VEGF Antagonists Decrease Barrier Function of Retinal Pigment Epithelium In Vitro: Possible Participation of Intracellular Glutathione

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Purpose. To investigate the influence of VEGF antagonists on the barrier function of the retinal pigment epithelium and underlying mechanisms.

Methods. Porcine RPE cells were cultured on six-well membrane inserts. The cells were exposed to bevacizumab (62.5 μg/mL) or ranibizumab (25 μg/mL) for 24 hours (short term) or 9 days (long term). Transepithelial flux of FITC-dextran and intracellular levels of reduced glutathione (GSH) at normal and low-glucose conditions were investigated at different points in time. The influence of the addition of triamcinolone acetonide (TA) was investigated. The effect of GSH depletion on RPE permeability was examined using L-buthionine sulfoximine (BSO), a γ-glutamylcysteine synthethase inhibitor.

Results. After short-term exposure, VEGF antagonists increased the transepithelial flux of FITC-dextran significantly on day 2. Bevacizumab, but not ranibizumab, increased permeability up to 9 days. Under long-term exposure, both drugs enhanced permeability for 7 days; bevacizumab had the stronger effect. The addition of TA inhibited this increase. At the ninth day of short- and long-term exposure, bevacizumab-exposed cells, but not ranibizumab-exposed cells, exhibited a significantly lower GSH level. In the low-glucose condition, both drugs accelerated the decrease of intracellular GSH for the first 48 hours. GSH depletion increased the permeability of retinal pigment epithelium. TA had no effect on BSO-induced GSH depletion.

Conclusions. The results suggest that bevacizumab and ranibizumab may decrease RPE barrier function, with bevacizumab exhibiting a prolonged and more profound effect. Combination with TA is thought to be beneficial because of its protective effect on stabilizing RPE junctional integrity. (*Invest Ophthalmol Vis Sci.* 2010;51:4848–4855) DOI:10.1167/iovs.09-4699

Antagonists of vascular endothelial growth factor (VEGF) have provided a revolutionary change in the treatment of age-related macular degeneration (AMD). The therapeutic principle is the regression of choroidal neovascularization (CNV), followed by a reduction of edema and an improvement of visual acuity. Anti-VEGF therapies with intravitreal injection of bevacizumab (Avastin; Genentech, South San Francisco, CA) and ranibizumab (Lucentis; Genentech) are promising new treatment options in patients with CNV secondary to AMD[1,2] (bevacizumab use is off label). Because vitreous VEGF levels are elevated in patients with diabetic macular edema or macular edema secondary to retinal vein occlusion, anti-VEGF therapy has been introduced as an alternative treatment of these disorders, too.[3–9]

In spite of the favorable results of these therapies, several adverse incidents related to their use, such as retinal pigment epithelial (RPE) tear[10–18] and intraocular inflammation,[19–21] have also been reported. The mechanisms of the incidents are still a matter of speculation. Some researchers have demonstrated that VEGF antagonists exhibited no toxicity on retinal cells when used in clinical concentrations.[22–24] There are still, however, few reports concerning the influence on retinal cells other than toxicity.[25,26] In particular, the influence on RPE cell function has not been well investigated.

RPE tight junctions function as the outer blood-retinal barrier, which contributes to a restricted diffusion barrier between retinal and choroidal perfusion. Because of adverse effects such as RPE tear and intraocular inflammatory reactions, it is possible that anti-VEGF treatment disturbs the barrier or some related cellular functions by, for example, influencing RPE permeability. We were previously able to show that oxidative stress induced increased permeability of cultured RPE cells that was accompanied by redox imbalance with reduced glutathione (GSH), one of the most important antioxidant components in all animal cells.[27] The increased permeability and redox imbalance can be prevented by triamcinolone acetonide (TA).[28]

In this study, therefore, we investigated the influence of bevacizumab and ranibizumab on the paracellular permeability of cultured retinal pigment epithelium and on intracellular GSH levels and the effect of TA in combination with VEGF antagonists.

Materials and Methods

Cell Culture

Porcine eyes were obtained from a local slaughterhouse, and the RPE cells were isolated as previously described.[29] Cells were cultivated with Dulbecco's modified Eagle's medium (DMEM high glucose 4.5 mg/mL; PAA, Cölbe, Germany) supplemented with penicillin/streptomycin, L-glutamine, sodium pyruvate, and 10% porcine serum (PAA). Cells were incubated at 37°C under 5% CO_2. The medium was changed every 2 days, and the cells were subcultured by trypsin-ethylenediaminetetraacetic acid (EDTA) digestion when they reached confluence, as assessed by phase-contrast microscopy. Second-passage cells were used in all experiments. The integrity and the stability of the RPE junction used in the study were confirmed by immunostaining of zonular occludence-1 (ZO-1) and the measurement of transepithelial resistance (TER). For immunostaining of ZO-1, rabbit polyclonal ZO-1 antibody (Hypromatrix, Inc., Worcester, MA) and goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as a first and a second antibody, respectively. TER was measured with an epithelial voltmeter (EVOM; World Precision Instruments, Sarasota, FL) until 5 weeks after plating. The TER of the filter alone was subtracted from the results obtained from the filters with RPE cells. Final resis-

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tance-area products were calculated by multiplying by the area of the cell growth ($\Omega \cdot cm^2$).

Permeability Assay

Transepithelial permeability was evaluated as previously described.[30] In brief, second-passage RPE cells were cultured on top of transwell-clear polyester membrane inserts for six-well plates (0.4-μm pore size; Corning Incorporated, Corning, NY) under the culture conditions described here and were used in the experiments 4 weeks after reaching confluence. Twenty-four hours before assay, the cells were exposed to bevacizumab (62.5 μg/mL) or ranibizumab (25 μg/mL) from the cell's apical side, with or without TA (10^{-5} M; Sigma, St. Louis, MO). The concentrations of VEGF antagonists were approximately one fifth those of clinical use. TA stock solution was made as previously described.[31] The assay was conducted using fluorescein isothiocyanate (FITC)-dextran (4FD; Sigma), which was added to the upper medium. Five hours after the addition of FITC-dextran, medium from the lower chamber was collected, and the amount of fluorescence was measured using a fluorescence spectrofluorophotometer (FP-550; Jasco, Tokyo, Japan) with an excitation wavelength of 488 nm and an emission wave length of 530 nm.

After collecting the lower medium at the first day (day 1), the medium was completely replaced by the new one, and the cells were cultured either without any treatment until the end of the test (short-term test) or with continuous exposition to bevacizumab or ranibizumab, with or without TA, during the entire experimental period (long-term test). The medium was exchanged every day, adding the antagonists and TA each time in the long-term test. Both short- and long-term tests lasted for 9 days. The transepithelial flux of FITC-dextran for 5 hours was investigated on days 1, 2, 3, 7, and 9, as described.

VEGF Immunoassay

For the measurement of VEGF concentration in the culture medium after the exposure of VEGF antagonists, the supernatant of the cell culture was collected in the permeability assay, as described. The VEGF concentration was measured with a VEGF-ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Determination of GSH

At the last day of the short- and long-term permeability assays (day 9), the cells on the membrane were collected by trypsinization, and the intracellular GSH levels were examined using a commercial kit according to the manufacturer's protocol (Glutathione Assay Kit; Trevigen Inc., Gaithersburg, MD). This kit uses a kinetic enzymatic recycling procedure of 5,5′-dithiobis-2-nitrobenzonic acid/glutathione reductase.

Given that GSH synthesis needs nicotinamide adenine dinucleotide phosphate (NADPH), which is synthesized in the pentose phosphate pathway using glucose as a source, the glucose-depleted condition significantly decreases the production of new GSH. To clarify the effect of bevacizumab and ranibizumab on the consumption of intracellular GSH, glutathione assay was performed under the two different glucose concentrations: the high-glucose condition (4.5 mg/mL), which is normally used for the culture of RPE cells, and the glucose-depleted condition, in which cells are cultured in low-glucose medium (1 mg/mL; DMEM low glucose; PAA). Second-passage RPE cells cultured on 35-mm dishes were exposed by either bevacizumab (62.5 μg/mL) or ranibizumab (25 μg/mL) and were cultivated for either 1 day or 2 days in the same medium. The cells were collected 1 or 2 days after exposure, and the GSH level was measured as described.

Permeability Assay with Glutathione Depletion

The influence of GSH depletion on paracellular permeability was investigated. Cells were treated with L-buthionine sulfoximine (BSO 1 mM; Sigma), an inhibitor of γ-glutamyl cysteine synthetase, which is the rate-limiting enzyme of GSH synthesis,[32] during the entire experimental period in permeability assay. To confirm the GSH depletion by BSO, the GSH level was measured with the GSH assay kit as described.

Statistical Analysis

Each experiment was conducted in triplicate and repeated three times. Statistical significance was determined by paired Student's *t*-test. $P < 0.05$ was considered significant.

Results

Characteristics of the Tight Junctions of the RPE Cell Culture

To confirm the quality of the junctional integrity of RPE cell cultures used in the present study, morphologic and functional characteristics of the junctions were evaluated. As shown in Figure 1A, RPE cells showed a typical epithelial morphology and formed a monolayer close to the in vivo situation. Figure 1B demonstrates immunostaining of ZO-1, one of the tight junction proteins. ZO-1 protein localized at the cell-cell border and showed clear continuous linear staining. Nuclear staining was also observed; its intensity was reduced gradually during cultivation but lasted weeks after plating. TER increased dramatically even after confluence around 4 days after plating, and it reached high resistance (around 1400 $\Omega \cdot cm^2$) around 3 weeks after plating and lasted for weeks (Fig. 1C).

Transepithelial Flux after Short-term Exposure of VEGF Antagonists

As shown in Figure 2A, cells that were exposed to the VEGF antagonists for the first 24 hours showed significantly higher flux on day 2 (1 day after the removal of bevacizumab and ranibizumab; 143% ± 19% and 126% ± 22% of the control, respectively; $P < 0.05$). After that (from day 3), the ranibizumab-exposed cells displayed flux comparable to that of control cells, whereas bevacizumab-exposed cells displayed significantly higher flux until day 9. TA suppressed this increase and stabilized the flux during the entire experimental period but did not have a significant effect on flux when applied alone.

Increased Transepithelial Flux by Bevacizumab and Ranibizumab in a Continuous Exposure (Long Term)

Cells were cultured under the conditions with bevacizumab or ranibizumab, with or without TA, during the entire experimental period. As shown in Figure 2B, in longer exposure, ranibizumab also induced increased permeability for several days, although the increase was less pronounced than it was in bevacizumab-exposed cells. Bevacizumab-exposed cells exhibited the peak of the flux on day 2 (254.4% ± 68.2% of control) and ranibizumab-exposed cells on day 3 (163.3% ± 35.6% of control), after which the flux gradually decreased. On day 9, the permeability of the ranibizumab-exposed cells returned to the control level, whereas the bevacizumab-exposed cells showed still significantly higher flux (157.9% ± 18.6% of control; $P < 0.01$). TA inhibited these flux increases.

VEGF Concentration after VEGF Antagonist Exposure

To confirm VEGF inhibition in all the ranibizumab- and bevacizumab-exposed cells and to investigate whether a relation existed between permeability and VEGF concentration, VEGF concentrations at each time point were measured.

Figure 3 shows the detectable VEGF concentrations in the culture medium after exposure of the VEGF antagonists or TA for the first 24 hours. On the second day, the medium was

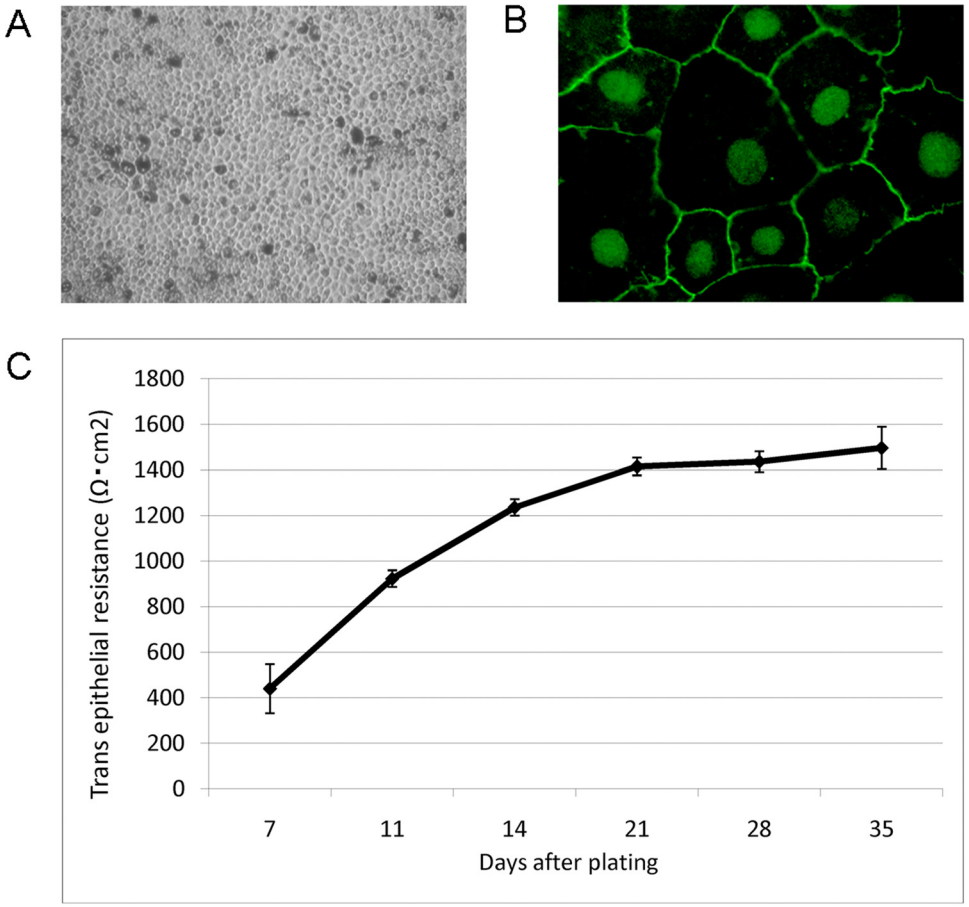

FIGURE 1. Morphologic and functional analysis of junctional integrity of RPE cell culture used in this study. (**A**) Phase-contrast microscope image of confluent second-passage RPE cell culture. The cells are epithelioid and form a monolayer. (**B**) Immunostaining of ZO-1 protein of second-passage RPE cells at 3 weeks after plating. ZO-1 protein is localized at the cell-cell border and forms clear linear staining. Nucleus accumulation was also observed that was reduced during cultivation but lasted for weeks. (**C**) TER increased dramatically even after reaching confluence around 4 days after plating and reached high resistance (around 1400 $\Omega \cdot cm^2$) around 3 weeks after plating and was maintained for weeks.

replaced by a new one without any of these agents. On the first day of treatment, there was no difference in VEGF concentration between bevacizumab- and ranibizumab-exposed cells (0 pg/mL). Twenty-four hours after the removal of bevacizumab or ranibizumab (day 2), bevacizumab-exposed cells secreted small amounts of VEGF (58 ± 15 pg/mL), and even less was secreted from TA plus bevacizumab-exposed cells (40 ± 5 pg/mL; $P < 0.05$). In the medium of ranibizumab-exposed cell cultures on day 2, little VEGF was detected (5 ± 2 pg/mL). TA plus ranibizumab-exposed cells secreted no VEGF in the medium on day 2. On day 3, ranibizumab-exposed cells started to secrete detectable amounts of VEGF in the medium (377.5 ± 144.5 pg/mL), whereas bevacizumab-exposed cells already secreted about three times as much (1052 ± 40.1 pg/mL; $P < 0.01$). TA-exposed cells secreted significantly less both in bevacizumab- and ranibizumab-exposed cell cultures (711 ± 90.5 pg/mL and 211 ± 133 pg/mL; $P < 0.01$ and $P < 0.05$, respectively). On day 9, the VEGF concentrations of both bevacizumab- and ranibizumab-exposed cell cultures were comparable to normal levels (1078 ± 60.3 pg/mL and 1290 ± 150.9 pg/mL, respectively), whereas TA-exposed cells still secreted slightly less VEGF (1000.22 ± 70.3 and 889 ± 126.4 pg/mL, respectively), in which the difference was significant between TA plus ranibizumab-exposed cells and the control ($P < 0.01$).

Change of Intracellular GSH Level after Bevacizumab and Ranibizumab Exposure (Early Phase for 2 Days) in High- and Low-Glucose Conditions

Intracellular GSH levels were investigated after the exposure of VEGF antagonists. They were measured using glutathione assay kit, as described in Materials and Methods. GSH levels of the cells cultured for 1 hour in a nontreated fresh culture medium were defined as the control level (100%). In the high-glucose condition (4.5 mg/mL), there was no significant difference in GSH levels between the control and the bevacizumab- and ranibizumab-exposed cells (Fig. 4A). However, GSH levels after the exposure of VEGF antagonists varied strongly among cultures derived from different donor animals, which resulted in the big standard variations shown at day 1 in the graph (bevacizumab 158.2% ± 100.8%, ranibizumab 137.5% ± 96.11%). We suspected, therefore, that an increase in GSH consumption was induced by the VEGF antagonists' exposure and that the difference in the potential ability of GSH new synthesis among the donor animals resulted in the large variations in GSH level. Low-glucose conditions, in which cells are less able to compensate for the loss of GSH by new synthesis, could disclose the GSH consumption (Fig. 4B). In this condition, GSH levels in bevacizumab- and ranibizumab-exposed cells declined significantly faster than in nontreated control cells in the first 2 days, and ranibizumab-exposed cells showed significantly faster decreases than bevacizumab-exposed cells (bevacizumab-exposed cells, 70.1% in 1 day and 45.9% in 2 days; ranibizumab-exposed cells, 44.6% in 1 day and 25.1% in 2 days; nontreated controls, 85.9% in 1 day and 73.1% in 2 days).

GSH Levels on Day 9 after Short- and Long-term Exposure of Bevacizumab and Ranibizumab

Figures 5A and 5B show the amount of GSH on day 9 after short- and long-term permeability assay, respectively. Total GSH levels in bevacizumab-exposed cells were significantly lower in both short- and long-exposure (80.2% ± 12.4% and

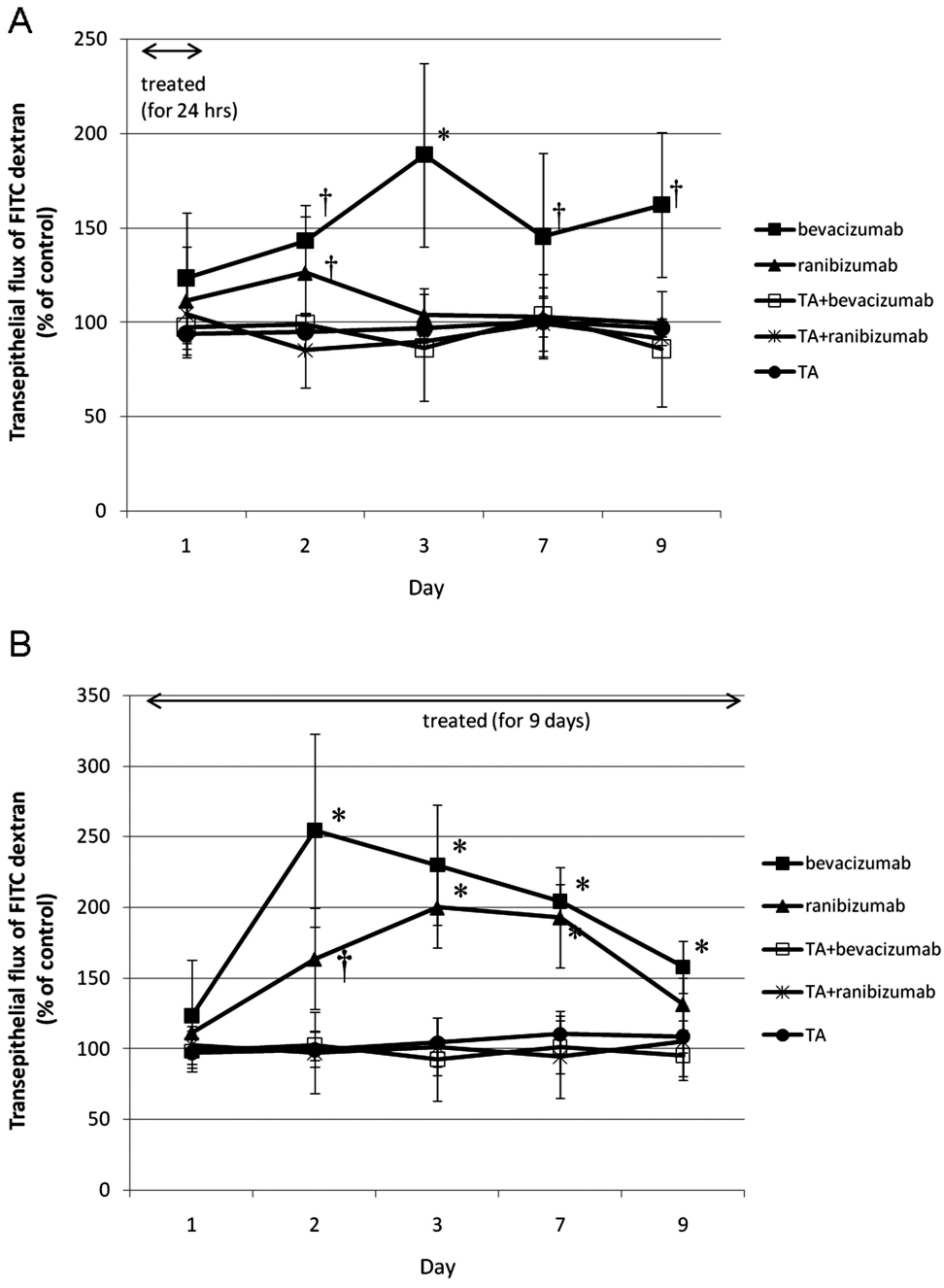

FIGURE 2. Permeability assay under short-term (24 hours) (**A**) and long-term (9 days) (**B**) exposure of VEGF antagonists. (**A**) After 24 hours of exposure, the flux of FITC-dextran was measured as day 1. The medium was completely replaced with fresh medium without antagonists or TA. On indicated days, FITC-dextran flux was measured. Both antagonists increased flux on day 2. Ranibizumab-exposed cells recovered to control level, whereas bevacizumab had a prolonged effect. (**B**) Under continuous exposure, both antagonists increased flux significantly, in which the effect by bevacizumab was larger than by ranibizumab and returned to control level at day 9 when treated with ranibizumab but not with bevacizumab (*$P < 0.01$, †$P < 0.05$ compared with the nontreated control).

63.4% ± 14.9%; $P < 0.05$ and $P < 0.01$, respectively). GSH levels in ranibizumab-exposed cells on day 9 were comparable to those in nontreated control cells.

Relation between GSH Depletion and RPE Permeability

The influence of GSH decrease on RPE permeability was investigated. The GSH-depleted condition was achieved by the use of 1 mM BSO, the inhibitor of γ-glutamyl cysteine synthethase, and the GSH level was measured using a glutathione assay kit. The GSH level declined 51.1% ± 2.3% by BSO 1 day after exposure (Fig. 6A). TA did not inhibit this decrease. The transepithelial flux of FITC-dextran was measured under this GSH-depleted condition. BSO and TA were exposed with a constant concentration during the entire experimental period. The transepithelial flux increased significantly after the exposure of BSO. With the peak on day 4 (119.54% ± 5.2% of control; $P < 0.01$), flux recovered to the control level (Fig. 6B). TA suppressed BSO-induced increase of permeability.

DISCUSSION

The present study shows that bevacizumab and ranibizumab induce increased permeability of cultured retinal pigment epithelium, with a stronger and longer lasting effect seen with bevacizumab, suggesting that these VEGF antagonists might decrease RPE barrier function; bevacizumab exhibited a stronger effect than ranibizumab. Cells exposed for 24 hours with ranibizumab returned to baseline at day 3, whereas bevacizumab-exposed cells exhibited elevated flux until day 9. Even cells that were continuously exposed to ranibizumab returned

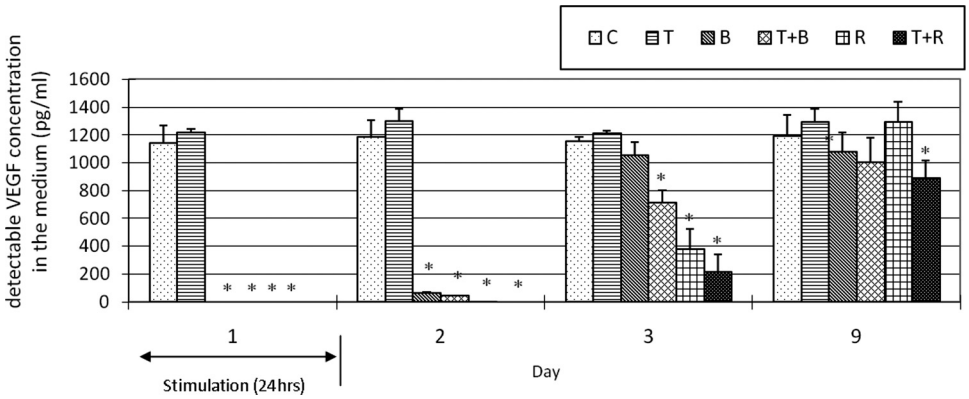

FIGURE 3. Detectable VEGF concentration after the exposure and removal of VEGF antagonists and TA. By the exposure of the antagonists, VEGF in the medium was completely neutralized. After the removal of these agents from the medium, VEGF was first detected in the medium from bevacizumab-exposed cells (day 2) and then from ranibizumab-exposed cells 1 day later (day 3). The VEGF amount in bevacizumab-exposed cells on day 3 was significantly higher than in ranibizumab-exposed cells. TA-added medium showed lower VEGF levels. On day 9, only the cells with TA plus ranibizumab-exposed cells still expressed significantly lower amount of VEGF compared with the control. C, control; T, TA; B, bevacizumab; T+B, TA plus bevacizumab; R, ranibizumab; T+R, TA plus ranibizumab. $^*P < 0.01$ compared with nontreated control.

to baseline at day 9. In addition, in general, the effect seen with bevacizumab was more profound than with ranibizumab.

The development of RPE tears has been reported as among the possible incidences to follow anti-VEGF treatment.[10–18] The tear can occur in eyes with classic or occult CNV, regardless of the existence of pigment epithelial detachment (PED).[10,17] It is, however, obvious that the incidence rate of RPE tear is significantly higher in eyes with PED.[10,16–18,33] It is assumed that the retinal pigment epithelium in an existing PED is already severely weakened because of the pathologic mechanism; therefore, the incidence rate is higher than in eyes

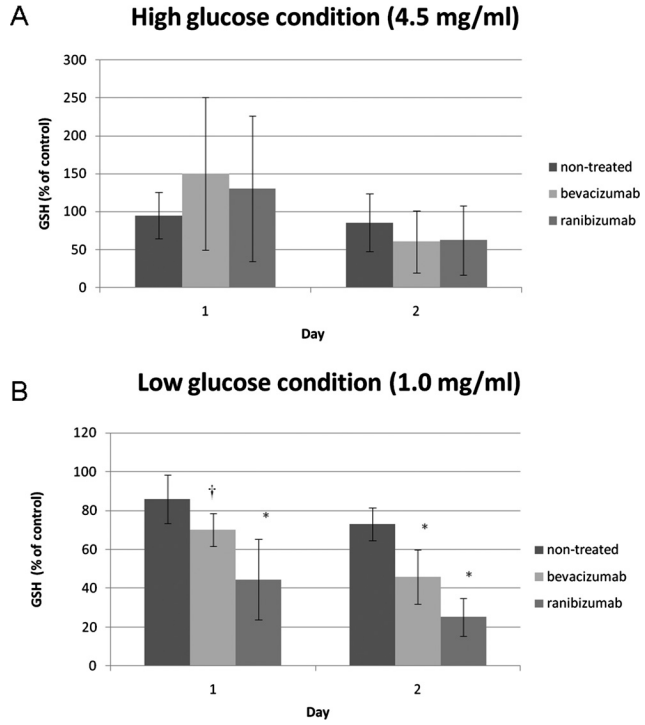

FIGURE 4. Intracellular GSH levels after the exposure of VEGF antagonists under high (4.5 mg/mL; **A**) and low (1.0 mg/mL; **B**) glucose conditions. GSH levels were measured up to 2 days after exposure. No medium exchange was conducted during the entire experimental period. (**A**) Under high-glucose conditions, a profound variation of GSH level among the individuals was detected after 1 day of exposure of the antagonists. On day 2, variation among the individuals decreased, and the mean GSH was lower than in the control, though this was not significant. (**B**) Under low-glucose conditions, GSH decreases during cultivation were observed even in control cells (86.0% in 1 day, 73.2% in 2 days). The antagonist-exposed cells significantly accelerated the GSH decrease in the first 2 days. Ranibizumab-exposed cells showed a faster GSH decrease than bevacizumab-exposed cells (bevacizumab-exposed cells, 70.1% in 1 day and 45.9% in 2 days; ranibizumab-exposed cells, 44.6% in 1 day and 25.1% in 2 days) ($^*P < 0.01$, $\dagger P < 0.05$ compared with the nontreated control of the same day).

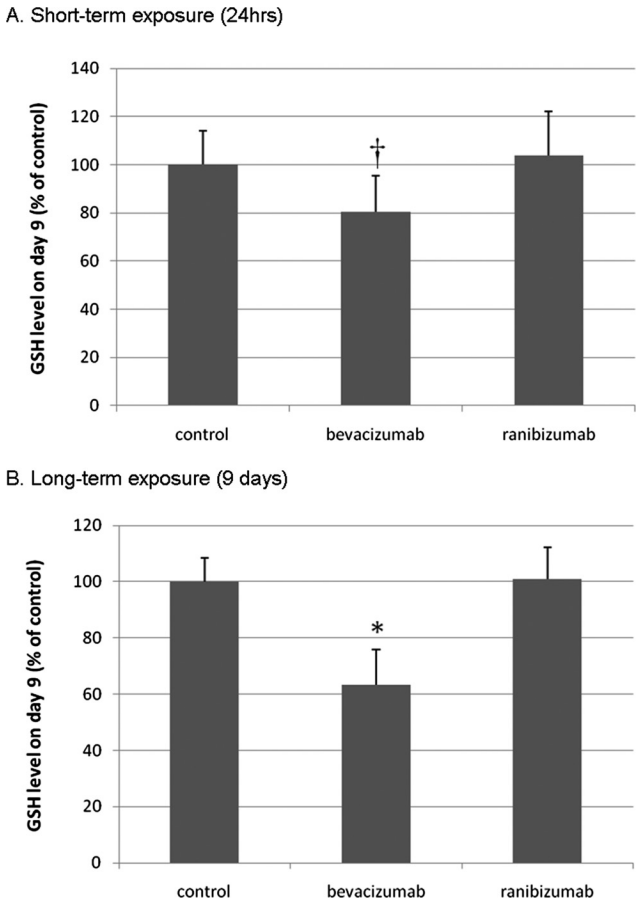

FIGURE 5. Intracellular GSH levels on day 9, after (**A**) short-term (24 hours) and (**B**) long-term (9 days) exposure of VEGF antagonists. (**A**) RPE cells on the membrane insert in permeability assay were collected on day 9, and the GSH levels were measured. Bevacizumab-exposed cells showed significantly lower amounts of intracellular GSH (80.2% after short-term exposure, 63.4% after long-term exposure). Ranibizumab-exposed cells showed no difference in GSH level ($^*P < 0.01$, $\dagger P < 0.05$ compared to the nontreated control).

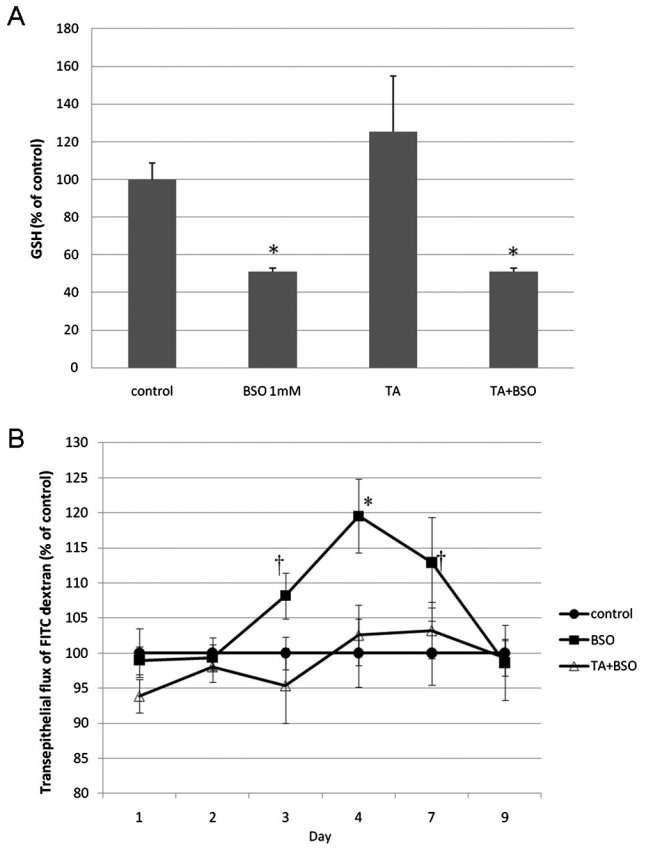

FIGURE 6. Effect of GSH depletion by BSO on RPE permeability. (**A**) GSH level was measured after BSO, TA, and TA plus BSO exposure. BSO (1 mM) decreased the GSH level to 51.4%. TA did not have any effect on this decrease. (**B**) Permeability assay under GSH-depleted condition by BSO (continuously exposed). BSO induced a temporal increase of transepithelial flux of FITC dextran, which had a peak on day 4 (119.5%) and recovered to the control level on day 9. Addition of TA suppressed this increase significantly (*$P < 0.01$, †$P < 0.05$ compared with the nontreated control).

without PED. We speculate that anti-VEGF treatment increases the natural risk for RPE tears. Several potential mechanisms have been proposed, including rapid resorption of the sub-RPE fluid and contraction of the CNV. Additionally, the disturbance of RPE barrier function, suggested in this study, might be one of the participating mechanisms. To the best of our knowledge, the difference in the incidence rate between bevacizumab- and ranibizumab-treated eyes has not been thoroughly investigated. More data have been published with bevacizumab than with ranibizumab, and, according to these previous reports, most researchers presented an incidence rate around 17% after the bevacizumab treatment,[16,18,33] whereas with ranibizumab, an incidence rate of 12.3% is presented.[17] The authors suggest that this rate is similar to the incidence rate in the natural course of PED (10%–12%)[34,35] and that ranibizumab injection might be associated with a lower tendency for inducing RPE tears compared with bevacizumab treatment. Chiang et al.[16] reported a retrospective study investigating the RPE tear incidence rate in eyes with preexisting PED. They presented a rate of RPE tear four times higher after bevacizumab treatment than after ranibizumab treatment. If the hypothesis holds true that bevacizumab induces RPE tear at a higher rate than ranibizumab, the difference between these two drugs presented in this study might be strongly related to the pathogenesis of RPE tear after anti-VEGF treatment.

Results from the concurrently performed intracellular GSH measurement suggested GSH consumption by VEGF antagonists. Given that redox imbalance after oxidative stress is accompanied by increased permeability in RPE cells,[28] we considered that GSH consumption might be related to the decrease of RPE barrier after the exposure of bevacizumab and ranibizumab. In the early phase (up to 2 days after exposure), these antagonists presented characteristic influences on the intracellular GSH levels. In high-glucose (4.5 mg/mL) conditions, which contain enough resources for new GSH synthesis, GSH levels after 1 day of exposure were not statistically significant, but they showed strong variation in GSH levels among cultures from different donor animals. The large standard deviation might be explained by the fact that different experiments were conducted with primary cells from different donor animals that seemed to differ in their ability to synthesize GSH. Experiments conducted with cells derived from the same animals always exhibited similar results. GSH compensation by new synthesis seemed to occur differently among the cultures, possibly reflecting the situation among patients. It is reported that the ability of GSH synthesis diminishes with aging[36] and varies with the individual, depending on the nutrient state, smoking habit,[37] and genetic factors. To circumvent this difference between cultures, we conducted experiments under low-glucose conditions (1 mg/mL), in which the source of GSH synthesis was insufficient. Results revealed that increased GSH consumption or loss of GSH by either VEGF antagonist occurs in the early phase after exposure.

Bevacizumab-exposed cells showed both prolonged increases of permeability and low GSH levels after 9 days. Based on this result, it is strongly suggested that bevacizumab has a prolonged influence on the functionality of RPE cells. Interestingly, this result agrees with those of a study[38] recently reported by our laboratory, in which we demonstrated the accumulation of bevacizumab, but not of ranibizumab, in RPE cells, treated for 24 hours, after 7 days of exposure. It is tempting to speculate that the intracellular bevacizumab accumulation might be connected to the GSH decrease of the late phase and thus that it affects the functionality of RPE cells, including barrier function.

Intracellular GSH is known to regulate endothelial/epithelial permeability.[39,40] To our knowledge, however, the relation between intracellular GSH level and RPE permeability has not been investigated. Our results show that GSH depletion by BSO induced the temporal increase of RPE permeability. If the GSH decrease after bevacizumab-exposure is related to its intracellular accumulation, as discussed, it is assumed that the decrease of GSH becomes significant sometime after exposure. Therefore, we consider that the prolonged increase of permeability after bevacizumab-exposure might be related to the decrease of GSH. As another possibility of the mechanism for the decrease of GSH, Ghibelli et al.[41] reported the nonoxidative loss of GSH associated with apoptosis through GSH extrusion. RPE viability 1 week after bevacizumab exposure has been investigated in vitro in our laboratory, in which no significant increase of cell death was detected.[38] We consider, therefore, apoptosis-induced GSH depletion to be unlikely. For whatever reason, bevacizumab-induced intracellular GSH decrease might be of concern because it might cause damage to the retinal pigment epithelium, which could be critical especially for AMD patients.

As an explanation of early GSH consumption suggested by the experimental results shown in Figure 4, slight oxidative stress by these VEGF antagonists might occur. Thus far, however, we have not detected any direct oxidative stress change, but this idea can be supported by a recent report Byeon et al.,[42] who show a protective effect of VEGF on RPE survival

under oxidative stress. Their results suggest that these VEGF antagonists may decrease the antioxidant potential in RPE cells.

It is well known that VEGF increases the permeability of vascular endothelial cells,[43,44] but the basic role of VEGF on RPE cell function has still not been well investigated, and results regarding the effect of VEGF on the permeability of the retinal pigment epithelium are controversial. In some studies, VEGF caused the decrease of RPE transepithelial resistance,[45] reflecting disturbed junctional integrity, whereas in others, VEGF stabilized the junction of retinal pigment epithelium.[46] In our present study, no significant direct relation between VEGF concentration and RPE permeability was found as an immediate response, though long-term effect of VEGF depletion is still to be elucidated. VEGF concentration in the culture medium was completely neutralized after treatment with bevacizumab or ranibizumab, regardless of the simultaneous exposure of TA. After removal of these agents, VEGF was detected again, and recovery of the secretion from ranibizumab-exposed cells was slower than recovery from bevacizumab-exposed cells, corresponding well to the higher efficacy of ranibizumab we previously confirmed in an organ culture model.[47] TA significantly slowed down the recovery of VEGF secretion, which is consistent with previous reports.[31]

TA stabilized RPE permeability, even in GSH-depleted conditions, indicating that TA has a protective effect on RPE junctions through the GSH-independent pathway. The possible mechanism, such as a direct influence of TA on the junctional protein, is still unclear and must be further investigated. From this protective effect of TA, it is conceivable that TA might reduce the rate of RPE tear induced by anti-VEGF drugs. Previously, we presented the protective effect of TA on RPE junctions from oxidative stress, which might also play a role in anti-VEGF treatment. Rebound or rapid reincrease of VEGF after anti-VEGF treatment, which might induce the recurrence of CNV or macular edema, could also be prevented by TA. Therefore, the administration of TA with anti-VEGF drugs is considered to be beneficial in various aspects, as supported by clinical reports in which combination therapy with TA achieved preferable clinical consequences.[48,49] The benefit of combination therapy with VEGF antagonists for AMD has been reported not only with TA. Photodynamic therapy (PDT) is reported to improve the therapeutic effect and thus can reduce the number of the treatments.[50,51] As new tools for combination therapy, anti–platelet-derived growth factor and integrin $\alpha5\beta1$ inhibitor are in early-phase clinical trials.[52,53] Regarding the incident of RPE tear, however, its rate has not been studied thus far in any of the other combination therapies tested.

In conclusion, the early- and late-phase events in RPE cells after bevacizumab and ranibizumab exposure shown in this study, especially considering RPE barrier function, exhibited a profound effect on the RPE function of bevacizumab and a difference between these two antagonists that might relate to clinical issues such as RPE tear or ocular inflammation. The involvement of GSH consumption was suggested as one of the mechanisms for these effects. Further clinical investigation is needed to clarify the difference between bevacizumab- and ranibizumab-treated eyes and to evaluate our hypothesis about the pathologic mechanisms of RPE tear. To preserve the outer blood-retinal barrier stability, TA might be beneficial if it is combined with VEGF antagonists.

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