. Pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit currents obtained before and after the addition of ouabain. (**B**) Pump function (μA/$cm^2$) attributable to Na,K-ATPase activity was determined in the absence (*open circles*) or presence (*closed squares*) of 0.1 μM insulin for the indicated times. Data are mean ± SD of values from of replicates from a representative experiment. *$P < 0.05$ compared with the corresponding value for cells incubated without insulin (Student's *t*-test). (**C**) Pump function (μA/$cm^2$) attributable to Na,K-ATPase activity was determined 6 hours after incubation of cells in the presence of the indicated concentrations of insulin. Data are mean ± SD of values of four replicates from four representative experiments. *$P < 0.05$ for the indicated comparisons (Student's *t*-test).

inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), expression of the total Na,K-ATPase $\alpha_1$-subunit did not change (Fig. 4B), and the insulin-induced dephosphorylation of Na,K-ATPase $\alpha_1$-subunit was diminished (Fig. 4C).

## Effect of Staurosporine, GF109203X, and Okadaic Acid on Insulin-Induced Na,K-ATPase Activation

To test whether the stimulatory effect of insulin on Na,K-ATPase activity was mediated by PKC, we examined the effects of staurosporine and GF109203X. The increase in Na,K-ATPase activity induced by insulin was significantly inhibited by staurosporine and GF109203X (Fig. 5). These results indicated that the increase in Na,K-ATPase activity induced by insulin at a concentration of 0.1 μM was mediated by PKC.

We next examined whether okadaic acid might affect the Na,K-ATPase activation induced by insulin. The activity of Na,K-ATPase at 0.1 μM insulin was significantly reduced in the presence of 1 μM okadaic acid (Fig. 5). These results suggest that the activity of PP1, PP2A, or both is essential to insulin-induced Na,K-ATPase activation.

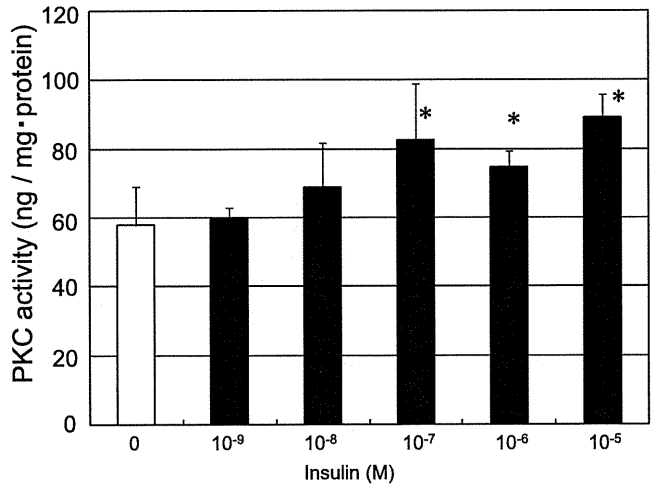


FIGURE 3. Effect of insulin concentration on PKC activity in cultured mouse corneal endothelial cells. Cells were incubated with the indicated concentrations of insulin for 30 minutes, after which the activity of PKC was measured in cell extracts. Data are mean ± SD of values of four replicates from four representative experiments. *$P < 0.05$ versus the value for cells incubated without insulin (Student's *t*-test).

## Effect of Insulin on Na,K-ATPase $\alpha_1$-Subunit Cell Surface Expression

To determine whether the effect of insulin changes the cell surface expression of the Na,K-ATPase $\alpha_1$-subunit, we examined the immunocytochemistry of the Na,K-ATPase α1-subunit after insulin treatment in the presence and absence of the inhibitors staurosporine, GF109203X, and okadaic acid. The staining was performed without permeabilization and the majority of observed staining was on the cell surface; thus, inactive Na,K-ATPase was not detected. Insulin-treated corneal endothelial cells expressed the Na,K-ATPase α1-subunit at their lateral cell membranes more than did control cells (Figs. 6A, 6B). In the presence of inhibitors, Na,K-ATPase α1-subunit expression of insulin-treated corneal endothelial cells was weakened at their lateral cell membranes (Figs. 6C–E).

## DISCUSSION

In the present study, we show that insulin increases Na,K-ATPase activity and its related pump function in cultured corneal endothelial cells. Changes in Na,K-ATPase activity and pump function under various experimental conditions were well correlated. Our results support the notion that Na,K-ATPase activity is an important determinant of the ability of corneal endothelial cells to maintain the water content of the corneal stroma.[50] Our results suggest that the observed effect of insulin on Na,K-ATPase activity in corneal endothelial cells is transient. A chronic lack of insulin in type 1 diabetes mellitus or a chronic reduced level of insulin signaling by insulin resis-

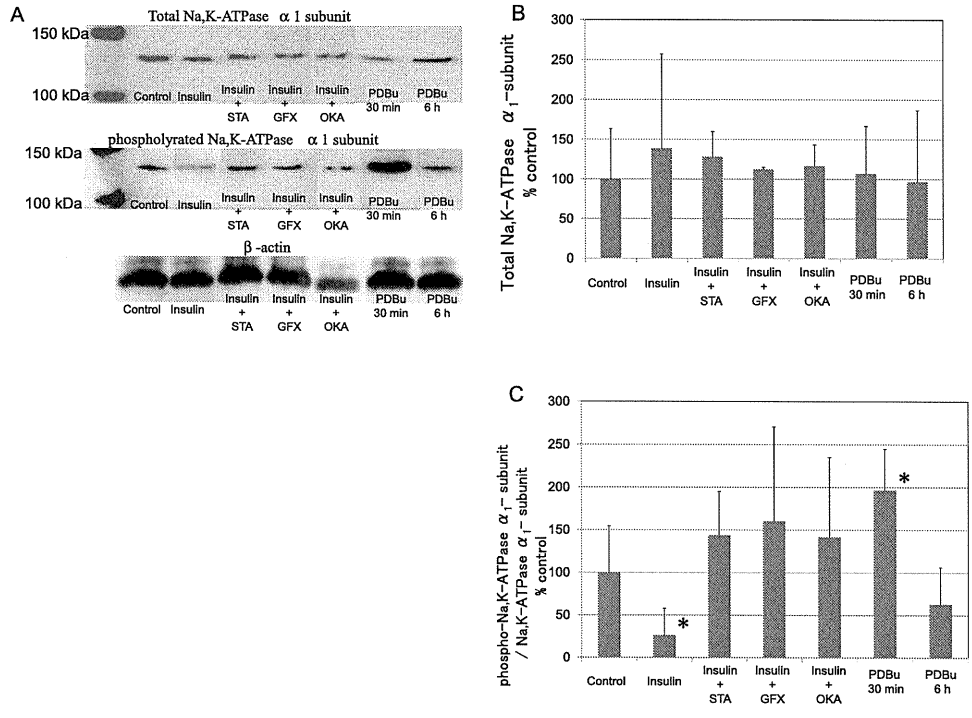


**FIGURE 4.** Western blot analysis of Na,K-ATPase $\alpha_1$-subunit and phospho-Na,K-ATPase $\alpha_1$-subunit expression. (**A**) Representative signals of expression. *Top*: Na,K-ATPase $\alpha_1$-subunit. *Middle*: phospho-Na,K-ATPase $\alpha_1$-subunit. *Bottom*: $\beta$-Actin. For the following, the relative intensity of each band to $\beta$-actin was measured by a densitometer as the expression of Na,K-ATPase $\alpha_1$ or phospho-Na,K-ATPase $\alpha_1$-subunit. (**B**) Cells were incubated in the absence (control) or presence of 0.1 $\mu$M insulin for 6 hours, 0.1 $\mu$M insulin for 6 hours with 30 minutes preincubation of 1 $\mu$M staurosporine (insulin+STA), 0.1 $\mu$M GF109203X (insulin+GFX), or 1 $\mu$M okadaic acid (insulin+OKA), 0.1 $\mu$M PDBu for 30 minutes as a positive control, and 0.1 $\mu$M PDBu for 6 hours and were then assayed for the expression of Na,K-ATPase $\alpha_1$-subunit. Data are mean ± SD from five experiments, expressed as a percentage of control. (**C**) The rate of inactive state of Na,K-ATPase $\alpha_1$-subunit with insulin, insulin+STA, insulin+GFX, insulin+OKA, and PDBu for 30 minutes and 6 hours. Values represent the ratio of phospho-Na,K-ATPase $\alpha_1$-subunit expression to Na,K-ATPase $\alpha_1$-subunit expression. Data are mean ± SD of values from five experiments. $^*P < 0.05$ versus the value for cells incubated without insulin (Student's *t*-test).

tance in type 2 diabetes mellitus is essential in the pathogenesis of corneal abnormalities in diabetes.

Insulin has been shown to stimulate the electrogenic sodium transport in a variety of cells.[27–37] In most cases, the increase in $Na^+$ transport is thought to be a result of the stimulation of the Na,K-ATPase. Various mechanisms of insulin action have been advocated, including changes of the kinetic properties of the enzyme,[28,29] an increase in the intracellular Na concentration, which in turn leads to a subsequent pump stimulation,[30–34] and an increase in the pump concentration at the cell surface by serum and glucocorticoid-dependent kinase (SGK).[35–37] Regardless whether insulin stimulates pump activity by a previous increase in cytosolic $Na^+$, in its affinity for $Na^+$, or in pump availability at the cell surface, the insulin receptor signaling cascades must be involved.[27] The signaling cascades include those mediated by protein kinases such as PKC. To date, PKC is regarded to trigger the rapid action of insulin on the Na,K-ATPase and to be involved in the stimulation of the Na,K-ATPase by insulin in muscle cells.[27] Our results suggest that the regulation of Na,K-ATPase activity by insulin in corneal endothelial cells is associated with the active state of the Na,K-ATPase $\alpha_1$-subunit, and Na,K-ATPase activation by insulin appears to be mediated by PKC and PP1 or PP2A.

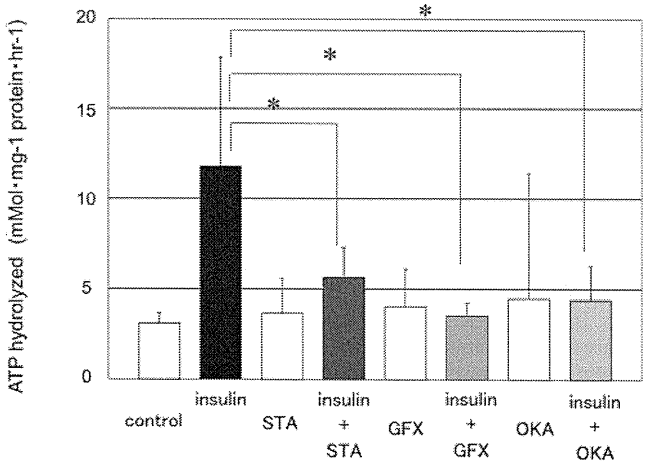


**FIGURE 5.** Effect of staurosporine (STA), GF109203X (GFX), and okadaic acid (OKA) on insulin-induced Na,K-ATPase activity in cultured mouse corneal endothelial cells. Cells were incubated first for 30 minutes in the absence or presence of 1 $\mu$M staurosporine, 0.1 $\mu$M GF109203X, or 1 $\mu$M okadaic acid and then for an additional 6 hours in the additional presence of 0.1 $\mu$M insulin before measurement of Na,K-ATPase activity. Data are mean ± SD of values of four replicates from four representative experiments. $^*P < 0.01$ versus the value for cells incubated with insulin alone (Student's *t*-test). Na,K-ATPase activity did not significantly increase in the presence of staurosporine + insulin, GF109203X + insulin, or okadaic acid + insulin compared with control.

Na,K-ATPase is the largest protein complex in the family of P-type cation pumps, and its minimum functional unit is a heterodimer of the $\alpha$- and $\beta$-subunits.[51] In the case of Na,K-ATPase $\alpha$-subunits, four isoforms ($\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$) are present in mammalian cells.[52] The $\alpha_2$ isoform appears to be involved in regulating $Ca^{2+}$ transients involved in muscle contraction, whereas the $\alpha_1$ isoform probably plays a more generalized role.[52] Huang et al.[53] reported that both the $\alpha_1$ and the $\alpha_3$ isoforms are expressed in human corneal endothelial cells. We examined Na,K-ATPase $\alpha_1$-subunit expression in corneal endothelial cells because of its generality. It remains to be investigated whether other isoforms play any role in corneal endothelial cells.

The anti-phospho-Na/K ATPase $\alpha_1$ antibody we used in the present study recognizes the Na,K-ATPase $\alpha_1$-subunit only when phosphorylated at Ser18. This phosphorylation triggers endocytosis of the Na,K-ATPase $\alpha_1$-subunit and results in inhibition of the Na,K-ATPase activity.[48,49] The phospho-Na,K-ATPase $\alpha_1$-subunit (Ser18) could be regarded as an inactive state of the Na,K-ATPase $\alpha_1$-subunit. Ser18 itself may be phos-

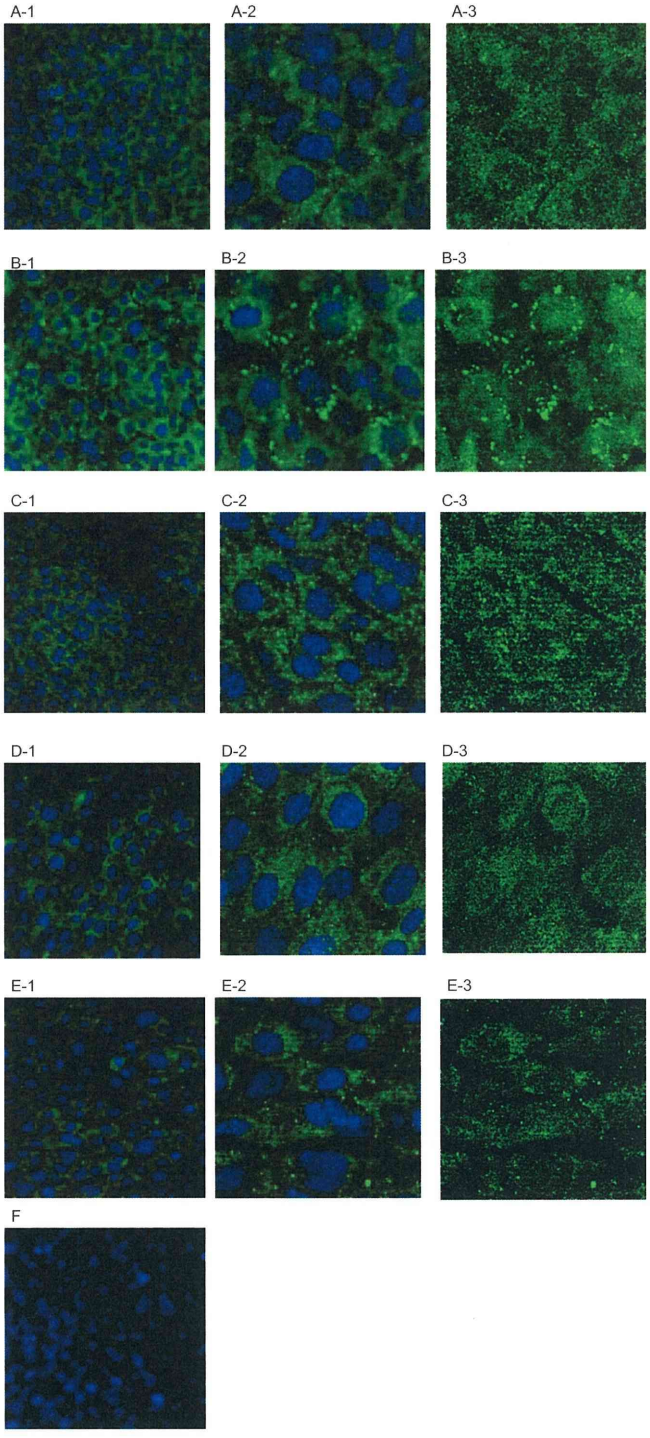


**FIGURE 6.** Effect of insulin on Na,K ATPase $\alpha_1$-subunit cell surface expression. Cells were incubated in the absence of insulin (**A**), presence of 0.1 $\mu$M insulin for 6 hours (**B**), 0.1 $\mu$M insulin for 6 hours with 30 minutes preincubation of 1 $\mu$M staurosporine (**C**), 0.1 $\mu$M GF109203X (**D**), or 1 $\mu$M okadaic acid (**E**) and then were assayed for the cell surface expression of Na,K-ATPase $\alpha_1$-subunit by immunocytochemistry. (**A-1–E-1**) Low magnification. (**A-2–E-2**) High magnification. (**A-3–E-3**) Without nuclear staining. (**F**) Negative control by using goat anti rabbit IgG (final concentration 2 $\mu$g/mL) as a primary antibody.

phorylated directly by PKC.[54–56] In our study, although insulin increased PKC activity, insulin decreased the ratio of phospho-Na,K-ATPase $\alpha_1$-subunit expression to total Na,K-ATPase $\alpha_1$-subunit expression. As we previously reported, PKC exerts bidirectional (stimulatory and inhibitory) regulation of Na,K-ATPase activity in mouse corneal endothelial cells, and PKC stimulates Na,K-ATPase activity by activating PP1, PP2A, or both, which dephosphorylates the Na,K-ATPase $\alpha_1$-subunit in corneal endothelial cells.[57] We also reported that PKC has an inhibitory effect on Na,K-ATPase activity,[57] and this effect may be attributed to Ser18 direct phosphorylation by PKC. In the present study, PDBu phosphorylated the Na,K-ATPase $\alpha_1$-subunit at 30 minutes; phosphorylation was decreased at 6 hours. The time-response curve of Na,K-ATPase activity by insulin (Fig. 1A) seemed to rise at 2 hours, and the effect became significant at 6 hours and 12 hours. There appears to be a time lag between PKC activation and Na,K-ATPase activation. Some time may be required for subsequent dephosphorylation and cell surface expression of Na,K-ATPase, and it may support our idea that PP1 or PP2A is subsequently activated by insulin-induced PKC. In addition, PP1- and PP2A-induced dephosphorylation of Na,K-ATPase may overcome direct phosphorylation by PKC in corneal endothelial cells. Previous reports also have shown that insulin activates phosphatidylinositol 3-kinase (PI-3 kinase) by insulin/IGF-I receptor, and that PI-3 kinase, presumably acting through PKC, subsequently activates PP1, PP2A, or both in porcine endometrial epithelial cells,[29] rat skeletal muscle cells,[58–60] and frog skin.[61] PP1 or PP2A subsequently dephosphorylates the $\alpha$-subunit of Na,K-ATPase and stimulates its enzymatic activity.[29,58–60] Ser18 is one of the phosphorylation sites of Na,K-ATPase. Other phosphorylation mechanisms, such as Ser11 dephosphorylation and Tyr10 phosphorylation, may also play roles in Na,K-ATPase activation.[62–64] We selected Ser18 dephosphorylation to prove that dephosphorylation by protein phosphatase 1 or 2A affects Na,K-ATPase activity. Although we did not examine the effect of protein phosphatases on Tyr10 phosphorylation, activation phosphatases should be synergistic and may not prevent the increase in activity by insulin. In the immunocytochemistry phase, insulin increased cell surface expression of the Na,K-ATPase $\alpha_1$-subunit, and the presence of inhibitors such as staurosporine, okadaic acid, and GF109203X decreased its expression. These results support our conclusions.

Although we did not measure the activity of other kinases, such as SGK or AKT/protein kinase B (PKB), in corneal endothelial cells, recent studies have reported that SGK also activates Na,K-ATPase by increasing the availability of the enzyme at the basolateral membrane and that SGK is under the control of insulin.[35–37] AKT/PKB has been reported to be activated by insulin-induced PI-3 kinase phosphorylation.[65] The ouabain-induced PI-3 kinase-AKT/PKB signaling pathway has been reported to upregulate Na,K-ATPase expression in rat cardiac myocytes[66] and pig kidney epithelial cells,[67] but whether the insulin-induced PI-3 kinase-AKT/PKB pathway activates Na,K-ATPase in corneal endothelium remains unknown. In our study, PKC inhibitors and the PP1/PP2A inhibitor significantly reduced the insulin-induced activation of Na,K-ATPase. This result suggests insulin-induced PKC and PP1/PP2A activation has a significant effect on Na,K-ATPase activation in corneal endothelial cells. However, a slight but insignificant difference existed between the inhibitory effects of PKC inhibitors and the PP1/PP2A inhibitor. In addition, for significant increases, differences in concentrations were seen between PKC activation and Na,K-ATPase activation by insulin, although each similarly reached a plateau at $>0.1$ $\mu$M insulin concentration. In corneal endothelial cells, PP1, PP2A, or both may be activated primarily by insulin-induced PKC activation, whereas the existence of other insulin-induced kinases such as SGK and AKT/PKB must be clarified in further studies. Thus, the mechanism of insulin action is complex, and further studies are necessary to elucidate the pathways by which the effect of insulin on corneal endothelial cells is mediated.

In conclusion, we have shown that insulin increases Na,K-ATPase activity and pump function in corneal endothelial cells. Furthermore, our results support a model in which PKC and PP1 or PP2A mediates the activation of Na,K-ATPase by insulin in corneal endothelial cells. A lack of insulin in type 1 diabetes mellitus or a reduced level of insulin signaling by insulin resistance in type 2 diabetes mellitus may play a role in the pathogenesis of corneal abnormalities in diabetes.

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CORNEA

# Surgical outcome of Descemet's stripping automated endothelial keratoplasty for bullous keratopathy secondary to argon laser iridotomy

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**Abstract**
*Background* To report the 6-month clinical outcome of Descemet's stripping automated endothelial keratoplasty (DSAEK) for bullous keratopathy (BK) secondary to argon laser iridotomy (ALI), and compare the results with those of DSAEK for pseudophakic bullous keratopathy (PBK) or Fuchs' endothelial dystrophy (FED).
*Methods* A total of 103 patients (54 with ALI, 28 with PBK, 21 with FED) undergoing DSAEK were retrospectively analyzed. Simultaneous cataract surgery was performed in 37 patients with ALI and 13 with FED. Preoperative ocular conditions, best spectacle-corrected visual acuity (BSCVA), spherical equivalent refraction (SE), induced astigmatism, keratometric value, endothelial cell density (ECD), and complications were determined over 6 months postoperatively.
*Results* Mean axial length in the ALI group (21.8±0.8 mm) was significantly shorter than that in the FED ($P$=0.02) or PBK groups ($P$=0.003). Severe corneal stromal edema ($n$=6), advanced cataract ($n$=10), posterior synechia ($n$=3), poor mydriasis ($n$=5), and Zinn zonule weakness ($n$=1) were found only in the ALI group. A significant improvement was observed in postoperative BSCVA in all groups. No significant difference was observed in BSCVA, SE, induced astigmatism, keratometric value, ECD, or complications among the three groups.
*Conclusions* Descemet's stripping automated endothelial keratoplasty for BK secondary to ALI showed rapid postoperative visual improvement, with similar efficacy and safety to that observed in DSAEK for PBK or FED.

**Keywords** Descemet's stripping automated endothelial keratoplasty · Argon laser iridotomy · Fuchs' dystrophy · Pseudophakic bullous keratopathy · Posterior lamellar keratoplasty

## Introduction

The cause of bullous keratopathy (BK), one of the main reasons for corneal transplantation worldwide, differs by region. For example, in addition to cataract surgery, Fuchs' dystrophy is a major cause of BK in western countries [1]. In Japan, on the other hand, argon laser iridotomy (ALI) is the second most common cause for BK according to a recent national survey [2, 3]. Bullous keratopathy secondary to ALI (ALI-BK) can occur long after ALI, and both eyes with angle-closure glaucoma and those that have undergone prophylactic ALI may be affected. Descemet's stripping automated endothelial keratoplasty (DSAEK), a lamellar corneal surgical procedure, allows selective replacement of the posterior layers of the cornea in the treatment of BK [4, 5]. The advantages of DSAEK over conventional penetrating keratoplasty include the need for only a small incision to be

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made, maintenance of structural integrity of the cornea, rapid visual recovery, and minimal induction of astigmatism [6–8]. However, when DSAEK is performed for ALI-BK, several challenges arise. These eyes characteristically have shallow anterior chambers which may render anterior chamber surgical maneuvers more difficult and risky. Therefore, DSAEK for ALI-BK is often technically challenging, even for well-experienced surgeons [9]. However, hitherto, reports on the surgical outcome of DSAEK for ALI-BK have only involved small patient samples [9, 10]. The incidence and management of the intra- and postoperative complications and visual outcomes of DSAEK for ALI-BK remain largely unknown. The aim of this study was to investigate the 6-month clinical outcome of DSAEK for ALI-BK, and to compare with those undergone DSAEK for other causes of BK (Fig. 1).

## Patients and methods

### Patients

The medical records of all consecutive patients undergoing DSAEK for BK resulting from ALI, Fuchs' endothelial dystrophy (FED), or pseudophakic bullous keratopathy (PBK) between April 2007 and December 2010 at Tokyo Dental College Ichikawa General Hospital were retrospectively reviewed (Table 1). Patients were excluded from the analysis because of the following reasons; combined causes suspected such as ALI and FED (three cases), previous history of penetrating keratoplasty (eight cases), macular dysfunction due to previous retinal detachment (two cases) or Axenfeld-Rieger syndrome (one case), and end-stage glaucoma without central visual field (one case), or BK due to birth injury with corneal stromal opacity (two cases). The present study adhered to the tenets of the Declaration of Helsinki.

### Surgical procedures and postoperative treatment

After sub-Tenon or retrobulbar injection of 2% lidocaine, a 5.0-mm temporal or superior corneoscleral incision was made (details in Table 2). An anterior-chamber maintenance cannula was inserted for paracentesis. Descemet's membrane stripping was performed with a diameter corresponding to the graft size, using a reverse-bent Sinsky hook (ASICO, Westmont, IL, USA) and an epithelial trephine marker. In most cases, the graft size was 8.0 mm, as shown in Table 2. Apart from seven eyes (13%) in the ALI group and four eyes (14%) in the PBK group in which nDSAEK were performed [10], the recipient's endothelium and Descemet's membrane were carefully removed by forceps. Pre-cut donor grafts were trephinated and the endothelial surface of the lenticle coated with a small amount of viscoelastic material (Viscoat®, Alcon, Fort Worth, TX, USA). Donor tissue was gently inserted into the anterior chamber using a Busin glide (ASICO, Westmont, IL, USA)/IOL glide and Shimazaki DSAEK forceps (Inami, Tokyo, Japan). Pull-through technique was used to insert the donor graft, except in six eyes (three eyes in the ALI group, two eyes in the PBK group and one eye in the FED group) in which the folding technique was used. Air was carefully injected into the anterior chamber to unfold the graft. Fluid from between the recipient's stroma and the graft was drained via small incisions in the midperipheral recipient cornea. Ten minutes after air injection, most of the air was replaced with balanced salt solution (BSS plus®, Alcon, Fort Worth, TX, USA). At the end of the procedure, subconjunctival tobramycin 4 mg (Tobracin®, J-Dolph, Shiga, Japan) and betamethasone 0.4 mg (Rinderon®, Shionogi, Osaka, Japan) were administered. In patients with significant lens opacity, standard phacoemulsification and aspiration, and implantation of an intraocular lens were performed prior to DSAEK using the phaco-chop technique. Postoperative medication included 0.1% levofloxacin (Cravit®, Santen, Osaka, Japan) and 0.1% betamethasone sodium phosphate (Sanbetazon®, Santen, Osaka), starting at 5 times a day for 3 months and then tapering off thereafter.

### Examinations

Best spectacle-corrected visual acuity (BSCVA), spherical equivalent (SE), induced astigmatism [11], keratometric value (K value), and endothelial cell density (ECD) were measured pre- and postoperatively at 1, 3 and 6 months. Preoperative corneal opacity was semi-quantitatively graded using slit-

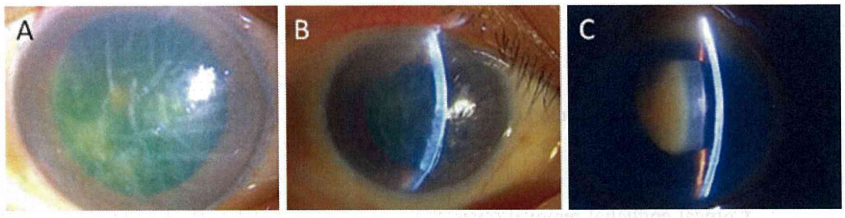


**Fig. 1** Slit-lamp photographs of three patients in ALI group taken preoperatively (a, b, c). Two cases (a, b) had severe corneal stromal edema with Descemet membrane fold with hard cataract. One case (c) had mild corneal stromal edema and rock-hard cataract with Zinn zonule weakness and poor mydriasis. DSAEK with cataract surgery had been performed safely on all patients

**Table 1** Patient demographics

| | ALI (N=54) | PBK (N=28) | *P*value | FED (N=21) | *P*value |
|---|---|---|---|---|---|
| Age (year) | 76±6.0 | 76±6.0 | NS* | 67±9.0 | NS* |
| Female/Male | 49/5 | 21/7 | 0.096† | 17/4 | 0.03† |
| Axial length (mm) | 21.8±0.8 | 23.1±2.4 | 0.018* | 22.8±1.5 | <0.001* |
| Presence of tube in anterior chamber (eyes, %) | 0 (0%) | 0 (0%) | - | 0 (0%) | - |
| Grade of corneal opacity (eyes, %) | | | | | |
| Grade 0 | 0 (0%) | 0 (0%) | - | 0 (0%) | - |
| Grade 1 | 14 (31.8%) | 8 (34.8%) | 0.68** | 9 (45%) | 0.22** |
| Grade 2 | 12(34.1%) | 8 (34.8%) | | 8 (35%) | |
| Grade 3 | 18 (34.1%) | 7 (30.4%) | | 4 (20%) | |
| Emery-Little Grading of cataract | | | | | |
| Mean of grade of cataract | 3.57±0.96 | - | - | 2.95±0.74 | 0.013* |
| Grade 1 cataract (eyes, %) | 0 (0%) | - | - | 0 (0%) | - |
| Grade 2 cataract (eyes, %) | 8 (14.8%) | - | - | 6 (28.6%) | 0.53† |
| Grade 3 cataract (eyes, %) | 17 (31.5%) | - | - | 10 (47.6%) | 0.81† |
| Grade 4 cataract (eyes, %) | 19(35.2%) | - | - | 5 (23.8%) | 0.12† |
| Grade 5 cataract (eyes, %) | 10 (18.5%) | - | - | 0 (0%) | 0.013† |
| Simultaneous cataract surgery (eyes, %) | 37 (68.5%) | 0 (0%) | - | 13 (61.9%) | 0.60† |
| Cataract surgery on beforehand (eyes, %) | 9 (7.4%) | 0 (0%) | | 0 (0%) | - |

* Mann–Whitney U test

** $\chi^2$ test † Fisher exact test

lamp biomicroscopy as follows: grade 0: clear and normal; grade 1: slightly hazy, but iris cleft visible; grade 2: iris cleft difficult to identify; grade 3: iris cleft impossible to identify. Cataracts were graded according to the Emery–Little

**Table 2** Details of surgical procedure of DSAEK in each groups

| | ALI (*n*=54) | PBK (*n*=28) | FED (*n*=21) |
|---|---|---|---|
| Descemet's stripping endothelial keratoplasty (eyes, %) | 47 (87%) | 24 (86%) | 21 (100%) |
| Non-Descemet's stripping endothelial keratoplasty (eyes, %) | 7 (13%) | 4 (14%) | 0 (0%) |
| Graft size (eyes) | | | |
| 7.0 mm | 1 | 0 | 0 |
| 7.5 mm | 1 | 0 | 0 |
| 7.75 mm | 1 | 1 | 1 |
| 8.0 mm | 51 | 26 | 19 |
| 8.25 mm | 0 | 0 | 1 |
| 8.5 mm | 0 | 1 | 0 |
| Location of corneoscleral incision (eyes, %) | | | |
| Temporal | 51 (94%) | 23 (82%) | 20 (95%) |
| Superior | 3 (6%) | 5 (18%) | 1 (5%) |
| Corneal epithelial removal (eyes, %) | 13/54 (24%) | 1/28 (3.6%) | 4 (19%) |
| Trypan blue staining (eyes, %) | 3/37 (8.1%) | 0 (0%) | 0 (0%) |
| Multiple sphincterotomy (eyes, %) | 3/37 (8.1%) | 0 (0%) | 0 (0%) |

classification. Poor preoperative mydriasis was defined as a pupil diameter of less than 5 mm after instillation of tropicamide and phenylephrine 3 times per 10 minutes. Decimal values of BCSVA were converted to logarithm for statistical analysis. Endothelial cell density was measured using a specular microscope (SP-3000P®, Tomey, Nagoya, Japan). Intra- and postoperative complications were also recorded.

### Statistical analysis

The Mann–Whitney U, Wilcoxon, Kruskal–Wallis, $\chi^2$ tests and Fisher's exact test were used for the statistical analysis. A $P$ value of less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed with the SSRI software (SSRI Co. Ltd., Tokyo, Japan).

## Results

### Preoperative ocular conditions

Table 1 summarizes the demographic data on the 103 eyes with BK analyzed in this study, which included 54 with ALI-BK (ALI group), 28 with PBK (PBK group), and 21 with FED (FED group). The proportion of eyes that had simultaneous cataract surgery is shown in Table 1. Mean axial length was significantly smaller in ALI group than in FED ($P$=0.02) or PBK groups ($P$=0.0003, Mann–Whitney U test). As shown in Table 1, the grade of corneal opacity was similar in each group. However, severe corneal stromal edema with descemet's membrane fold occurred in six eyes (11%) in ALI group. Moreover, posterior synechia ($n$=3) and poor mydriasis ($n$=5) occurred more frequently in ALI group. Mean grade of cataract was significantly higher in ALI group than in FED group, with grade 5 cataract found only in ALI group (Table 1). Higher than grade 4 cataract occurred significantly more often in the ALI group than in the FED group ($P$=0.023; Fisher exact test). As shown in Table 2, various types of intraoperative manipulation, including corneal epithelium removal, trypan blue-assisted phacoemulsification and multiple sphincterotomy, were required only in ALI group.

### BSCVA, induced astigmatism, SE and K value

All eyes were followed up for a minimum of 6 months postoperatively. Clarity was maintained in the donor graft at postoperative month 6 in 50 eyes (92.6%) in ALI group, 26 eyes (92.9%) in PBK group, and 20 eyes (95.2%) in FED group. Table 3 summarizes BSCVA at preoperative and postoperative months 1, 3 and 6. With regard to postoperative BSCVA, 53 eyes (98.1%) in ALI group, 26 eyes (92.9%) in PBK group and 19 eyes (90.4%) in FED group showed improved BSCVA. Forty-four eyes (81.5%) in ALI group, 19 eyes (67.9%) in PBK group and 14 eyes (66.7%) in FED group achieved a BSCVA of 20/40 or better at 6 months. A significant improvement was observed in BSCVA from 1 month after DSAEK in each group ($P$< 0.0001 in ALI group, $P$=0.0006 in PBK group, and $P$= 0.0004 in FED group; Wilcoxon test). In ALI and PBK groups, BSCVA improved significantly from 1 month to 3 months postoperatively ($P$<0.0001 in ALI group and $P$= 0.0065 in PBK group; Wilcoxon test). Table 4 shows the results of refractive data. No significant difference was observed in induced astigmatism throughout the postoperative observation period in each group. No significant difference was observed in SE between the preoperative and postoperative 1-month values in any group. A significant difference was observed in SE between postoperative month 1 and 6 in FED group ($P$=0.046, Wilcoxon test). No significant differences were observed in preoperative and 1-, 3-, and 6-month postoperative K values in any group.

### Endothelial cell loss

Table 5 summarizes ECD of donor preoperatively and ECD at postoperative month 1, 3 and 6. Preoperative ECD

**Table 3** The average of best spectacle-corrected visual acuity (BSCVA)

| BSCVA (LogMAR±SD) | ALI (range) | PBK (range) | FED (range) | $P$ value |
|---|---|---|---|---|
| Preoperative | 1.27±0.64 (0.16 to 3) | 1.41±0.59 (0.52 to 2.7) | 0.88±0.49 (0.3 to 2) | |
| 1 m | 0.60±0.63 (0.16 to 3) | 0.78±0.64 (0.3 to 2.7) | 0.44±0.46 (0.16 to 2) | NS* |
| 3 m | 0.43±0.57 (0 to 2.7) | 0.65±0.67 (0.1 to 2.7) | 0.39±0.44 (0.05 to 2) | NS* |
| 6 m | 0.34±0.60 (−0.08 to 2.7) | 0.50±0.65 (0 to 2.7) | 0.21±0.30 (0 to 1) | NS* |
| Eyes showing improved BSCVA (eyes) | 53 (98.1%) | 26 (92.9%) P=0.27† | 19 (90.4%) P=0.19† | |
| Postoperative BSCVA>20/40 (eyes) | 44 (81.5%) | 14 (66.7%) P=0.18†† | 19 (67.9 %) P=0.22†† | |

*Kruskal–Wallis test comparing the difference among three groups

† Fisher's test comparing the number of eyes showing improved BSCVA between in ALI group and PBK group or FED group

†† Fisher's test comparing the number of eyes showing postoperative BSCVA>20/40 between in ALI group and PBK group or FED group

**Table 4** Graft survival rate and refractive data

| | ALI | PBK | FED | *P* value |
|---|---|---|---|---|
| Graft survival rate at postoperative 6 m (eyes, %) | 50 (92.6%) | 26 (92.9%) | 20 (95.2%) | |
| Induced astigmatism (D) | | | | |
| 1 m | 1.4±1.2 | 2.5±2.1 | 2.3±1.4 | 0.007* |
| 3 m | 1.5±1.1 | 2.3±1.1 | 2.0±1.6 | 0.036* |
| 6 m | 1.5±1.0 | 1.8±0.7 | 1.7±1.3 | NS* |
| Spherical equivalence (D) | | | | |
| Preoperative | 0.69±2.6 | −0.74±1.86 | −0.25±1.5 | NS* |
| 1 m | 0.31±1.6 | −0.48±2.2 | −0.39±2.2 | NS* |
| 3 m | 0.17±1.2 | −0.94±2.0 | 1.6±5.26 | NS* |
| 6 m | −0.15±0.78 | −0.94±2.0 | −2.7±4.3 | NS* |
| Keratometric value | | | | |
| Preoperative | 44.5±2.4 | 43.8±2.0 | 44.5±2.3 | NS* |
| 1 m | 43.3±2.4 | 43.3±2.2 | 44.0±2.2 | NS* |
| 3 m | 44.0±2.2 | 43.6±1.9 | 43.5±2.6 | NS* |
| 6 m | 44.0±2.1 | 43.7±2.1 | 43.8±1.4 | NS* |

Data were presented as mean± SD. *Kruskal–Wallis test compared the difference among three groups

significantly decreased 1 month after DSAEK ($P<0.0001$ in ALI group, $P=0.043$ in PBK group, and $P=0.012$ in FED group; Wilcoxon test). In addition, postoperative ECD significantly decreased from month 3 (1174±361 cells/mm$^2$) to month 6 (944±386 cells/mm$^2$) in PBK group ($P=0.017$; Wilcoxon test), although no rejection episode occurred between 3 and 6 months in this group.

### Complications

Complications are summarized in Table 6. No significant differences were observed in incidence of intra- or postoperative complications among the three groups. Three eyes out of 37 eyes (8.1%) in ALI group showed posterior capsule rupture intraoperatively. In two of these three eyes, an intraocular lens was inserted into the capsular bag, while in the remaining eye the lens was fixed to the sulcus. Twelve out of 103 eyes (11.5%) (six eyes in ALI group, three eyes in PBK group and three eyes in FED group) showed dislocation of the donor corneal lenticle at day 1 postoperatively, with each undergoing successful reattachment with one or a pair of air bubble tamponades. Pupillary block glaucoma secondary to anterior chamber air bubble occurred in three out of 103 eyes (2.9%) (one eye in ALI group and two eyes in PBK group) on the day of surgery, and was successfully treated by air removal. One out of 103 eyes (0.97%) (PBK group) developed acute graft rejection characterized by mild inflammation in the anterior chamber and keratic precipitates on the donor endothelium. In this case, graft rejection was treated with intensive topical and intravenous corticosteroid therapy. No case underwent re-keratoplasty within 6 months. Postoperative ocular hypertension developed in six eyes (three eyes in ALI group, one eye in PBK group and two eyes in FED group) and was treated with antiglaucoma eye drops. Cystoid macular edema was found in one eye in ALI group, and was treated with intensive instillation of 0.1% diclofenac sodium (Diclod®, Wakamoto Pharmaceuticals, Tokyo, Japan).

**Table 5** Endothelial cell density (ECD)

| ECD(/mm$^2$±SD) | ALI | PBK | FED | *P* value |
|---|---|---|---|---|
| Donor preoperatively | 2339±284 | 2600±341 | 2701±332 | NS* |
| 1 m | 1360±467 | 1023±260 | 1158±487 | NS* |
| 3 m | 1217±485 | 1174±361 | 1388±612 | NS* |
| 6 m | 1124±427 | 944±386 | 1230±560 | NS* |

*Kruskal–Wallis test comparing endothelial cell density among three groups

## Discussion

We evaluated the 6-month clinical outcome of Descemet's stripping automated endothelial keratoplasty (DSAEK) for BK secondary to ALI and compare the results with those of DSAEK for pseudophakic bullous keratopathy (PBK) or Fuchs' endothelial dystrophy (FED). We demonstrated that DSAEK for BK secondary to ALI showed rapid postoperative visual improvement, with similar efficacy and safety to that observed in DSAEK for PBK or FED.

Bullous keratopathy secondary to ALI is becoming increasingly common in Asian countries, especially in Japan [2, 3, 12–15], where a national survey revealed that ALI-BK accounted for approximately one-fourth of BK cases

**Table 6** Complications

| | | ALI | PBK | *P* value* | FED | *P* value* | Total |
|---|---|---|---|---|---|---|---|
| Intraoperative | Posterior capsule rupture (eyes, %) | 3 (8.1%) | 0 (0%) | 0.54 | 0 (0%) | 0.56 | 3 (2.9%) |
| Postoperative; early | Dislocation (eyes, %) | 6 (11%) | 3 (11%) | 1 | 3 (14%) | 0.70 | 12 (12%) |
| | Pupillary block (eyes, %) | 1 (1.9%) | 2 (7.1%) | 0.27 | 0 (0%) | 1 | 3 (2.9%) |
| Postoperative; chronic | Rejection (eyes, %) | 0 (0%) | 1 (3.6%) | 1 | 0 (0%) | 1 | 1 (0.97%) |
| | Ocular hypertension (eyes, %) | 3 (5.5%) | 1 (3.6%) | 1 | 2 (9.5%) | 0.61 | 6 (5.8%) |
| | Cystoid macular edema (eyes, %) | 1 (1.9%) | 0 (0%) | 1 | 0 (0%) | 1 | 1 (0.97%) |

*Fisher exact test compared with ALI group

undergoing keratoplasty. Most cases of ALI-BK develop long after laser iridotomy, in which an argon laser is used [2]. However, the underlying mechanism of ALI-BK remains unclear. Several hypotheses have been postulated, including increased temperature in the local aqueous humor [16], high energy delivered during ALI breakdown of the blood–aqueous barrier, and change in aqueous humor fluid dynamics [17, 18].

This study demonstrated that surgery for ALI-BK is technically challenging, mainly due to the small size of the eyeball shallow anterior chamber and challenging simultaneous cataract surgery in ALI group. In the present study, the mean axial length of eyes receiving DSAEK for ALI-BK was less than 22 mm. In addition, advanced cataract is often associated with this disorder, and physicians are reluctant to perform surgery due to decreased endothelial density. Moreover, a shortage of donor grafts in Japan often gives rise to delayed keratoplasty, which makes the procedure even more challenging. There were also associated problems such as poor mydriasis and weak Zinn zonule in some cases. To overcome difficulties in surgery, various supporting surgical procedures are used, and simultaneous cataract surgery. Epithelial removal technique was performed in case of invisible anterior chamber by strong corneal epithelial edema. In addition, trypan blue staining was performed to stain anterior capsule for continuous curvilinear capsulorhexis (CCC) in the case of severe cataract. In this study, all trypan blue staining was combined with epithelial removal procedure. The postoperative outcome in eyes with ALI-BK was comparable to other groups, suggesting that combined DSAEK and cataract surgery can be performed without severe complications with the above-mentioned surgical technique in most cases.

In this study, the average BSCVA 6 month after DSAEK for ALI-BK was 20/29 with low induced astigmatism. Seven eyes (12.9%) achieved postoperative BSCVA of 20/20 or better. Clarity was maintained in the donor graft at postoperative month 6 in 50 eyes (92.6%). No case underwent re-keratoplasty within 6 months. These results are comparable with those of Gorovoy [5], Koenig [6], and Kobayashi [9] (Table 7). Endothelial cell loss was 52% at postoperative 6 months, similar to 6-month data reported previously [6]. Postoperative endothelial loss after DSAEK for ALI-BK was not significantly different from that in the other two groups. We were concerned about the possibility that the ALI group may experience a faster decrease in ECD than the other groups after DSAEK, because of the pathogenesis mechanism of ALI. However, the results of this study showed that the 6-month clinical outcomes of ECD in DSAEK for ALI-BK were comparable with those of DSAEK for BK caused by FED or PBK [20, 21]. One of the explanations may be that simultaneous cataract surgery eliminates the cause of endothelial cell damage by deepening the anterior chamber.

Our previous report on PK for ALI-BK showed a similar result in BSCVA to that of the present study (78.6% of patients achieved 20/40 or better); however, incidence of rejection (8.2%) and postoperative glaucoma (18.4%) was relatively high [22]. The refractive outcome at postoperative 6 months of our previous study indicated that PK for ALI-BK resulted in similar SE (the average was 0.19±4.6 D) to the present study, and higher induced astigmatism (the average; 3.3±2.4 D) (unpublished data). Although there was no report that showed postoperative refraction data of PK for ALI-BK in detail, PK usually results in unstable refraction as long as sutures are present, and even after their removal [23–26]. Bahar et al. reported the comparison of 12-month surgical outcomes of DSAEK and PK [8]. Stability of the refraction is a major advantage of all endothelial keratoplasty techniques as compared with PK. In current study, the postoperative refraction after DSAEK for ALI-BK was stable during follow-up terms.

In summary, this study of 6-month outcomes of DSAEK for BK secondary to ALI showed rapid postoperative visual improvement, with similar efficacy and safety to that observed in DSAEK for PBK or FED. Although many of the eyes in ALI group presented technical challenges during surgery, those challenges could be successfully managed by modification of the procedures and implements used.

**Table 7** Comparison of clinical outcome with previous reports

| Author Journal | Gorovoy et al. Cornea, 2006 [5] | Koenig et al. Cornea, 2007 [6] | Koenig et al. Ophthalmology, 2007 [7] | Bahar et al. Ophthalmology, 2008 [8] | Kobayashi et al. Cornea, 2008 [9] | Kobayashi et al. Am J O, 2008 [10] | Price et al. Ophthalmology, 2010 [19] | Hirayama et al. |
|---|---|---|---|---|---|---|---|---|
| Operation | DSAEK | DSAEK | DSAEK | DSAEK | DSAEK | nDSAEK | DSAEK | DSAEK/ nDSAEK |
| Study design | Retrospective | Prospective | Prospective | Prospective | Prospective | Prospective | Prospective | Retrospective |
| Number of eyes (eyes) | 16 | 34 | 26 | 45 | 14 | 6 | 173 | 54 |
| Disease (eyes) | FED 9 | FED 11 | FED 12 | FED 28 | ALI-BK 14 | ALI-BK 6 | FED 147 | ALI-BK |
| | PBK 7 | P/ABK 23 | P/ABK 14 | PBK 12 | | | P/ABK 23 | |
| | | | | ICE syndrome 2 | | | Other endothelial failure 3 | |
| | | | | Failed graft 3 | | | | |
| M:F | 7:9 | 6:28 | 6:20 | 20:25 | 1:13 | 2:4 | 69:104 | 5:49 |
| Mean age (years) | 66 | 73.8 | 75.9 | 70.2 | 74.2 | 74.5 | 72 | 76 |
| Follow-up time | 1 year | 6 months | 3 months | 9.8 months | 228 ±132 days | 6 months | 1 year | 6 month |
| Postoperative BSCVA | 20/40 or better (except three eyes with macular scar, optic dystrophy, primary graft failure) | Mean 20/42 (62% achieved 20/40 or better) | Mean 20/45 | Mean 20/44 | 20/40 or better (23% achieved 20/20) | More than 20/32 (33% achieved 20/20) | – | Mean 20/29 (13% achieved 20/20) |
| Postoperative SE (D) | – | 0.97 | 0.82 | 0.96 | – | – | – | −0.15 |
| Postoperative astigmatism (D) | – | 1.80 | 2.12 | 1.36 | 0.53 | 0.85 | – | 1.5 |
| Postoperative ECD (/mm$^2$) | 1,714 (except one eye of primary graft failure) | 1,396 | – | 1,735 | 1,654 | 2,391 | 1,743 | 1,124 |
| Cell loss | 41% | 50% | – | 36% | 45% | 26% | 38% | 52% |
| Complications during operation (eyes) | – | – | – | – | Vitreous prolapse 2 (14%) | None | – | Posterior capsule rupture 3 (5.5%) |
| Postoperative complications (eyes) | | | – | | | | | |
| Graft failure | 1 (6%) | 3 (9%) | – | 1 (2%) | – | 0 (0%) | – | – |
| Dislocaton | 4 (25%) | 9 (27%) | 9 (35%) | 7 (16%) | 2 (14%) | 1 (17%) | 10 (6%) | 6 (11%) |
| Acute rejection | – | 6 (18%) | 3 (12%) | 1 (2%) | – | – | 9 (5%) | 0 (0%) |
| Elevated IOP | – | – | – | 3 (7%) | – | – | 27 (16%) | 3 (6%) |
| Papillary block | – | 1 (3%) | 1 (4%) | – | – | 0 (0%) | 0 (0%) | 1 (2%) |
| Others | | | | Interface opacity 2 (4%) CME 1 (2%) | | Subclinical endothelial rejection 1 (17%) | Retinal detachment 1(<1%) Anterior synechiae 2 (1%) | CME 1 (2%) |

DSAEK; Descemet's stripping automated enthothelial keratoplasty, nDSAEK; non-Descemet's stripping automated enthothelial keratoplasty, FED; Fuchs' endothelial dystrophy, PBK; pseudophakic bullous keratopathy, ABK; aphakic bullous keratopathy, ALI-BK; bullous keratopathy secondary to argon laser iridotomy, ICE syndrome; iridocorneal endothelial syndrome, SE; spherical equivalent, ECD; endothelial cell density, IOP; intraocular pressure, CME; cystoid macular edema

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