The Anti-oxidative Role of ABCG2 in Corneal Epithelial Cells

Miyuki Kubota, Shigeto Shimmura, Hideyuki Miyashita, Motoko Kawashima, Tetsuya Kawakita, and Kazuo Tsubota

PURPOSE. ABCG2 is a putative marker of progenitor cells, including the corneal epithelium. The authors investigated whether ABCG2 functions in the homeostasis of corneal epithelial cells using abcg2 knockout (KO) mice and corneal epithelial cell lines.

METHODS. abcg2 KO mice and a spontaneously immortalized murine corneal epithelial cell line (TKE2) were used for experiments. Flow cytometry was used to determine the presence of side population (SP) cells based on the ability of ABCG2 to efflux Hoechst 33342 dye. Expression of ABCG2 was also examined by RT-PCR. Cytotoxicity assay (IC50) and propidium iodide staining were performed in semiconfluent cells treated with hypoxia (1% O2) or with the pro-oxidant mitoxantrone.

RESULTS. abcg2 KO mice had a normal corneal epithelial phenotype; however, cultured abcg2 KO epithelial cells were prone to oxidative damage by mitoxantrone. TKE2 cells were resistant to mitoxantrone at low doses, but higher concentrations were toxic in a dose-dependent manner. Coculture with the ABCG2 inhibitors reserpine and Ko143 inhibited resistance to mitoxantrone, with a statistically higher cell death ratio. abcg2 KO cells were also significantly more sensitive to hypoxia than were wild-type control cells.

CONCLUSIONS. ABCG2 may protect corneal epithelial progenitor SP cells against oxidative stress induced by toxins and hypoxia.

Keywords: ABCG2; Corneal epithelial cells; Hypoxia; Mitoxantrone; Side population cells.

The study of adult stem cells was pioneered in the bone marrow, which hosts the niche for multipotent hematopoietic stem cells (HSCs). The purification of HSCs was greatly enhanced by the use of the fluorescent DNA-binding dye, Hoechst 33342, which has the property of being selectively effluxed by immature cells by a transporter of the multidrug-resistant gene family. The specific gene was found to be the ATP-binding cassette transporter ABCG2/Bcrp1, and a subset of these immature cells isolated by flow cytometry was labeled side population (SP) cells to differentiate them from the majority of cells (main population cells) that do not have this property. Further studies demonstrated the presence of ABCG2 in cells other than HSC, and SP cells are now known to exist in skeletal muscle, umbilical cord blood, liver, lung, pancreas, brain, skin, retina, and mammary gland.

SP cells have also been identified in the corneal limbus, where epithelial stem cells are believed to reside. limbal SP cells may contain an enriched fraction of stem cells because the ABCG2 protein is expressed mainly in basal limbal cells, which are also negative for the differentiation marker cytokeratin 12 (K12). Furthermore, de Paiva et al. have demonstrated the coexpression of deltaNp63 with ABCG2 in SP cells, which also demonstrated a high colony-forming efficiency when cocultured with 3T3 feeder cells. Although the available data suggest that the SP fraction of the limbal epithelium may be enriched with epithelial stem cells, there is no information as to whether ABCG2 functions in the maintenance or protection of these cells. Therefore, we sought to elucidate the possible role of ABCG2 in corneal epithelial cells using abcg2 KO mice and an established murine corneal epithelial cell line.

MATERIALS AND METHODS

Animals

abcg2 KO mice (129/C57BL6 background) were provided as a kind gift from Brian Sorrentino (Division of Experimental Hematology, St. Jude Children’s Hospital) and housed in Keio University School of Medicine. All experimental procedures were approved by the ethics committee of Keio University and were conducted in accordance with the National Institutes of Health guidelines. Human animal care conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture

Murine corneal epithelial cells (TKE2) were cultured in defined-keratinocyte serum-free medium (DKSFM; Life Technologies Corporation, Carlsbad, CA) supplemented with EGF (10 ng/mL) and cholera toxin (0.1 µg/mL). Cells were fed every 3 days and passaged every 7 days at a density of 5 × 10^5 cells/75 cm^2 flask. Mouse corneal epithelial cell lines were established from abcg2 KO mice and wild-type (WT) littermate by a modified protocol from our previous report. In brief, corneal explants including the limbus were cultured in six-well plates with DKSFM. After 5 days to 1 week, cells were treated with dissociation medium (TrypLE Express; Life Technologies, Grand Island, NY) for 7 to 10 minutes at 37°C to remove contaminating fibroblasts and differentiated epithelial cells. This process was repeated three times every 5 days until culture plates were confluent with epithelial cells. Confluent cells were then dissociated with dissociation medium (TrypLE Express; Life Technologies) for 15 minutes, passaged into 75-cm^2 flasks, and maintained as a stable cell line.

Detection of Side Population Cells

Dissociated TKE2 cells were suspended in DKSFM (10^6 cells/mL) and were incubated at 37°C for 15 minutes with or without the ABCG2 blocker verapamil hydrochloride (50 µM; Sigma-Aldrich, St. Louis, MO).
MO). Subsequently, cells were treated with Hoechst 33342 (5–10 μg/mL; Dojindo Laboratory, Kumamoto, Japan) at 37°C for 90 minutes. After washing, the cells were stained with propidium iodide (PI; 2 μg/mL, Dojindo Laboratory) and were analyzed (FACS Vantage; Becton-Dickinson, Franklin Lakes, NJ).

RT-PCR Assay

Genomic DNA was isolated with a blood and tissue kit (DNeasy; Qiagen, Germantown, MD) according to the supplied protocol. RNA was isolated with a purification kit (RNasy; Qiagen), and cDNA was synthesized (ReverTra Ace; Toyobo, Osaka, Japan). Polymerase chain reaction (PCR) was performed (Blend Taq [Toyobo] and Gene Amp 9700 [Applied Bioscience Inc., Foster City, CA]). Primers used for PCR were as follows: abcg2 WT allele (forward, AGGCCACTCTTCCAAGACT; reverse, GCAGCGCATCGCCTTCTATC), abcg2 KO allele (forward, AGGCCACTCTTCCAAGACT; reverse, GCAGCGCATCGCCTTCTATC), and β-actin (forward, TGTTACCAACTGGGACGACA; reverse, TCTACGTGTGGTTGTAAG). PCR products were analyzed by agarose gel electrophoresis.

Immunostaining

Cryosection of murine corneas and TKE2 cells cultured in four-well chamber slides (Nunc, Thermo Fisher Scientific, Waltham, MA) was fixed with ice-cold 4% paraformaldehyde in PBS for 5 minutes. Cell cultures were permeabilized with 0.25% nonionic surfactant ( Triton-X; Promega, Madison, WI) in PBS at room temperature (RT) for 10 minutes. After blocking, cryosections were reacted with an anti–cytokertatin-12 (K12) antibody (4 μg/mL, sc-17101; Santa Cruz Biotechnology, Santa Cruz, CA), anti–K15 antibody (4 μg/mL, LHK15, MS-10668-P1, Neomarkers; Thermo Fisher Scientific, Waltham, MA), and cell cultures were stained with an anti–ABCG2 antibody (10 μg/mL, BXP21, MAB4146; Chemicon, Millipore, Billerica, MA) at RT for 1 hour. After washing, samples were stained with Cy3- or FITC-conjugated secondary antibody (Chemicon) and counterstained with DAPI (1 μg/mL; Dojindo Laboratory). Immunofluorescence was examined by epifluorescence microscopy (Axio Imager; Zeiss, Oberkochen, Germany).

Cell Viability Assay

Cells were seeded into 96-well plates (5 × 10⁴ cells/well) and were cultured overnight. Cells were fed with new medium containing 1.2% ovoalbumin (Sigma-Aldrich) with or without the ABCG2 blocker reserpine (10 μM; ApoBio; Astek, Fukuoka, Japan) at 37°C for 72 hours. After washing, cells were dissociated by enzyme treatment (TrypLE Express; Life Technologies) and were filtrated with 70-μm cell strainer (Becton-Dickinson). Centrifuged cells were suspended in PBS with 1.5 μM PI (10⁶ cells/mL) and were analyzed (Alta HyperSort System; Beckman Coulter, Fullerton, CA).

Cell Viability under Hypoxia

Cells were seeded into 96-well plates and cultured overnight under normoxia. Then they were cultured under either normoxia or hypoxia (1% O₂) with the use of an N₂/CO₂ multigas incubator (APM-30D; Astek, Fukuoka, Japan) for an additional day. PI (final concentration, 5 μM) was added to stain dead cells. The center of each well was photographed with a fluorescence microscope, and PI-positive cells were counted with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

RT-PCR and RT-PCR Results

Mice treated with 10 ng/mL, 0.1 μg/mL, and 1 μg/mL of the ABCG2 blocker reserpine (10 μM; ApoBio; Astek, Fukuoka, Japan) at 37°C for 72 hours. After washing, cells were dissociated by enzyme treatment (TrypLE Express; Life Technologies) and were filtrated with 70-μm cell strainer (Becton-Dickinson). Centrifuged cells were suspended in PBS with 1.5 μM PI (10⁶ cells/mL) and were analyzed (Alta HyperSort System; Beckman Coulter, Fullerton, CA).

Detection of Reactive Oxygen Species

Reactive oxygen species (ROS) were detected by using 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate (CM-DCHDFA; Life Technologies), which is a 2', 7'-dichlorofluorescein (DCF) derivative. The reduced form of DCF derivative becomes fluorescent by oxidation. Cells were seeded in six-well plates (1.5 × 10⁶ cells/well) and cultured overnight. Cells were fed with new medium containing 80 μM MIT, with or without 10 μM reserpine, and the antioxidant N-acetyl-L-cysteine (NAC; 300 μM; Nakarai-Tesque, Kyoto, Japan) at 37°C for 72 hours. After washing with PBS, cells were stained with CM-H₂DCFDA (10 μM) and PI (1.5 μM) at 37°C for 5 minutes. After washing twice with PBS, immunofluorescence was examined by epifluorescence microscopy (Axio Observer; Zeiss) or was measured by flow cytometry. For flow cytometry, CM-H₂DCFDA-stained cells were dissociated by enzyme treatment (TrypLE Express; Life Technologies) and were filtrated with 70-μm cell strainer (Becton-Dickinson). Centrifuged cells were suspended in PBS with 1.5 μM PI (10⁶ cells/mL) and were analyzed (Alta HyperSort System; Beckman Coulter, Fullerton, CA).

Cell Viability under Hypoxia

Cells were seeded into 96-well plates and cultured overnight under normoxia. Then they were cultured under either normoxia or hypoxia (1% O₂) with the use of an N₂/CO₂ multigas incubator (APM-30D; Astek, Fukuoka, Japan) for an additional day. PI (final concentration, 5 μM) was added to stain dead cells. The center of each well was photographed with a fluorescence microscope, and PI-positive cells were counted with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Detection of Reactive Oxygen Species

Reactive oxygen species (ROS) were detected by using 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate (CM-DCHDFA; Life Technologies), which is a 2', 7'-dichlorofluorescein (DCF) derivative. The reduced form of DCF derivative becomes fluorescent by oxidation. Cells were seeded in six-well plates (1.5 × 10⁶ cells/well) and cultured overnight. Cells were fed with new medium containing 80 μM MIT, with or without 10 μM reserpine, and the antioxidant N-acetyl-L-cysteine (NAC; 300 μM; Nakarai-Tesque, Kyoto, Japan) at 37°C for 72 hours. After washing with PBS, cells were stained with CM-H₂DCFDA (10 μM) and PI (1.5 μM) at 37°C for 5 minutes. After washing twice with PBS, immunofluorescence was examined by epifluorescence microscopy (Axio Observer; Zeiss) or was measured by flow cytometry. For flow cytometry, CM-H₂DCFDA-stained cells were dissociated by enzyme treatment (TrypLE Express; Life Technologies) and were filtrated with 70-μm cell strainer (Becton-Dickinson). Centrifuged cells were suspended in PBS with 1.5 μM PI (10⁶ cells/mL) and were analyzed (Alta HyperSort System; Beckman Coulter, Fullerton, CA).
RESULTS

abcg2 KO Mice Showing Normal Corneal Phenotype

abcg2 KO mice are born without an apparent phenotype; however, they are sensitive to phototoxicity when fed with chow enriched with pheophorbide. We were able to reproduce skin lesions; however, the same mice did not show any abnormalities in the ocular surface, including the expression of K12 and K15 (Fig. 1). Primary cultures of corneal epithelial cells were successfully passaged from abcg2 KO corneas with no apparent difference in proliferative capacity compared with littermate WT control corneas.

Effect of Inhibition of ABCG2 on ROS Production in Cultured Epithelial Cells

The TKE2 cell line was enriched in SP cells, as shown by flow cytometry (Figs. 2A, 2B). ABCG2 expression was confirmed by immunocytochemistry and RT-PCR (Figs. 2C, 2D). When TKE2 cells were pretreated with the oxidant marker CM-H2DCFDA, background fluorescence was observed after oxidation of CM-H2DCFDA to the fluorescent form, DCF (Fig. 3A). Treatment with the pro-oxidant MIT enhanced oxidative stress-related fluorescence (Fig. 3B), which was further enhanced by treatment with the ABCG2 inhibitor reserpine (Fig. 3C). Enhanced fluorescence by the addition of reserpine was confirmed by flow cytometry (Fig. 3D, dotted line) compared with MIT treatment alone (solid line) or with the addition of the antioxidant NAC (gray peak).

ABCG2 Conferring Resistance to Oxidative Damage-Induced Cell Death

To investigate the effects of ROS on cell viability, WST-1 assay was performed using TKE2 cells and passaged abcg2 KO corneal epithelial cells. MIT showed a dose-related increase in cell death in TKE2 cells that was significantly enhanced with simultaneous treatment with the ABCG2 inhibitor Ko143 (Fig. 4A) or reserpine (Fig. 4C). By using two different inhibitors, we showed that cell toxicity was not an intrinsic property of the inhibitor. A significant increase in sensitivity to MIT was also observed in abcg2 KO cells compared with WT control (Fig. 4B). The IC50 of WT was 135 μM and that of IC50 of KO was 106 μM. Hypoxia was chosen as an intrinsic source of ROS formation for cytotoxicity assays. WT cells were resistant to hypoxic culture conditions of 1% O2 (54.5 ± 30.9 PI-positive cells per field), whereas a significant increase in cell death was observed after hypoxic culture of abcg2 KO cells (108.4 ± 63.7 PI-positive cells per field; Fig. 5).

DISCUSSION

ABC transporters are plasma membrane proteins that can cause multidrug resistance in cells by actively extruding a wide variety of structurally diverse compounds from them. ABCG2 was also discovered as a candidate gene for resistance to antitumor drugs, including MIT. When it was first discovered that the ability of ABCG2 to efflux the Hoechst 33342 dye could be used to isolate hematopoietic stem cells from the bone marrow, researchers attempted to find abnormalities in the developmental process of mice with defects in the abcg2 gene. Contrary to expectations, studies using knockout mice did not reveal any hematologic or developmental deficits. Despite the apparent normal phenotype of abcg2 KO mice, Jonker et al. found that the loss of transporter function of porphyrins renders these mutant mice sensitive to phototoxicity. Alterations in the metabolism of low molecular weight metabolites such as heme or drugs may cause ABCG2 (+) progenitor cells to become sensitive to xenobiotics or oxidative stress related to such compounds.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932968/)
Since the cornea is located at the body surface, unlike the bone marrow, we hypothesized that ABCG2 may function to protect against phototoxicity or other forms of oxidative stress. Although we were able to reproduce phototoxic skin lesions in the abcg2 KO mice, as reported by Jonker et al.21 (and data not shown), these mice showed normal cytokeratin expression in the cornea (Fig. 1), possibly because of protection by the eyelids or a higher tolerance of corneal epithelial cells compared with the skin. However, higher doses of pheophorbide were lethal and, therefore, impossible to examine. We resorted to an in vitro system using TKE2 murine epithelial cells18 and passaged corneal epithelial cells from abcg2 KO mice.

The expression of ABCG2 was confirmed in TKE2 cells (Fig. 2) by PCR and flow cytometry. When TKE2 cells were treated with the pro-oxidant MIT, DCF-related fluorescence was observed, reflecting the production of intracellular ROS. Fluorescence intensity was enhanced by the addition of the ABCG2 inhibitor reserpine (Fig. 3). This suggests that the application of drugs that inhibit ABCG2 function may increase oxidative stress within progenitor cells expressing ABCG2. Furthermore, treatment with MIT induced cell death in TKE2 and WT epithelial cells in a dose-dependent manner, and a significant increase in cell death was observed by the addition of Ko143 or reserpine or by knocking out the abcg2 gene (Fig. 4). Cell

**Figure 3. Detection of mitoxantrone-induced ROS in TKE2 cells.** We used the fluorescent probe CM-DCH, which only becomes fluorescent after cleavage by intracellular ROS. Untreated control cells show only background fluorescence (A) compared with cells treated with MIT (B). Fluorescence was further enhanced when the ABCG2 inhibitor reserpine was added to the medium (C). ROS formation was also demonstrated by flow cytometry (D). Fluorescence intensity of MIT-treated cells (solid line) was enhanced by the addition of reserpine (dotted line). The addition of the antioxidant NAC reduced ROS-related fluorescence in cells treated with MIT and reserpine (gray peak).

**Figure 4. MIT-induced cell death in TKE2, abcg2 KO, and WT cells.** (A) TKE2 cells treated with both MIT and ABCG2 inhibitor Ko143 significantly decreased IC50 compared with MIT alone, indicating that inhibiting ABCG2 with Ko143 increases cell death from MIT. (B) WT and KO cells from passages 7 to 20 were used for experiments. Cell viability of abcg2 KO cells decreased with the addition of MIT, with a significantly lower IC50 compared with WT cells. (C) A significant increase in cell death was also observed with the ABCG2 inhibitor reserpine.
culture under hypoxia (1% O₂) also induced a significantly higher rate of cell death in \( \text{abcg2} \) KO cells compared with WT cells, further showing that loss of function of ABCG2 enhances susceptibility to oxidative stress (Fig. 5).

Although ABCG2 does not seem to play a key role in the embryonic development of the corneal epithelium, the gene may play a role in the maintenance of the progenitor cell pool. Stem cells in the bone marrow are sensitive to oxidative stress, and regulation of ROS formation by the ATM gene is vital in maintaining self-renewing properties in HSCs. Given that the corneal epithelium is constantly under oxidative stress because of ambient oxygen concentrations and environmental UV radiation, several antioxidative measures are known to exist. For example, the limbal stem cell niche is rich in melanocytes that provide melanin to basal limbal epithelial cells to form “melanin caps.”

Corneal epithelial cells are also equipped with antioxidant defense systems such as superoxide dismutase and the glutathione/glutathione peroxidase systems. The results of our study also have several implications regarding the possible role of loss of function in the \( \text{abcg2} \) gene as a trigger of corneal epithelial progenitor depletion. For example, inhibition of ABCG2 may be related with the onset of drug-induced stem cell depletion causing pseudo-ocular cicatricial pemphigoid. Drugs applied topically to the ocular surface are relatively concentrated compared with drugs administered systemically. Clinically used compounds such as \( \beta \)-blockers may have an affinity to ABCG2, altering transporter function. On the other hand, increased drug metabolism by the cytochrome P450 pathway leads to an increase in the turnover of porphyrin, which, like pheophorbide, contains a
heme moiety that can cause an accumulation of ROS within the cell. Given that loss-of-function single nucleotide polymorphisms have been identified in the human \textit{abcg2} gene, an unfortunate combination of factors may cause a chronic loss of stem cells in susceptible individuals.

Further studies are required to elucidate the function of ABCG2 in the maintenance of the corneal epithelium. If a relationship between ABCG2 function and drug-induced cell damage does indeed exist, the WST-1 assay combined with \textit{abcg2} KO epithelial cells may be used as a toxicity-screening tool for the development of new topical drugs. Studies to confirm such possibilities are under way.

**Acknowledgments**

The authors thank Yumi Matsuzaki of the Department of Physiology and Akira Sonoda of the Central Research Laboratory of the Keio University School of Medicine for valuable advice on flow cytometry techniques.

**References**


