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LABORATORY INVESTIGATION

Subretinal Delivery of Immunoglobulin G with Gold Nanoparticles in the Rabbit Eye

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Abstract

Purpose: To examine the feasibility of subretinal delivery of immunoglobulin G (IgG) adsorbed onto gold nanoparticles (GNPs) and its histologic distribution in the rabbit retina after the injection.

Methods: Goat IgG was adsorbed onto GNPs electrostatically. Goat IgG-adsorbed GNPs or buffer with goat IgG was injected into the subretinal space of rabbit eyes and followed up for 3 months by examination of fundus photographs, immunohistochemistry against goat IgG, and transmission electron microscopy (TEM). Human retinal pigment epithelial cells (ARPE-19 cells) were cultured, and cell proliferation with or without GNPs was assayed.

Results: At 1 week after the subretinal injection of goat IgG-adsorbed GNPs, retinal degeneration was observed in the outer retina, and goat IgG was immunolabeled in the retinal pigment epithelium (RPE) and the photoreceptor cells. TEM showed GNPs located in the outer segments and in the lysosomes in the RPE at 1 month and no apparent cytotoxicity of the RPE. There were no inhibitory effects of GNPs on proliferation of ARPE-19 cells.

Conclusions: Goat IgG was successfully delivered into photoreceptor cells and RPE using GNPs, though retinal degeneration in the outer retina occurred in this model. This might be an alternative drug delivery method to photoreceptors and RPE. **Jpn J Ophthalmol** 2009;53:249–256 © Japanese Ophthalmological Society 2009

Key Words: IgG, gold nanoparticle, rabbit, retina, subretinal delivery

Introduction

Colloidal gold has been used for immunogold labeling in cytochemistry for several decades.[1] Recently, researchers have also been studying the biological properties of naked gold nanoparticles (GNPs) and using them as diagnostic and therapeutic tools, such as X-ray contrast agents and antiangiogenic agents.[2–4] Moreover, GNPs have been engineered for delivery of biomolecules such as nucleic acids and proteins for target-specific delivery by conjugation of GNPs with specific antibodies such as anti-epidermal growth factor (EGF) receptor antibody.[5–7]

Inhibition of ocular angiogenesis is important for preserving vision in eyes with neovascular diseases such as proliferative diabetic retinopathy and neovascular maculopathy.[8] Intravitreal injections of anti-vascular endothelial growth factor (VEGF) antibody have been shown to dramatically inhibit ocular angiogenesis.[8] To enhance the delivery of anti-VEGF antibody or other antiangiogenic agents to specific sites in the eye, GNPs may have some advantages because gold is an inert metal and nano-sized particles have a large surface area.[5,9,10]

In this study, we examined the feasibility of subretinal delivery of goat immunoglobulin G (IgG) adsorbed onto GNPs into rabbit eyes.

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Materials and Methods

Preparation of Gold Nanoparticles

Gold nanoparticles were prepared by citrate reduction of chloroauric acid as previously described.[1,7] Briefly, after 100 ml of auric acid solution (1 mM) was heated to boiling, and 10 ml of sodium citrate solution (38.8 mM) was added and stirred. Then, the GNP solution was cooled to and stored at room temperature. Visible spectrophotometry and transmission electron microscopy (TEM) were employed to examine the homogeneity and size of the prepared GNPs.

Adsorption of Goat IgG onto Gold Nanoparticles

Nonspecific goat IgG (#I5256; Sigma-Aldrich, St. Louis, MO, USA) was electrostatically adsorbed onto GNPs as described previously.[7] Briefly, the GNPs were diluted in 20 mM HEPES buffer (pH 10) to a final gold concentration of 1 mM with an optical density of about 0.8 at 520 nm. The goat IgG was dissolved in the 20 mM HEPES buffer at a concentration of 1 mg/ml. To adsorb the goat IgG onto the GNPs, 100 µl of the goat IgG solution was added to 2 ml of the diluted GNP solution and incubated for 1 h at room temperature. Then, the solution was centrifuged at 10 000 *g* for 10 min and the supernatant discarded. The goat IgG-adsorbed gold pellet was resuspended in 0.01 M phosphate-buffered saline (PBS) buffer (pH 7.4). The goat IgG-adsorbed GNPs were prepared for each subretinal injection.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

To detect the goat IgG electrostatically bound to the GNPs, the goat IgG-adsorbed GNPs were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a NuPAGE electrophoresis system (Invitrogen, Grand Island, NY, USA) as described previously.[11] Briefly, goat IgG-adsorbed GNPs with three different concentrations of goat IgG were prepared. The prepared GNPs were centrifuged and resuspended in a NuPAGE LDS sample buffer. Then, NuPAGE reducing agent was added to the sample. The samples were boiled in water for 5 min and then put on ice. Each sample was centrifuged at 14 000 *g* for 15 min, and the supernatants were isolated. The supernatants were loaded on a 4%–12% Bis–Tris gradient gel according to the manufacturer's manual. After electrophoresis, the gel was stained with Coomassie brilliant blue dye and photographed.

Animals

Pigmented Dutch-belted rabbits weighing 1–1.5 kg were purchased from Myrtle's Rabbitry (Thompson's Station, TN, USA). Twenty-two rabbits were used for this study. All animal procedures adhered to the provisions of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of San Francisco Veterans Medical Center.

Surgical Procedures

The animals were anesthetized with an intramuscular injection of 35 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride. The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride eye drops. Before the surgical procedures, the ocular surface was anesthetized with topical instillation of 0.5% proparacaine hydrochloride eye drops.

Under an operating microscope, paracentesis was performed using a 28-gauge needle attached to an insulin syringe. Then, the sclera was punctured at 2 mm from the limbus using a 25-gauge needle. A RetinaJect subretinal cannula (SurModics, Eden Prairie, MN, USA) was inserted through the sclerotomy site into the vitreous cavity.[12] A contact lens was put on the cornea, and the fundus observed under an operating microscope. About 0.15–0.2 ml of the suspension of goat IgG-adsorbed GNPs in 0.01 M PBS or goat IgG dissolved in 0.01 M PBS was injected into the subretinal space, which resulted in retinal detachment of about 3–5 optic disc diameters inferior to the disc. No vitrectomy was performed. The sclerotomy site was not sutured, and bacitracin ophthalmic ointment (500 U/g) was put in the cul-de-sac at the end of the surgery. Only one eye of each rabbit was operated on and used for the study.

Fundus photographs of the rabbit eyes were taken at 1 week, 1 month, and 3 months after the surgery before euthanasia of rabbits.

Histologic Procedures

The rabbits were euthanized at 1 week, 1 month, or 3 months after the surgery, and the eyes were removed for histologic examinations, as described previously.[13] Six rabbits were euthanized at each time point. The eyes were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The injected area was excised and processed for paraffin sections. For TEM, the eyes were fixed in Karnovsky's fixative (1% paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). Two rabbits were euthanized at 1 month and 3 months after the surgery, respectively, and the eyes were processed for TEM.

Transmission Electron Microscopy

The methods for TEM have been previously described.[13] Briefly, the tissues were fixed in Karnovsky's fixative at

4°C. Fixed tissues were then rinsed in water, postfixed in reduced OsO_4 (2% OsO_4 + 1.5% potassium ferrocyanide), and stained en bloc with uranyl acetate. The tissues were then dehydrated in ethanol, cleared in propylene oxide, and embedded in Eponate 12 (Ted Pella, Redding, CA, USA). Thick sections were cut and stained with toluidine blue. Then, they were examined under a light microscope to select the area for thin sections. The thin sections were cut using a Leica Ultracut UCT microtome (Leica Microsystems, Bannockburn, IL, USA) and examined under a Philips Tecnai 10 electron microscope (Philips, Eindhoven, The Netherlands).

Immunohistochemistry Against Goat IgG

After the eyes were fixed in the fixative solution, the retina–choroid–sclera complex was excised and embedded in paraffin. The paraffin sections were prepared, mounted on the slides, and dried. After deparaffinization, the slides were put in the microwave to retrieve the antigen. The slides were then soaked in 0.3% H_2O_2 for 10 min and washed in 0.1 M PBS containing 0.05% Tween-20. The slides were then incubated with a biotinylated anti-goat IgG antibody (Vector Laboratories, Burlingame, CA, USA) overnight at room temperature. On the next day, after the slides were rinsed in 0.1 M PBS containing 0.05% Tween-20, they were incubated with ABC reagent solution (PK-6105-Vectastain Elite ABC kit; Vector Laboratories) according to the manufacturer's manual. Vector NovaRED substrate, a peroxidase substrate, was used to color the immunopositive reactions in the slides red. After the slides were counterstained with hematoxylin, they were dehydrated in graded alcohols, cleared, and mounted. The slides were observed under a microscope and photographed.

Cell Culture

Human retinal pigment epithelial cells (ARPE-19 cells) were purchased from ATCC (CRL-2302; Manassas, VA, USA) and cultured as previously described.[14] The cells were plated at a density of 2.0×10^3 cells/cm^2 on culture dishes and grown in the growth medium, Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (SH30071.03; HyClone, Logan, UT, USA), and 100 U/ml of penicillin G and 100 µg/ml of streptomycin at 37°C in 5% CO_2 in air. The growth medium was changed every 2 to 3 days, and cells cultured between passages 5 and 8 were utilized.

To examine the effects of GNPs on the proliferation of ARPE-19 cells, ARPE-19 cells were plated at 1.0×10^5 cells per well on six-well culture plates and cultured in the growth medium at 37°C in 5% CO_2 in air with or without GNPs. Three different concentrations of GNP were added to the growth medium at the time the ARPE-19 cells were plated. The final concentrations of the GNPs in the growth medium were 10 µM, 100 µM, and 1 mM, respectively. The growth medium with GNPs was changed at 3 days after plating.

To examine the proliferation curves of ARPE-19 cells in the growth medium with different concentrations of GNPs, the cells in the culture plates were washed with calcium- and magnesium-free PBS three times and trypsinized at 24, 48, 72, and 96 h after plating. The cells were counted with a hemocytometer. At each time point, three separate culture wells were counted three times each. The control proliferation curve of ARPR-19 cells was obtained by counting the numbers of ARPE-19 cells cultured in the growth medium only.

Results

Visible Spectrophotometry and Size of the GNPs

As shown in Fig. 1, the GNPs showed a typical absorption curve with maximum absorption at 520 nm. This absorption

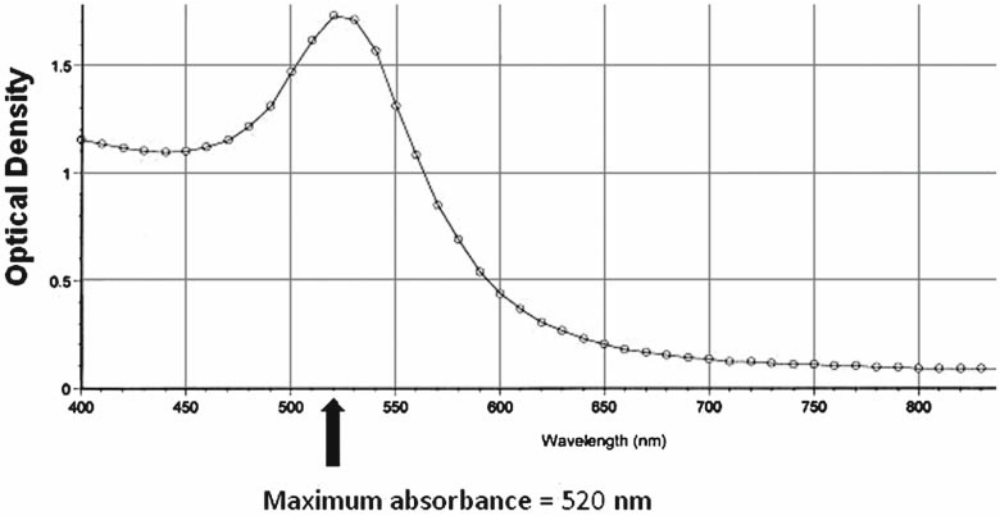

Figure 1. Visible spectrophotometer curve of gold nanoparticles (GNPs) prepared by the chemical reduction of chloroauric acid. The maximum absorption was 520 nm (*arrow*).

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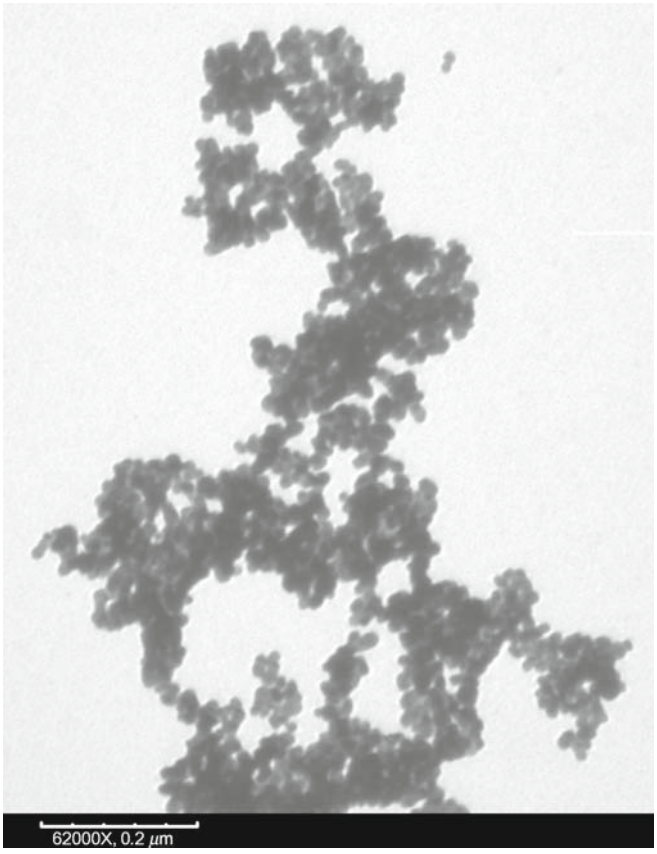

Figure 2. Photomicrograph of GNPs by transmission electron microscopy (TEM). GNPs were homogenous with diameter of 12 nm.

curve and maximum absorption were consistent on every occasion. TEM of the GNPs showed that the GNPs were homogenous with an average size of 12 nm (Fig. 2).

SDS-PAGE

To examine whether the goat IgG was adsorbed onto the GNPs, SDS-PAGE and Coomassie blue staining of the gel were performed. As shown in Fig. 3, 1 mg of goat IgG was loaded in lane 2 as a positive control, and two strong bands of 26 and 51 kDa were detected. Lane 1 was a molecular marker. These 26 and 51 kDa bands corresponded to denatured IgG. Lane 3 of GNPs not incubated with IgG showed no protein bands and served as a negative control. The same two 26- and 51-kDa bands were detected in lanes 4, 5, and 6, with GNPs incubated with 100 μg, 300 μg, and 1 mg of goat IgG, respectively. To examine unadsorbed goat IgG in the supernatant after the incubation of goat IgG with GNPs, the supernatants were collected and prepared for SDS-PAGE. Lanes 7 and 8 were the supernatants after incubation of 300 μg and 1 mg of goat IgG with GNPs, respectively. No protein bands were detected in lanes 7 and 8.

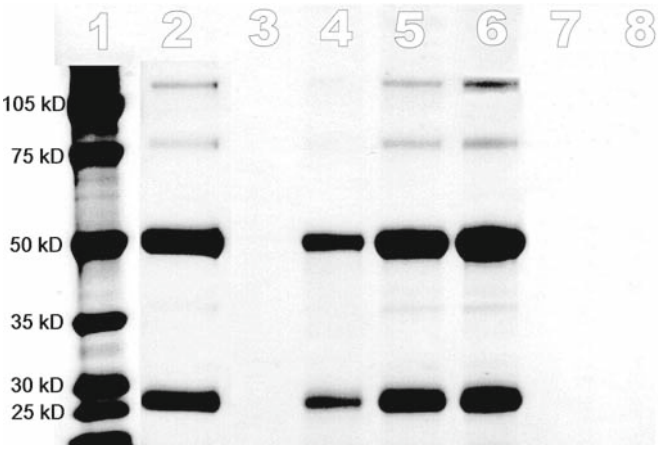

Figure 3. Photograph of the gel after Coomassie blue staining. *Lane 1*, molecular marker; *lane 2*, 1 mg goat IgG (positive control); *lane 3*, GNPs only (negative control). In *lanes 2, 4, 5*, and *6*, two strong bands were detected at 26 and 51 kDa, which correspond to denatured IgG. *Lanes 4, 5*, and *6* contained goat IgG-adsorbed GNPs after incubation with 100 μg, 300 μg, and 1 mg of goat IgG with GNPs, respectively. *Lanes 7* and *8* contained the supernatants after incubation of 300 μg and 1 mg of goat IgG with GNPs, respectively. No protein bands were detected in lanes 7 and 8.

Fundus Photographs After the Subretinal Injection

Fundus photographs were taken after the subretinal injections. Figure 4a shows a fundus photograph taken immediately after the subretinal injection of goat IgG-adsorbed GNPs (arrows). At 1 month after the injection of goat IgG-adsorbed GNPs, the retinal detachment had been completely resolved, and the retinal pigment epithelium (RPE) in the injected area showed mild pigmentation and retinal degeneration (arrows in Fig. 4b). At 3 months after the injection of goat IgG-adsorbed GNPs, the pigmentation of the RPE and retinal degeneration in the injected area was increased, and a clear demarcation line between the injected area and the intact area was observed (arrows in Fig. 4c). A similar pigmentation of the RPE and retinal degeneration was observed at 3 months after the injection of goat IgG with PBS (arrows in Fig. 4d) and after the injection of GNPs only (data not shown).

Immunohistochemistry Against Goat IgG in the Retina

To localize the delivered goat IgG in the retina, immunohistochemistry against goat IgG was performed in the eyes at 1 week and 1 month after the subretinal injections. As shown in Fig. 5a, at 1 week after the injection of goat IgG-adsorbed GNPs, the goat IgG was detected in the RPE (arrowhead), the photoreceptor layer (arrow), the outer nuclear layer (asterisks), and partially in the outer plexiform layer, and retinal degeneration was observed in the outer nuclear layer and the photoreceptor layer.

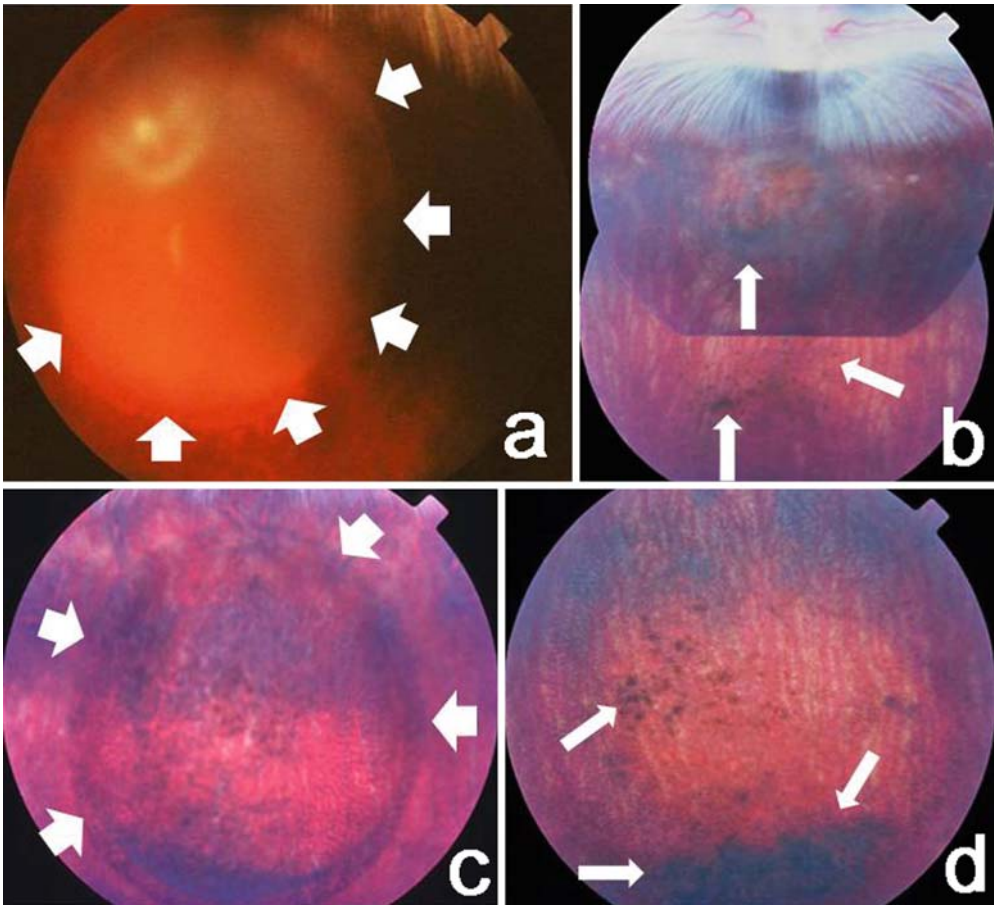

Figure 4. a Fundus photograph immediately after the subretinal injection of goat IgG-adsorbed GNPs. A retinal detachment was created (*arrows*). **b** Fundus photograph at 1 month after the injection of goat IgG-adsorbed GNPs. The retinal pigment epithelium (RPE) in the injected area showed mild pigmentation (*arrows*). **c** Fundus photograph at 3 months after the injection of goat IgG-adsorbed GNPs. Pigmentation of the RPE in the injected area was increased and clearly visible (*arrows*). **d** Fundus photograph at 3 months after injection of goat IgG with phosphate-buffered saline (PBS). A similar pigmentation of the RPE in the injected area was observed (*arrows*).

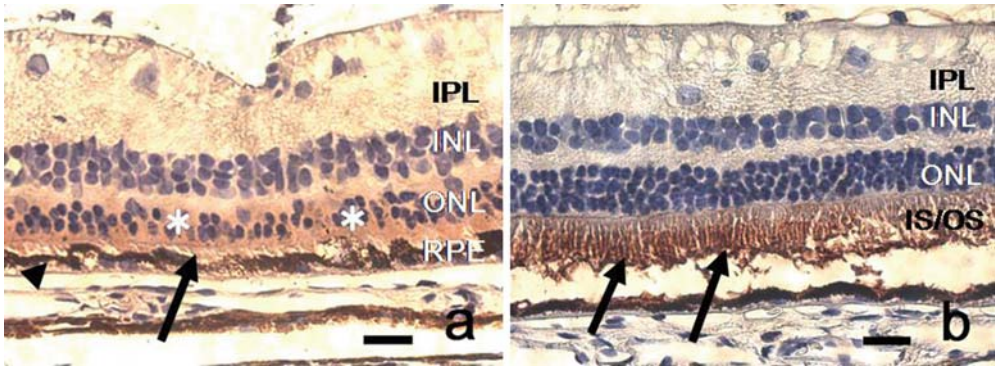

Figure 5a, b. Photomicrographs of immunohistochemistry against goat IgG. **a** At 1 week after the injection of goat IgG-adsorbed GNPs, immunopositive reactions were detected in the RPE (*arrowhead*), the photoreceptor layer (*arrow*), the outer nuclear layer (*ONL*) (*asterisks*), and partially in the outer plexiform layer. Retinal degeneration was observed in the ONL and the photoreceptor layer. Bar = 20 µm. **b** At 1 week after the injection of goat IgG with PBS, immunopositive reactions were observed only in the subretinal spaces (*arrows*). The subretinal spaces included spaces between the inner and outer segments (*IS*, *OS*) of the photoreceptor layer and between the photoreceptor layer and the RPE. *IPL*, inner plexiform layer; *INL*, inner nuclear layer. Bar = 20 µm.

In contrast, at 1 week after the injection of goat IgG with PBS (Fig. 5b), positive immunoreactions were observed only in the subretinal spaces between the inner and outer segments of the photoreceptor layer and in the subretinal space between the photoreceptor layer and the RPE (arrows in Fig. 5b). Less retinal degeneration of the outer retina was observed after the injection of goat IgG with PBS.

At 1 month after the injection, no positive immunoreaction was detected in the retina after the injection of either goat IgG-adsorbed GNPs or goat IgG with PBS because of

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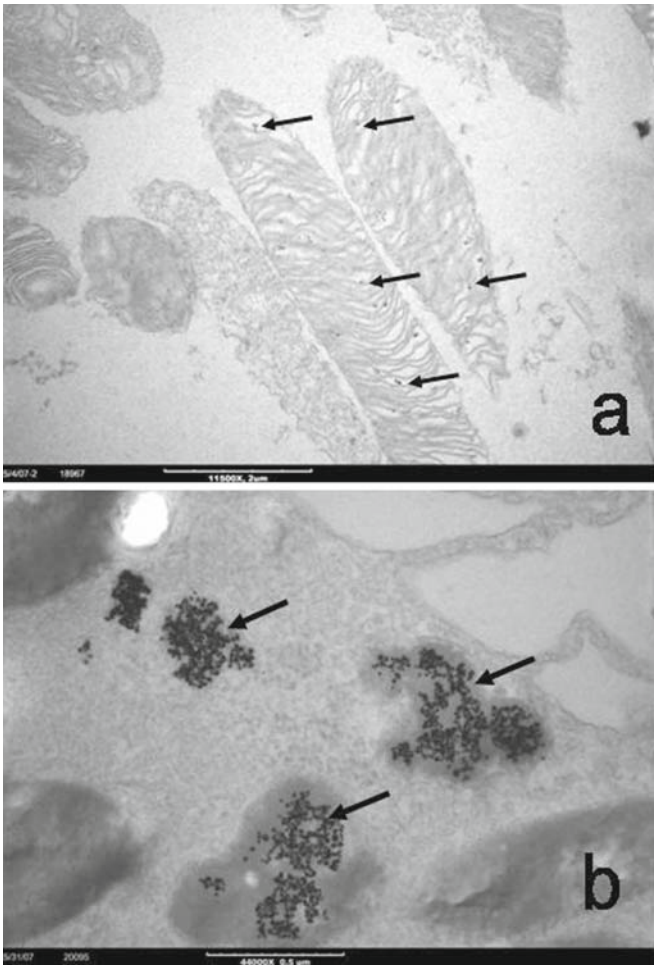

Figure 6a, b. TEM photomicrographs at 1 month after the injection of the goat IgG-adsorbed GNPs. **a** The GNPs were observed in the outer segments of the photoreceptors (*arrows*). **b** The GNPs were accumulated in the lysosomes in the RPE (*arrows*).

extensive degeneration of the outer retina, including the photoreceptor and outer nuclear layers (data not shown).

Transmission Electron Microscopy

To detect intracellular localization of the injected GNPs, TEM was performed at 1 month and 3 months after the subretinal injection of the goat IgG-adsorbed GNPs. At 1 month after the injection, the GNPs were observed in the outer segments of photoreceptors (Fig. 6a, arrows), and the GNPs were also accumulated in the lysosomes in the cytoplasm of the RPE (Fig. 6b). At 3 months after the injection, the GNPs were observed only in the lysosomes of the RPE (arrows in Fig. 7a), and no GNPs were transported outside of the basal side of the RPE (arrowheads in Fig. 7b). No apparent cytotoxicity was observed in the intracellular organelles such as the endoplasmic reticulum (asterisk in Fig. 7a) or the nucleus of the RPE cells.

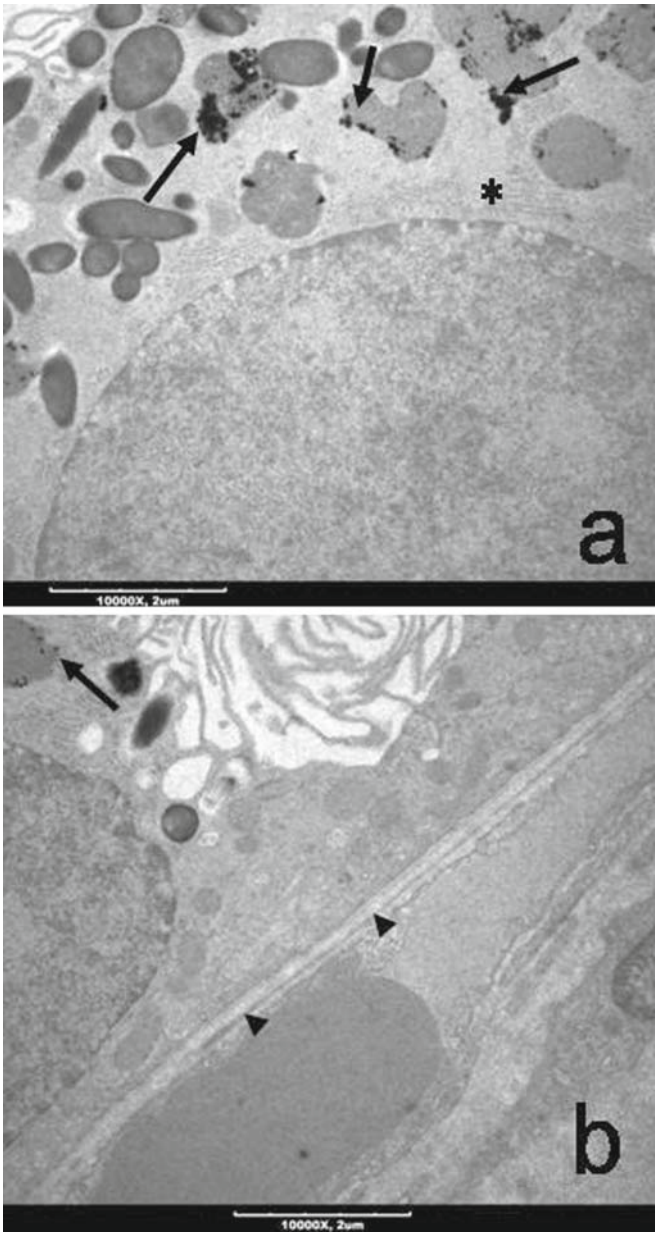

Figure 7a, b. TEM Photomicrographs at 3 months after the injection of the goat IgG-adsorbed GNPs. **a** The GNPs were observed only in the lysosomes in the RPE (*arrows*). No cytotoxicity was observed in intracellular organelles such as the endoplasmic reticulum (*asterisk*) or the nucleus of the RPE cells. **b** No GNPs were transported outside of the basal side of the RPE (*arrowheads*).

ARPE-19 Cell Culture

To examine the effects of GNPs on the proliferation of RPE cells, ARPE-19 cells were cultured and proliferation curves were examined with or without GNPs in the growth medium. As shown in Fig. 8, no statistical difference was detected among the three concentrations of GNPs (10 μM, 100 μM, and 1 mM) and the control. The presence of GNPs in the

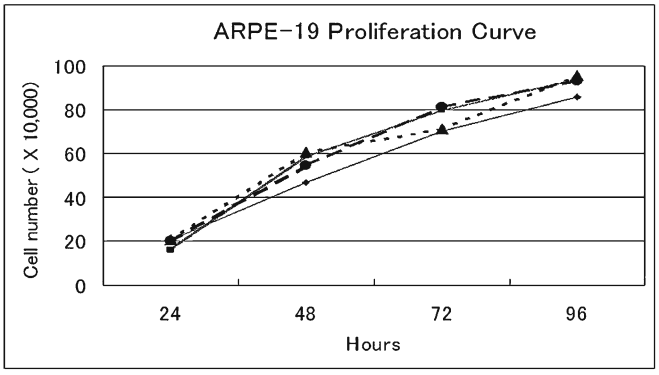

Figure 8. Proliferation curves of ARPE-19 cells in growth medium with 10 μM (·····), 100 μM (------), or 1 mM (——) of GNPs or in growth medium only (control, ——). No statistical differences were detected among these four proliferation curves.

growth medium did not affect proliferation of ARPE-19 cells.

Discussion

The surfaces of GNPs produced by the chemical reduction of chloroauric acid are known to be negatively charged; therefore, GNPs can adsorb positively charged groups of proteins electrostatically.[1,5,7] Because of this characteristic of GNPs, specific antibodies and proteins have been used to produce protein–gold complexes for immunoelectron microscopy and other biological purposes. El-Sayed et al.[7] showed that GNPs were efficiently delivered into cancerous cells with high expression of EGF receptor on their surfaces by adsorption of anti-EGF receptor antibody onto the GNPs. They delivered 600% to 700% more GNPs to those cells than did a nonmalignant cell line.[7] In this study we examined the feasibility of efficiently delivering IgG to the RPE and photoreceptor cells by using GNPs as a carrier. We demonstrated by electrophoresis that goat IgG was successfully adsorbed onto GNPs and that the goat IgG-adsorbed GNPs were delivered into the subretinal space, which resulted in immunopositive reactions against goat IgG in the RPE and photoreceptor cells in the retina. Cytotoxicity of the GNPs was not detected in the RPE by TEM; however, hyperpigmentation of the RPE and retinal degeneration were observed after the injection of either goat IgG-adsorbed GNPs, goat IgG with PBS, or GNPs only. The retinal degeneration might have been caused by the surgical retinal detachment because the rabbit retina has been shown to rapidly degenerate after experimental retinal detachment, even at 2 days after the detachment.[15,16] This might be due to the anatomical characteristics of the rabbit retina, most of which is avascular and fully depends on the choroidal circulation.[16] Other possible causes of the retinal degeneration include mechanical damage of the RPE induced by the stream from the fine needle at the time of the subretinal injection, toxic effects of the GNPs to the retina, and additional effects of immune responses against the goat IgG. To examine interspecies differences, other animal models should be studied in the same manner and an improved method for the subretinal injection should be developed to avoid damage not only to the RPE but also to the photoreceptor cells.

There was a clear difference between the retinas in the immunoreactivity against the goat IgG after the injections of goat IgG-adsorbed GNPs and of goat IgG with PBS. Since we used nonspecific goat IgG, goat IgG-adsorbed GNPs were nonspecifically taken up by the RPE and the photoreceptor cells, which suggests that uptake of the goat IgG-adsorbed GNPs occurred through nonspecific endocytosis and not through receptor-mediated endocytosis.[10] In addition, it is unknown whether the delivered goat IgG was in a functional state because the goat IgG was nonspecifically adsorbed onto the GNP surfaces, which may have induced conformational changes after adsorption and during delivery.[10] These issues should be addressed by further studies.

We demonstrated that IgG was successfully delivered to the photoreceptor cells and the RPE by subretinal injection of IgG-adsorbed GNPs. The GNPs in the cytoplasm of the RPE were observed in lysosomes by TEM. The GNPs in the lysosomes were probably derived from nonspecific endocytosis and phagocytosed outer segments and were still observed in the cytoplasm of the RPE at 3 months after the injection. In addition, the GNPs in the cytoplasm of the RPE cells did not seem to be cytotoxic because intracellular organelles such as the endoplasmic reticulum and the nucleus were observed by TEM to be intact. No exocytosis of the GNPs was observed at the basal side of the RPE, and the GNPs seemed stable in the lysosomes. Obviously, more studies regarding the cytotoxicity of GNPs in the RPE are needed. As a next step, when specific antibodies against receptors of photoreceptors or RPE are adsorbed or chemically conjugated onto GNPs, they might be taken up by receptor-mediated endocytosis and show a different intracellular distribution in the RPE or the photoreceptor cells.

GNPs are known to remain inert in cells.[9,17] Following intravenous injection of GNPs, no chronic or hematological toxicity was detected in mice for 1 month.[2] In the rabbit eye, Bakri et al.[18] showed no toxicity in the retina or the optic nerve at 1 month after intravitreal injection of 670 μmol of GNPs. In this study, we injected 1 μmol of GNP into the subretinal space, an amount that we expected to be within nontoxic concentrations. To examine one aspect of the cytotoxicity of GNPs, we examined their effects on proliferation of a human RPE cell line, ARPE-19. Similar to a previous study, we did not detect any inhibiting or toxic effects of GNPs on proliferation of ARPE-19 cells.[17]

In this study, we demonstrated that IgG-adsorbed GNPs could be delivered to the subretinal space and that the IgG was delivered to photoreceptor cells and RPE. Despite the adverse effect of retinal degeneration observed in this model, GNPs might provide a new drug delivery tool for eye diseases, but further studies are required.

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