Therapeutic Effect of Stealth-Type Polymeric Nanoparticles with Encapsulated Betamethasone Phosphate on Experimental Autoimmune Uveoretinitis

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PURPOSE. The therapeutic effects of betamethasone phosphate (BP) encapsulated in biocompatible and biodegradable blended nanoparticles of poly(lactic acid) (PLA) homopolymers and PEG-block-PLA copolymers (stealth nanosteroids) were examined in an experimental autoimmune uveoretinitis (EAU) model in Lewis rats.

METHODS. EAU was induced by S-antigen peptide in Lewis rats. Accumulation of systemically administered Cy7-labeled stealth nanoparticles in inflamed eyes of rats with EAU was assessed using in vivo fluorescence imaging, and the therapeutic effect of stealth nanosteroids, nonstealth nanosteroids, or saline on EAU was examined. The eyes were obtained 7 days after the treatment, and the histologic score was determined using pathologic findings. The expression of inflammatory cytokines in the retina of EAU.

RESULTS. Cy7-stealth nanoparticles accumulated in inflamed eyes of rats with EAU and remained in situ for a 3-day period. Systemically administered stealth nanosteroids (100 μg of BP) reduced the clinical scores of rats with EAU within 1 day and maintained the effect for 2 weeks. This treatment also decreased the histologic scores and the expression of inflammatory cytokines in the retina of EAU.

CONCLUSIONS. The strong therapeutic benefit on EAU obtained with the stealth nanosteroids may have been due to prolonged blood circulation and targeting to the inflamed uvea and retina, in addition to sustained release in situ. (Invest Ophthalmol Vis Sci. 2011;52:1516–1521) DOI:10.1167/iovs.10-5676

Glucocorticoids are effective in treatment of uveitis,1,2 but their systemic application is limited because of a high incidence of serious adverse effects, particularly in long-term treatment.3 Since intravenously administrated glucocorticoids distribute throughout the body and rapidly disappear, a relatively high dose is needed to achieve an effective concentration at an inflamed target site. Moreover, the physiological activity of glucocorticoids in many different tissues increases the risk of adverse effects. These problems require the development of a delivery system for glucocorticoids that enhances localization to the target site and sustains drug release.4–6

In previous studies, we have described the efficient preparation of poly(lactic acid) (PLA) nanoparticles (NPs) and shown the therapeutic effect of these agents in rats with experimental autoimmune uveoretinitis (EAU).7,8 The NPs exhibited significantly anti-inflammatory activity but were rapidly removed from systemic circulation by phagocytosis, resulting in accumulation in the liver and spleen. Meanwhile, poly(ethylene glycol) (PEG) is used for surface modification of NPs to reduce opsonization and prevent interactions with the mononuclear phagocyte system (MPS).9 NPs prepared from a mixture of PEG-PLA block copolymers and PLA (stealth NPs) have a prolonged retention time in vivo,10 which results in accumulation of the NPs at disease sites and enhances the effectiveness in the treatment of rheumatoid arthritis and asthma.11,12 Thus, PLA NPs with PEG grafting might escape renal exclusion and the MPS, resulting in an increased half-life in plasma.

In the present study, we determined the distribution of stealth NPs in the inflamed uvea and retina and examined the anti-inflammatory activity of BP encapsulated in biocompatible and biodegradable blended nanoparticles of PLA homopolymers and PEG-block-PLA copolymers (stealth nanosteroids) in rats with EAU.

MATERIALS AND METHODS

Materials

PLA with an average molecular weight (Mw) of 6170 was purchased from Wako Pure Chemicals Industries (Osaka, Japan). PEG-PLA was synthesized by ring-opening polymerization of D,L-lactide (Purac America, Lincolnshire, IL) in the presence of monomethoxy-PEG (Mw, 5580; NOF Co., Tokyo, Japan). PEG-PLA with an average Mw of 15,010 was used in this study. Betamethasone phosphate (BP), polyoxyethylene-polyoxypropylene block copolymer (Pluronic F68), diethanolamine (DEA), and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO); 4-(dimethylamino) pyridine was obtained from Merck (Hohenrung, Germany); and cyanidine (Cy7) mono-N-hydroxysuccinimide ester was purchased from GE Healthcare (Amersham, UK). Zinc chloride, polyethylene glycol sorbin monoolecte (Tween 80), acetic acid, dimethyl sulfoxide, and n-dodecylamine hydrochloride were purchased from Wako Pure Chemicals.

Preparation of NPs

NPs were prepared using the oil-in-water solvent diffusion method, as reported previously.13,15 The stealth nanosteroids (diameter, approximately 120 nm), which were manufactured by us, were composed of the PLA (Mw, 6170) homopolymer and a block copolymer of PEG (Mw, 5580) and PLA (Mw, 9430) (PEG content in the polymer blend, 10 wt%).11

Vehicle-only NPs without BP were also prepared as controls. Conventional PLA nanosteroids (nonstealth nanosteroids) formed from PLA homopolymers alone without PEG-PLA copolymers were also prepared.
by addition of an acetone solution of 50 mg PLA, 7.5 mg DEA, 68 µL of 1 M zinc chloride, and 28 µL of 350 mg/mL BP to 0.5% Pluronic F68.7

**Animals and Anesthesia**

Male Lewis rats weighing 200 to 250 g were obtained from Sankyo Labo Service Co. (Tokyo, Japan). All experiments were conducted in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. The rats were anesthetized with a 1:1 mixture of ketamine hydrochloride (10 mg/kg; Wako Pure Chemicals) and xylazine hydrochloride (4 mg/kg; Wako Pure Chemicals).

**Induction and Evaluation of Experimental Autoimmune Uveoretinitis**

Each rat was immunized with a SC injection of 50 µg of peptide 1-20 S-antigen (purity >95%; Takara-Bio, Ohtsu, Japan) emulsified (2 mg/mL) in complete Freund’s adjuvant containing Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI). The rats were examined every other day for clinical signs of EAU, and the clinical severity was graded from 0 to 4 using a previously described protocol8,16,17 with some modifications: grade 0, no inflammation; grade 1, iris vessel engorgement and minimal retinal vasculitis; grade 2, anterior chamber cells and mild retinal vasculitis; grade 3, fibrous exudates at the pupil margin and moderate retinal vasculitis; grade 4, retrolitrat hypopyon and severe retinal vasculitis. Topical 1% tropicamide and 2.5% phenylephrine hydrochloride were instilled to induce mydriasis to wash out floaters. The rats were anesthetized with ketamine hydrochloride (10 mg/kg; Wako Pure Chemicals) or xylazine hydrochloride (4 mg/kg; Wako Pure Chemicals). The animals were examined every other day for clinical signs of EAU, and the clinical severity was graded from 0 to 4 using a previously described protocol8,16,17 with some modifications: grade 0, no inflammation; grade 1, iris vessel engorgement and minimal retinal vasculitis; grade 2, anterior chamber cells and mild retinal vasculitis; grade 3, fibrous exudates at the pupil margin and moderate retinal vasculitis; grade 4, retrolitrat hypopyon and severe retinal vasculitis. Topical 1% tropicamide and 2.5% phenylephrine hydrochloride were instilled to induce mydriasis to wash out floaters. The rats were anesthetized with ketamine hydrochloride (10 mg/kg; Wako Pure Chemicals) or xylazine hydrochloride (4 mg/kg; Wako Pure Chemicals). The disease reached maximum severity on day 15 after immunization, after which the inflammation gradually resolved.

**Biodistribution of Nanoparticles in EAU Rats**

Accumulation of stealth NPs labeled with Cy7 in inflamed eyes was assayed by in vivo fluorescence imaging. A 500 µL aliquot of stealth or nonstealth NPs containing Cy7-dodecylamine (2.6 µg/mL) or free Cy7-dodecylamine was injected into the tail vein of EAU rats (n = 3 in each group) on day 13 after immunization. After 24 h, the rats were anesthetized with sodium pentobarbital and placed into a whole-body animal in vivo imaging system (Optix, GE Healthcare) equipped with band-pass excitation at 750 nm and long-pass emission filters at 770 nm to obtain near-infrared fluorescence images of the eyes.

On the other hand, rats (n = 5 in each group) with EAU were injected in the tail vein with stealth or nonstealth nanosteroids on day 13 after immunization. After 24 hours, the rats were killed by cervical dislocation in the anaesthetic chamber, and the exposed eyes were excised and washed quickly with cold water. The concentration of BP was quantified using a time-resolved fluoroimmunoassay kit.7,11,14,15 The detection limit was 0.01 µg/mL.

**Drug Treatment**

An intravenous injection of 0.5 mL of stealth nanosteroids (containing 100 µg BP), nonstealth nanosteroids (containing 100 µg BP), or saline was given to rats with EAU via the tail vein on day 13 after immunization (n = 8, each). The treatment was carried out when the clinical score reached a mean of 2, which is about half the maximum score in the course of the study. The clinical score was examined until day 35 after immunization in four rats in each group.

**Histopathology and Immunocytochemistry**

Four rats in each group were killed with sodium pentobarbital (intravenous; Wako Pure Chemicals) 7 days after the intravenous injection described above. Eyes were enucleated and immersion fixed for 10 minutes in 4% paraformaldehyde in a 0.1 N sodium cacodylate buffer (pH 7.4; Wako Pure Chemicals). After the cornea and lens were removed, the eyecup was cut in half. One half of the tissue was stored in the fixative solution, and small areas of retina were excised and embedded in low-melting-point agarose for immunocytochemical analysis by confocal microscopy. Sections (100 µm) were cut on a vibratome, blocked overnight in normal donkey serum (1:20; Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C, and then incubated with primary antibodies overnight at 4°C on a rotator. The primary antibodies were mouse monoclonal antibodies to IL-6 (1:100; R&D Systems, Minneapolis, MN), IL-17 (1:100; R&D Systems), and gluta mine synthetase (1:100; Chemicon, Temecula, CA) and a rabbit polyclonal antibody to VEGF (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Mouse and rabbit immunoglobulin G (IgG; Sigma) were used as controls. All antibody solutions were made in PBTA (0.1 M phosphate-buffered saline containing 0.5% bovine serum albumin [Fisher Scientific, Pittsburgh, PA], 0.1% Triton X-100 [Boehringer-Mannheim, Indianapolis, IN], and 0.1% sodium azide [Sigma]). After rinsing with PBTA, the sections were incubated with Cy3-conjugated donkey anti-mouse IgG (IL-17), Cy5-conjugated donkey anti-mouse IgG (glutamate synthetase) or Cy2-conjugated donkey anti-rabbit IgG (IL-6, VEGF; Jackson ImmunoResearch Laboratories) overnight at 4°C on a rotator. The sections were mounted in medium for fluorescence ( Vectashield, Vector Laboratories, Burlingame, CA) and viewed by laser scanning confocal microscopy.

For high-resolution transmitted light microscopy, the other half of the eyecup was fixed in 1% glutaraldehyde (Wako Pure Chemicals) and 1% paraformaldehyde (Wako Pure Chemicals) in a 0.086 M sodium phosphate buffer (pH 7.3) overnight at 4°C, fixed in 2% phosphate-buffered osmium tetroxide (Nissin-EM Co., Tokyo, Japan) for 1 h, and then embedded in epoxy resin (Nissin-EM Co.). Sections of 1 µm were prepared and stained with toluidine blue (Wako Pure Chemicals). The histologic severity of EAU was graded in five histologic sections from each animal in a blinded fashion by two ophthalmologists (TS and GA) using a previously described semiquantitative system.18, no destruction, no cell infiltration, and 1-7, limited or total destruction of the various layers of the retina: grades 1–2, destruction of outer segments of rods and cones; grades 3–4, destruction of the outer nuclear layer; grades 5–6, destruction of the inner nuclear layer; grade 7, destruction of the ganglion cell layer. Severity of disease was calculated as the mean value for all rats from three separate experiments.

**Statistical Analysis**

The clinical score for EAU was compared among the groups using a nonparametric Mann–Whitney U test. P < 0.05 was considered to be significant.

**RESULTS**

**Biodistribution of Nanoparticles in EAU Rats**

In vivo imaging showed accumulation of Cy7-labeled stealth NPs in inflamed eyes of rats with EAU at 24 hours after administration (Fig. 1A), whereas nonstealth Cy7 was not found in the eyes (Fig. 1B). The eyes were excised, and accumulation of Cy7-labeled stealth NPs in the back of the eye was evaluated (Fig. 1C). The NPs showed significant accumulation in inflamed eyes compared with nonstealth Cy7 (counts: 2317 ± 475 vs. 1747 ± 70, P < 0.05; Fig. 1D). Meanwhile, the average BP concentrations in inflamed eyes of rats with EAU were 0.13 ± 0.03 and 0.05 ± 0.01 mg/kg at 24 and 72 hours, respectively, after treatment with stealth nanosteroids (Fig. 2).

The BP levels at 24 and 72 hours after treatment with nonstealth nanosteroids were 0.02 ± 0.01 mg/kg and below the detection limit, respectively.

**Effects of Stealth Nanosteroids**

The clinical scores for rats with EAU (Fig. 3) reached a maximum on day 15 after immunization (3.29 ± 0.78), after which...
the inflammation gradually resolved. Rats with EAU treated with stealth nanosteroids showed reduced scores on day 15 to 33 after immunization compared with rats treated with nonstealth nanosteroids ($P < 0.01$ or $P < 0.05$). Significant differences between treatments with stealth and nonstealth nanosteroids were observed in both the early (day 15 after immunization, $1.33 \pm 0.29$ vs. $1.94 \pm 0.73$, $P < 0.05$) and the late (day 27 after immunization, $0.42 \pm 0.52$ vs. $1.65 \pm 0.54$, $P < 0.01$) stage. The scores for rats with EAU treated with 100 mg of nonstealth nanosteroids were lower than those for saline-treated rats (Fig. 3).

**Histopathology**

Representative histopathologic features of the retina of rats with EAU 7 days after treatment are shown in Figure 4. Disruption in the inner segment and outer segment of all surviving photoreceptors was observed in all areas in saline-treated rats (Fig. 4A). Rats treated with stealth nanosteroids displayed marked preservation of structural integrity (Fig. 4C), whereas those treated with nonstealth nanosteroids showed mild infiltration of inflammatory cells and disruption in the outer nuclear layer (Fig. 4B). Histologic scores for rats treated with stealth nanosteroids were significantly lower than those for rats treated with nonstealth nanosteroids ($1.50 \pm 0.49$ vs. $3.00 \pm 0.89$, $P < 0.05$; Fig. 4D).

**Immunocytochemistry**

Representative images of immunohistochemical staining of the retina of rats with EAU at 7 days after the treatment are shown
in Figure 5. In rats with EAU treated with saline, expression of IL-6 (green) and IL-17 (red) was found in all layers of the retina (Fig. 5A). Most of the infiltrated cells expressed both cytokines. Treatment with nonstealth nanosteroids reduced the number of infiltrated cells and the level of cytokines in the outer retina (Fig. 5B), but inflammatory cytokines remained in the inner nuclear layer. In rats with EAU treated with stealth nanosteroids, expression of IL-17 and infiltration of cells were almost completely absent in the retina (Fig. 5C). In Figures 5A–C, there was some light immunoreactivity with the IL-17 antibody in the retinal pigment epithelium and IL-6 antibody in the inner limiting membrane, but this was the same in all preparations.

Immunohistochemical results for retinas collected 7 days after the treatment from rats treated with saline, nonstealth nanosteroids, and stealth nanosteroids are shown in Figure 6. In the saline- and nonstealth nanosteroids-treated retinas, GS (magenta) and VEGF (green) were expressed in activated Müller cells (Figs. 6A, 6B). Enhanced activation of Müller cells on the outer retina was also observed in saline-treated retinas (Fig. 6A). In contrast, rats treated with stealth nanosteroids showed less VEGF expression and greatly reduced Müller cell activation (Figs. 6C, 6F).

**DISCUSSION**

This study shows that systemic administration of stealth nanosteroids results in higher anti-inflammatory activity than nonstealth nanosteroids in EAU rats. A 40% decrease in intraocular inflammation was obtained within a few days and maintained for 20 days after a single injection of stealth nanosteroids, while the same dose of nonstealth nanosteroids gave a significantly weaker response. Inflammation-dependent accumulation of stealth NPs in EAU rats was demonstrated using in vivo imaging, suggesting that stealth NPs escape from hepatic uptake and have a prolonged blood half-life. Thus, the strong
therapeutic benefit of stealth nanosteroids in EAU rats may be due to targeting to inflamed eyes, in addition to sustained release in situ and prolonged blood circulation.

Stealth NPs were specifically designed to enable systemic delivery of steroids.11,12,14,15 To avoid entrapment by the reticuloendothelial system, the outer layer of the NPs is PEGylated, which is essential for accumulation of NPs at the inflammatory site. This suggests that a long half-life in the circulation is critical for the overall efficacy of targeting of stealth NPs in vivo, and we have previously shown that the half-life of stealth nanosteroids is markedly longer than that of nonstealth nanosteroids.15 To facilitate accumulation at an inflammatory site via an enhanced permeability and retention effect16 and to suppress renal excretion and reticuloendothelial uptake, the diameter of the NPs was set at approximately 120 nm. With these properties, the stealth NPs can deliver steroids to inflammatory sites after systemic administration in vivo.1,11,12,15

Preferential accumulation and longer residence of BP in inflammatory sites may enhance its therapeutic effects, while rapid clearance of BP in conventional therapeutic strategies abolishes these effects. In the present study, BP delivered by stealth NPs preferentially accumulated in target sites of EAU rats after 24 hours, compared with delivery using nonstealth NPs. In addition, BP delivered by stealth NPs remained at these sites at 72 hours after administration, at which time BP delivered by nonstealth NPs was not detectable, suggesting that the stealth nanosteroids had longer residence times. Taken together, these results suggest that BP delivered by stealth NPs may be useful for treatment of inflammatory disorders such as chronic uveitis.

Previous studies have suggested that IL-6 and IL-17 play major roles in the pathogenesis of EAU.20,21 IL-6 is required for differentiation of Th17 cells, a recently discovered IL-17-producing helper CD4+ T-cell subset.22,23 Differentiated Th17 cells may recruit inflammatory cells into the retina, which then produce proinflammatory cytokines and chemokines.24 In this study we found that stealth nanosteroids reduced the inflammatory cells and inhibited the production of increased IL-6 and IL-17 in EAU rats. These cytokines contribute to development of EAU and retinal degeneration secondary to inflammation, and inhibition of their production may offer an important advantage for visual outcome.

Since VEGF is an inflammatory cytokine that induces ocular neovascularization and vascular hyperpermeability, they result in retinal or choroidal neovascularization (CNV) secondary to uveitis and uveitic edema. Recent reports have indicated the efficacy of anti-VEGF therapy for CNV secondary to uveitis.24–27 Fine et al.28 reported increased VEGF levels in the aqueous humor of patients with uveitic cystoid macular edema (CME), and Vinores et al.29 showed that VEGF expression is upregulated in the inner retina of EAU rats. In the present study, we showed that stealth nanosteroids inhibited the increase in VEGF expression in EAU rats. Since CNV and CME secondary to uveitis cause significant visual impairment, reduction of VEGF expression by stealth nanosteroids may result in a significant improvement in vision.

Overall, our results show that systemic administration of stealth nanosteroids inhibits the development of EAU through targeting and sustained delivery. Therefore, systemic delivery of stealth nanosteroids may offer a new therapeutic strategy against intraocular inflammation in humans.

References


