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Treatment of experimental arthritis with PLGA nanoparticles encapsulating betamethasone sodium phosphate

Extended report

¹ Megumu Higaki, MD, PhD, Mitsuharu Takatsu, MD, Yutaka Mizushima, MD, PhD: Institute of Medical Science, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8512, Japan

² Nobuo Izumo, PhD, Tsutomu Ishihara, PhD, Yutaka Mizushima, MD, PhD: DDS Institute, The Jikei University School of Medicine, 3-25-8 Nishi-Shinbashi, Minato, Tokyo 105-8461, Japan

Corresponding author: Megumu Higaki, MD, PhD, Institute of Medical Science, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8512, Japan, Tel and Fax: 81-44-978-2035, Mail address: megumu@dd.iij4u.or.jp

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Key Words: PLGA, Nanoparticles, BSP, AA rats, AblA mice

Short title: Treatment of arthritis with PLGA-nanosteroid

Abstract

Objective. The therapeutic activity of hydrophilic glucocorticoid encapsulated in PLGA nanoparticles, which have shown slow release and are targeted to inflamed joints after intravenous administration, is examined in experimental arthritis models.

Methods. Betamethasone sodium phosphate encapsulated in PLGA nanoparticles with a size of 100 - 200 nm (PLGA-nanosteroid) was prepared using a modified oil-in-water emulsion solvent diffusion method with Zn ions and coated with lecithin. Rats with adjuvant arthritis (AA rats) and mice with anti-type II collagen antibody induced arthritis (AbIA mice), were treated intravenously with PLGA-nanosteroid after the initial sign of arthritis.

Results. In AA rats, a 30% decrease in paw inflammation was obtained in 1 day and maintained for 1 week with a single injection of 100 μ g of PLGA-nanosteroid. Soft X-ray examination 7 days after the treatment showed the decreased soft tissue swelling with this treatment. Moreover, the PLGA-nanosteroid proved to be highly effective in AbIA mice. A single injection of 30 μ g of the PLGA-nanosteroid resulted in an almost complete remission of the inflammatory response after 1 week. In contrast, the same dose of free BSP after three administrations moderately reduced the severity of inflammation. In addition, the histological examination at 7 days after the treatment showed a significant decrease of the inflammatory cells in the joints.

Conclusion. The observed strong therapeutic benefit obtained with the PLGA-nanosteroid might be due to the targeting of the inflamed joint and the prolonged release *in situ*. Targeted drug delivery using a sustained release PLGA-nanosteroid successfully intervened experimental arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint synovial inflammation and progressive cartilage and bone destruction, while its pathogenesis is not yet clearly understood (1).

Glucocorticoids can be highly effective in treating joint inflammation, but their systemic application is limited because of a high incidence of serious adverse effects, especially related to long-term treatment (2,3). As intravenously administered glucocorticoids are distributed in the whole body and rapidly cleared, high and frequent dosing is necessary to achieve an effective concentration at inflamed target sites. Moreover, profound physiological activity of glucocorticoids in many different tissues increased the risk of adverse effects in patients. It is necessary therefore, to develop a drug delivery system (DDS) for glucocorticoids incorporated in particular carriers, which show an enhanced localization in the target site and a sustained drug release (4, 5).

Since the long-circulating liposome system for DDS had already been introduced into clinics (6, 7), a report has been recently published using PEG-liposome containing prednisolone phosphate for the treatment of arthritis (8). However, the allergic reaction against liposome preparation even in the pegylated form becomes a problem to overcome (9). Meanwhile many researchers have developed poly (D, L-lactic/glycolic acid) (PLGA) or poly (D, L-lactic acid) (PLA) microparticles and nanoparticles that encapsulate water-insoluble, low-molecular weight pharmaceutical agents, which are employed for clinical use (10-13). We also have developed nanoparticles, in which a water-soluble, non-peptide, low-molecular weight pharmaceutical agent is made hydrophobic by a metal ion and encapsulated in nanoparticles formed of PLGA/PLA, with a surfactant applied to the surface of the nanoparticles whose diameter are 100-200 nm. They are capable of targeting a specific lesion site after intravenous administration and are less likely to burst at an early stage of administration so that they can gradually release the agent at the lesion site over a prolonged time period (J Controlled Release, submitted).

Whereas PLGA-nanosteroid for intra-articular preparation has been reported (14, 15), the encapsulating rate of steroids is low and the size is not suitable for intravenous administration. We therefore have prepared a PLGA-nanosteroid for intravenous preparation by efficiently encapsulating betamethasone sodium phosphate (BSP) by the modified oil-in-water solvent diffusion method as described before.

We studied the therapeutic activity of the intravenous PLGA-nanosteroid in arthritis models of adjuvant arthritis (AA) rats (16,17) and Type II collagen Antibody (Ab)-induced (AbIA) mice (18-20) to evaluate whether this preparation can improve the delivery and therefore the efficacy of glucocorticoids.

Materials and Methods

(1) Preparation of the PLGA-nanosteroid

We prepared the PLGA-nanosteroid as described before (submitted). In brief, PLGA

with a lactic acid /glycolic acid ratio of 50 / 50 (MW 13,000) was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Betamethasone sodium phosphate (BSP) and lecithin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). BSP-loaded nanoparticles were prepared using the modified emulsion solvent diffusion method in oil. One ml of 0.5 M Zinc Acetate (Wako) was added to 4 mg of BSP in 500 μ l of DDW and the pellets were collected. After centrifugation at 20,000 x g for 10 min, the pellets were dissolved in 0.6 ml of acetone with 20 mg of PLGA (MW 8,000) and 10 μ l of 0.5 M Zn-acetate was added. Then, 5 ml of dispersing DDW containing 0.5% lecithin were added and purified by ultra-filtration (YM-50, Millipore Co., Billerica, MA.), following gel filtration with a PD-10 column (Amersham Bioscience, Tokyo, Japan)

(2) Animals

Lewis rats (7 wks old male, weighing 200-250 g) and BALB/c mice (6 wks old female, weighing 20-25 g) obtained from SLC (Shizuoka, Japan) were used for this study. The animals were housed in an SPF environment and allowed free access to food and water. The Institution Committee of Animal Experiments in St. Marianna Medical University approved all animal studies.

(3) Induction of adjuvant arthritis

Arthritis was induced in Lewis rats by injecting, under ether anesthesia, 50 μ l of incomplete Freund's adjuvant solution (DIFCO, Detroit, Mich) containing 6 mg/ml of *Mycobacterium Butyricum* into the subplantar region of the left hind paw (21,22). Fourteen days after administration of the adjuvant, when the joint inflammation for all rats reached the maximal in the experiment, the animals were divided into groups (n = 7, each) so that there were no significant differences between the groups in terms of the volume of the left hind leg of the animals. In addition, a single dose of PLGA nanosteroid, blank PLGA nanoparticles, or saline was administered intravenously to each group. As controls, a single dose of BSP (100 μ g or 300 μ g) was subcutaneously administered to the respective groups of rats (n = 7, each). The development of arthritis in left hind paw was monitored plethysmographically by recording changes in paw volume by water displacement with model TK-101 (Muromachi Co., LTD. Tokyo). The inflammation rate was calculated by the following equation; inflammation rate (%) = (measured leg volume – leg volume without adjuvant)/(leg volume on Day 14 – leg volume without adjuvant) x 100. The average (\pm SD) of the leg volume without the adjuvant is 1.5 ml (\pm 0.2 ml). Body weights were also monitored daily during the experiment. The swelling in the paw was also assessed using soft tissue X-P on Day 21 (23). Normal and arthritic rats with the treatment were anesthetized with sodium pentobarbital (45 mg/kg, ip), placed on a radiographic box, and radiographs of the hind paws were obtained with a Philips x12 machine (40 kW for 0.01 seconds).

(4) Induction of Antibody-induced arthritis

Arthritis was induced using the methods of Terato *et al.* (18). Balb/c mice were injected intravenously with 2 mg/kg of an arthritogenic mAb cocktail (Chondrex, LLC, Seattle, WA) on Day 0, and LPS (2.5 mg/kg) was intraperitoneally injected on Day 3. This protocol induces severe arthritis within 48h following LPS injection and persisted for more than 1 week. Intravenous treatment with a PLGA-nanosteroid (100 μ g 1x), BSP (100 μ g in PBS, once or three times daily), blank PLGA nanoparticles, or saline (n = 7, each group) started 2 days after LPS administration (On Day 5). Mice were weighted daily and examined for visual signs of inflammation. The clinical severity was graded as follows: 0, normal; 1, erythema; 2, slight swelling; and 3, severe swelling or deformity. Each limb was graded, allowing a maximum clinical score of 12 for each animal (24).

On Day 12, animals were anesthetized with sodium pentobarbital(45 mg/kg, ip) and killed. The distal one-third of the limbs were immersion-fixed in formalin, decalcified in HCl, processed by routine methods, and embedded into paraffin. The specimens were sectioned at 4-6 μ m, stained with hematoxylin and eosin, and examined by light microscopy (19, 20). The extent of cellular infiltration and the erosion of joint cartilage was separately determined and graded from 0 to 3 (3 is the severest) in each paw.

(5) Statistics

The Mann-Whitney U-test was conducted for statistical analysis. *P* values lower than 0.05 were considered significant.

Results

(1) AA rat

Three days after the administration of the adjuvant, the first signs of joint inflammation became apparent (paw volume; 3.0 ± 0.2 ml), in conjunction with a loss of body weight due to the onset of adjuvant-induced arthritis. 14 days after, the disease reached its maximal severity (paw volume; 4.8 ± 0.3 ml), after which the inflammation process gradually resolved. Although vehicle PLGA nanospheres as well as saline did not show any anti-inflammatory effect, the PLGA-nanosteroid exhibited, as early as 1 day after administration, a high anti-inflammatory effect (62 ± 3 %) and continued to exhibit the effect over a succeeding 7-day-period; meanwhile the anti-inflammatory effect of free BSP was gradually lost over time (Fig. 1). An anti-inflammatory effect of the PLGA-nanosteroid was better than the vehicle control ($P < 0.01$, Day 15-21) and the three times dose of free BSP ($P < 0.05$, Day 16-21). There was no difference in body weight change among the four groups.

In the soft X-ray, a marked reduction of swelling in the left paw was seen in the PLGA-nanosteroid treated rats (Fig. 2). Meanwhile, the cartilage erosion could not be prevented completely in this experiment, since the treatment was started after the maximum severity of arthritis.

(2) AblA mice

AblA developed rapidly in immunized mice, and clinical signs (periarticular erythema and edema) of the disease first appeared in the front paws 2 days after the LPS challenge (on Day 5) with a 100 % incidence. Erythema and swelling in the hind and front paws increased in frequency and severity in a time-dependent manner, reaching maximum arthritis indices of 5.5 ± 0.2 on Day 7 in the vehicle-treated mice. The clinical score of the vehicle-treated mice was as severe as that of the saline-treated mice (data not shown). In contrast, PLGA-nanosteroid treated mice on Day 7 had a macroscopic evidence of disease with a reduced score of 3.8 ± 0.1 on Day 7 ($P < 0.01$). In addition, PLGA-nanosteroid treated mice demonstrated a significant reduction in joint inflammation over a succeeding 5 days ($P < 0.01$ vs. vehicle control; $P < 0.05$ vs. three times dose of BSP) (Fig. 3).

Histological examination on Day 12 showed that treatment with PLGA-nanosteroid completely inhibited the influx of inflammatory cells, whilst a severe inflammatory reaction that was seen in the control animals (Fig. 4). There was no macroscopic evidence of either paw erythema or edema in the sham-treated mice. The rate of incidence and the absolute increase in body weight were comparable in normal mice and AblA mice during the experiment.

The arthritic mice treated with saline or vehicle PLGA revealed extensive cellular infiltration ($3.9 + 0.9$), although cartilage erosion was negligible even in the severe arthritis mice. In contrast, PLGA-nanosteroid treated mice showed only mild cellular infiltration ($0.9 + 0.7$) ($p < 0.01$ vs. vehicle control) (Fig. 5).

Discussion

The results indicate that PLGA encapsulation with this new method can strongly increase the therapeutic efficacy of BSP to employ intravenous treatment for

inflammatory diseases including RA. The preparation method of the PLGA nanoparticles encapsulating a water-soluble bioactive molecule such as BSP and designed for sustained release and intravenous injection has been previously described (J Controlled Release, submitted). Hydrophilic BSP is converted to be hydrophobic by the interaction with a zinc ion and was encapsulated in the nanoparticles formed of PLGA with a size of 100-200 nm. The resulting nanoparticles ranging from 90 to 120 nm in diameter with a BSP content of 2-3 weight % was used in this study. BSP was gradually released from the nanoparticles in the diluted serum in vitro over 20 days without any apparent initial burst(J Controlled Release, submitted). More than 95 % of the PLGA-nanoparticles with rhodamine was trapped in the spleen and the liver, whereas 2-3% of them accumulated in the inflamed, but not in the intact joint (data not shown). The PLGA-nanosteroid incorporated in the spleen and the liver did not exhibit anti-inflammatory-effect, since we could not show any detectable amounts of free BSP in the serum after the PLGA-nanosteroid treatment, and this treatment did not affect neither the delayed type hypersensitivity nor the T cell proliferation in rats (data not shown).

They are capable of targeting a specific lesion site and are less likely to burst at an early stage of administration, therefore enabling them to gradually release the agent at the lesion site over a prolonged period. It has been demonstrated that the nanoparticles encapsulating betamethasone dipropionate, a hydrophobic steroid, released a significant fraction of betamethasone at an early stage with approximately 90% or more of betamethasone having been released after several days. However, the nanoparticles prepared according to the method of the present study, in which the steroid's initial bursting out is significantly reduced, released the steroid in a more gradual manner and were able to release it over an extended period of time. A targeted intravenous drug delivery approach using the PLGA-nanosteroid could be a highly attractive alternative to current glucocorticoid treatment strategies such as intravenous pulse therapy or local intra-articular injections. We could reduce the number of times for steroid administration using PLGA whose safety has been clinically proven (10-13, 28). Although the effectiveness of liposome-steroid for the treatment of arthritis has been described (8, 29), it is difficult to maintain the prolonged release by liposome preparation. The PLGA-nanosteroid provides the opportunity to achieve high concentrations of glucocorticoid delivered selectively to all inflamed arthritis joints by a simple intravenous injection.

In a preliminary study in AA rats, the different size (70, 100, and 190 nm) and the different surfactants (lecithin, polyvinylalcohol, and Pluronic F68) of the PLGA nanosteroid have given the same anti-inflammatory effects. Moreover, there were no significantly different anti-inflammatory effects between PLGA (MW 13,000), PLA (MW14, 000), and PLGA (MW 8,000) nanosteroids. Meanwhile, It has also been demonstrated that the nanoparticles made of PLGA or PLA with small molecular weights, tend to release the steroid at an earlier stage, and that the nanoparticles made of PLGA tend to release the steroid earlier than those made of PLA.

Furthermore, selective accumulation of 100 - 200 nm, but not 500 nm PLGA nanoparticles at an inflammation site has been shown in rats using fluorescence-labeled experiments (data not shown). Hepato-splenic uptake is largely responsible for the elimination of nanoparticles in the case of 500 nm from the circulation and appears to be mediated by residual macrophages in these organs. We then used PLGA (MW 13,000) nanosteroid of 90-120 nm in size with lecithin as a surfactant in these experiments. A single intravenous injection of the nanosteroid can induce a strong, rapid, and long-lasting therapeutic benefit. About 30% remission of joint inflammation can be accomplished within 1 day of treatment and the therapeutic benefit of the injection lasts for up to 1 week, whereas the equivalent dose of limethasone (lipid emulsion; dexamethasone palmitate), that is currently employed for the treatment of RA (25,26), gradually loses an anti-inflammatory effect over time (data not shown). The earlier treatment with the PLGA-nanosteroid might also prevent the cartilage erosion in AA rats.

In AblA mice, the same prolonged anti-inflammatory effect could also be obtained with the PLGA-nanosteroid within 1 day and lasted for 7 days. The anti-inflammatory effect might be equivalent to PEG-liposomal glucocorticoid as described before (8). PLGA nanoparticles might protect BSP against conversion and degradation in the circulation as is seen in the liposome. It may also prevent it from the rapid and extensive tissue distribution that occurs with free BSP. PLGA nanoparticles may not just enhance the concentration of the drug at the target site, but also lower drug concentration at non-target tissues. The possibility of reduced toxicity may further improve the therapeutic index of the glucocorticoid upon PLGA nanoparticles.

In conclusion, the results in this study indicate that a single intravenous dose of hydrophilic BSP encapsulated in PLGA-nanoparticles can lead to a rapid, complete, and durable resolution of joint inflammation due to the enhanced and preferential localization of BSP in the inflamed joints. This novel approach could offer important advantages over existing therapies in arthritis, such as pulse therapy and intra-articular injection and liposome preparations of steroids. These results might apply to other inflammatory or immune diseases where a steroid is effective. We have also observed the effectiveness of this preparation in autoimmune uveitis models in rats (in preparation). As compared with free BSP, the slow-release, PLGP-based BSP nanoparticles is safe and markedly reduces the clinical score of experimental arthritis in rodents.

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Figure Legends

Fig. 1 Paw inflammation rate of AA rats

Arthritis was induced in Lewis rats as described in the Materials and Methods section.

Inflammation rate (%) = (measured leg volume – leg volume without adjuvant)/(leg volume on Day 14 – leg volume without adjuvant) x 100.

The average rate of 7 rats from Days 14 to 21 in each group is shown. SEM is within 10 %. The treatment is as follows: □; Saline (iv), ○; Vehicle-PLGA nanoparticles (iv), ●; PLGA-nanosteroid (100 □g, iv), □; BSP(100 □g, sc), ▲; BSP(300 □g, sc) (n = 7, each). **, *P* < 0.05 (PLGA-nanosteroid vs. 300 □g BSP), #, *P* < 0.01 (PLGA-nanosteroid vs. vehicle-PLGA nanoparticles)

Fig. 2 Representative soft tissue X-P of the hind paw of AA rats

The swelling in the paw is assessed by soft tissue X-P on Day 21 as described in the Materials and Methods section. (A) Normal rat control, (B) PLGA-nanosteroid (100 □g, iv) treated AA rat, (C) BSP (100 □g) treated AA rat, (D) Vehicle-PLGA nanoparticles treated AA rat

Fig. 3 Paw inflammation score of AblA mice

Arthritis was induced in Balb/c mice as described in the Materials and Methods section. The average clinical score is shown. SEM is within 10 %. ○; Treated with vehicle-PLGA nanoparticles, ●; Treated with PLGA-nanosteroid (30 □g 1x, iv), ▲; Treated with BSP (30 □g, 3 x, iv) (n = 7, each group). **, *P* < 0.05 (PLGA-nanosteroid vs. BSP) #; *P* < 0.01 (PLGA-nanosteroid vs. vehicle-PLGA nanoparticles)

Fig. 4 Representative histopathology of the front paw of AblA mice

(A) Normal mouse control, (B) Vehicle PLGA-nanoparticles treated AblA mouse, (C) PLGA-nanosteroid (30 □g 1x, iv) treated AblA mouse. An arrow indicates cellular infiltrations. A yellow bar indicates 5 mm.

Fig. 5 Histology Score of AblA mice

The extent of cellular infiltration in AblA mice treated with PLGA-nanosteroid (30 □g 1x, iv), BSP (30 □g, 3 x, iv), or vehicle PLGA nanoparticles was determined and graded from 0 to 3 in each paw. Data represents mean ± SEM (n = 7, each group).

#; *P* < 0.01 (PLGA-nanosteroid vs. vehicle-PLGA nanoparticles).

**; *P* < 0.05 (PLGA-nanosteroid vs. BSP)

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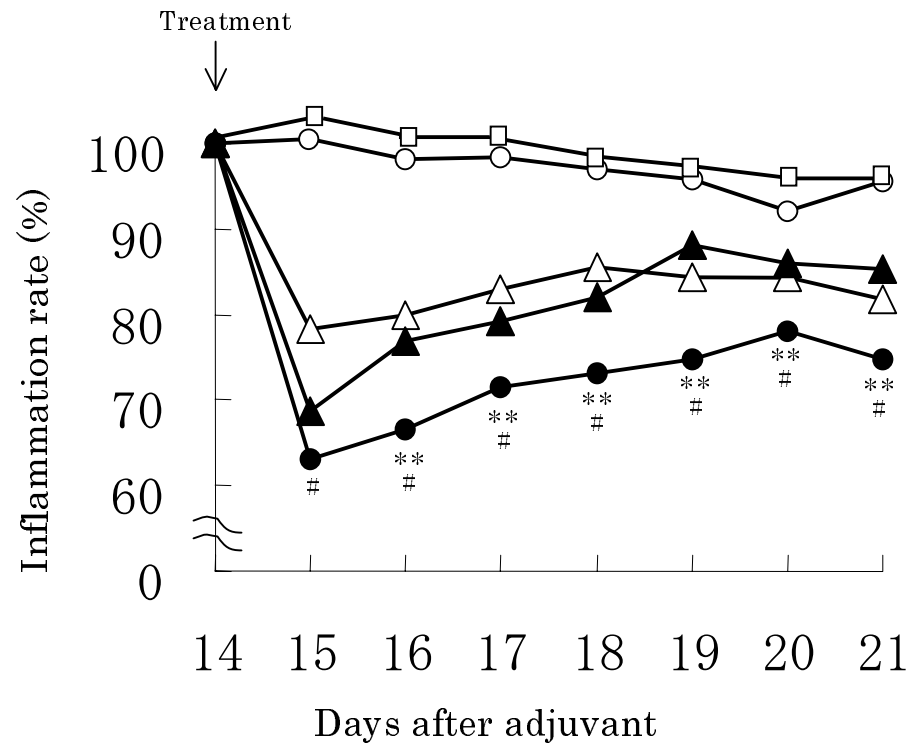




Fig.2

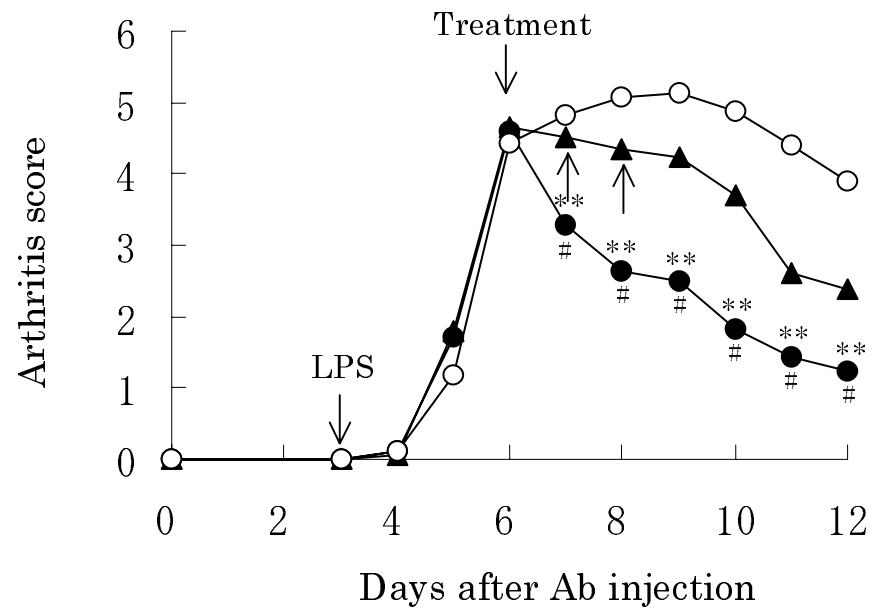


Fig.3

