Conjugate between Chondroitin Sulfate and Prednisolone with a Glycine Linker: Preparation and *in Vitro* Conversion Analysis

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A conjugate between prednisolone (PD) and chondroitin sulfate (CS) with glycine as a linker was prepared in order to obtain an effective macromolecular prodrug against inflammatory disease, especially rheumatoid arthritis. First, PD was converted to the *N*-trityl-glycine ester (Tr-GP), and the glycine ester of PD (GP) was obtained by detritylation of Tr-GP. Then, GP and CS were condensed with water-soluble carbodiimide to yield CS-GP. The obtained conjugate had a PD content of 2.24% (w/w). Conversion characteristics were investigated for GP and CS-GP to evaluate their potential as a prodrug. In the stability test of GP, PD was released well in the buffer at pH 6–7.4, but degraded rapidly at pH 8 without sufficient release of PD. As to CS-GP, PD was released more slowly than in GP, and the release rate rose with the increase in the medium pH. PD was released gradually from CS-GP over 24 h at a physiological pH. The conversion profiles of both GP and CS-GP almost followed pseudo-first order kinetics. The calculated conversion rate constants supported the gradual and effective release from CS-GP. The release rate of PD from GP and CS-GP was accelerated by the addition of rat plasma, but the promotion of release from CS-GP was small, suggesting that PD should be released gradually from CS-GP in the systemic circulation. It was demonstrated from the preliminary pharmacological study using rats with adjuvant-induced arthritis that CS-GP had high anti-inflammatory potential against arthritis.

Key words prednisolone; chondroitin sulfate; conjugate; prodrug; conversion rate; adjuvant-induced arthritis

Rheumatoid arthritis (RA) is a chronic disease that causes inflammation of the synovial membrane located inside the articular capsule. It is characterized by progressive destruction of the joints, involving synovial hyperplasia, synovial cell activation and articular inflammation.[1,2] Currently, the disease is a major problem worldwide, with many people suffering. The symptoms of the disease, including persistent pain, stiffness and joint swelling, lead to deterioration of the quality of life (QOL). Both genetic and environmental factors are considered to be involved in the etiology.[3]

The treatment of RA includes surgery to improve joint function, physiotherapy to recover motor function, and pharmacotherapy to ameliorate inflammation and joint destruction. As pharmacotherapy, non-steroidal anti-inflammatory drugs (NSAIDs),[4–6] disease-modifying anti-rheumatic drugs (DMARDs)[7] and glucocorticoids have been mainly used to manage various disease states.[4,5,8] In addition, recently, many antibody drugs have been developed actively, and their use is spreading progressively[9,10]; however, the aforementioned conventional drugs still play an important role in the treatment of RA. In particular, glucocorticoids are very highly potent and fast-acting agents in the treatment of RA; however, their chronic use often causes severe systemic side effects such as diabetes, osteoporosis and adrenal failure, resulting in their limited use.[11–14]

Therefore, recently, various attempts have been made to improve the use of glucocorticoids in the treatment of RA.[3,15–19] As their efficacy is associated with the drug concentration at the diseased site, specific delivery of the drug to the target site is considered to be a key to the promotion of efficacy. At the same time, such specific delivery to the target site is considered to enable the reduction of systemic side effects by decrease in their transfer to the other parts and/or reduction of the dose. These approaches are known as targeted delivery. For example, liposomal formulations, polymer–drug conjugates and nanoparticles have been delivered to inflammatory sites such as swelling joints.[3,15,16,20] These systems are based on the enhanced permeability and retention (EPR) effect, which is caused physiologically because neovascular vessels develop at the diseased site of RA.[15,16,20]

In this study, we focused on chondroitin sulfate (CS) as a carrier macromolecule, because CS is highly safe and can be applied intravenously and intramuscularly.[21–23] To date, no toxic or adverse effects have been reported with the use of CS. Although CS, injected intravenously, is excreted into urine to a large extent, some of the excreted CS-related molecules exhibit a high molecular weight similar to that of the original polymer, and some of them appear in the degradation form of oligosaccharides or inorganic sulfate ions.[19,24] From these pharmacokinetic features, CS is considered to behave as a polymer to a certain extent in the systemic circulation. Namely, CS is expected to contribute to systemic retention. In fact, prolonged systemic circulation and elevation of the area under the plasma concentration–time curve (*AUC*) were reported in the CS–cisplatin conjugate.[25] These safety and pharmacokinetics of CS suggest that CS should be a useful drug carrier for a delivery system of RA treatment.

As far as we know, conjugates between CS and glucocorticoids for the treatment of RA have hardly been reported. Regarding CS–drug conjugates for anti-inflammatory therapy, Peng *et al.* reported conjugates of CS and NSAIDs, which were tested for their effect on carrageenan-induced edema.[22] These conjugates were found to exhibit a prolonged effect. In the present study, prednisolone (PD), used often for the treatment of RA, was chosen as the glucocorticoid agent. The design of the conjugate of PD with CS was performed by taking into account the following matters. i) In order to enable the hydrolysis at the inflammatory acidic pH, the chemical bond

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such as hydrazone and *cis* acotinyl group could be proposed as an adequate linker because they are susceptible to hydrolysis at acidic pH with the fairly high stability at physiological pH.[26,27] Also, a peptide linker is another choice because they can be hydrolyzed by various peptidases in the tissues or cells after the localization there.[28] However, in the case of glucocorticoid drugs with hydroxy groups, those chemical combinations are not necessarily easy to synthesize. In the case of PD, being one of the glucocorticoid drugs, the ester linkage is the most simple. In fact, various ester prodrugs for glucocorticoid drugs, including macromolecule–glucocorticoid conjugates, have been examined by many researchers.[29–33] ii) If macromolecule–glucocorticoid conjugates are used for the delivery to the arthritic inflammatory site, it is required that they are stable to a fair extent at the systemic physiological condition and that they can release the active drug at an adequate rate at the target site. Although an ester bond is generally more susceptible to hydrolysis at higher pH, such requirements are considered to be achieved to a fair extent with the ester linkage of the macromolecular prodrugs. Namely, the ester bond is generally fairly stable at the systemic pH of 7.4, though it depends on the chemical structure. Furthermore, the ester linkage in the macromolecular prodrugs is generally stable against enzymatic hydrolysis due to the steric hindrance to the esterase.[18,34] These stability characteristics of the ester bond in the macromolecular prodrugs suggest that the pH-dependent drug release might be caused simply at the tissues, which enables *in vivo* release to be more predictable as compared to other designs of prodrugs. iii) In addition, although the pH value tends to be acidic at the arthritic inflammatory site, it is reported to vary from pH 7.4 to 6.0, dependent on the diseased states,[35] suggesting that the ester bond, subjected to hydrolysis widely at weakly basic, neutral and weakly acidic pH, should be adequate as a linker for the activation. iv) In the case of design of the ester prodrug of PD with CS as a carrier, a bi-functional linker with a carboxy group and an amino group is considered to be useful for the combination with the hydroxyl group of PD and the carboxy group of CS. Therefore, amino acids and peptides are proposed as an available linker; in particular, a simple amino acid, glycine, is suggested as a candidate for the linker. For instance, Conover *et al.* produced the conjugate of polyethylene glycol and camptothecin using a glycine linker, and investigated the pharmacokinetics and antitumor effect; the conjugate showed the gradual release in the conditions of physiological pH and rat plasma and achieved the fairly fast delivery to the diseased site, resulting in high antitumor effect.[36] Considering these chemical and biological conditions on the arthritis pharmacotherapy, glycine was employed as a linker in the present study. The produced conjugate was examined here for the detailed characteristics for the stability, drug release and pharmacological potential. The preparation of the conjugate was attempted as follows. First, PD was derivatized to a glycine ester of PD, named GP, and then the conjugate between CS and GP, called CS-GP, was prepared by a carbodiimide coupling method. GP and CS-GP were analyzed for *in vitro* conversion behaviors in different pH media and for the effect of plasma on the conversion. Also, a preliminary pharmacological study was conducted using rats with adjuvant-induced arthritis[4,37] to confirm the effectiveness of CS-GP.

Experimental

Materials PD, *N*,*N*′-carbonyldiimidazole (CDI), 4-dimethylaminopyridine (DMAP), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSC) and CS C sodium salt (CS-Na) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The CS-Na was derived from shark cartilage and contained 2.6% N and 6.6% S. For its composition, the ratio of 6-sulfate to 4-sulfate was 9 : 1, that is, 6-sulfate is major and the molecular weight (MW) was 40000–80000. *N*-Tritylglycine (Tr-G) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Heat-killed and desiccated *Mycobacterium tuberculosis* M37Ra was obtained from Difco Laboratories (Detroit, MI, U.S.A.) and used as an adjuvant. All other chemicals were of reagent grade.

Physical Measurements A UV-VIS absorption spectra were recorded using a Beckman DU640 spectrophotometer. NMR spectra were measured with a JEOL Lambda-500 (500 MHz) spectrometer, in which tetramethylsilane (TMS) was used as a reference with chemical shift of 0 ppm. MS spectra were obtained with a JEOL JSM600 mass spectrometer for electron ionization-mass spectrometry (EI-MS) and with an AXIMA–CRF PLUS mass spectrometer (Shimadzu Corp., Kyoto, Japan) for matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS).

Animals Male Wistar rats (7 weeks old; weighing 200–210 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Lewis female rats (8 weeks old, 150–160 g) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were bred on the breeding diet MF supplied by Oriental Yeast, Co., Ltd. (Tokyo, Japan) with water *ad libitum* at 23±1°C and relative humidity of 60±5%. They were used for the experiments a few days after purchase. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Japan. The animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

Preparation of CS-PD Conjugate with a Glycine Linker Glycyl-prednisolone (GP) was synthesized by the two step method. First, the Tr-G ester of PD, named Tr-GP, was synthesized as follows. Tr-G (477 mg, 1.5 mmol) and CDI (243 mg, 1.5 mmol) were dissolved in 10 mL tetrahydofuran (THF) at 0°C, and stirred for 30 min at 0°C. DMAP (15 mg, 0.12 mmol) and PD (270 mg, 0.75 mmol) were added to the solution, and the mixture was stirred at room temperature for 4.5 h. The solvent was evaporated, and the resultant residue was dissolved in several milliliters of a mixture of chloroform and methanol (90 : 1, v/v). The resultant solution was added to a silica gel column [3 cm (inner diameter)×30 cm (length); silica gel: 230–400 mesh size (Merck KGaA, Darmstadt, Germany)], and eluted using a mixture of chloroform and methanol (90 : 1, v/v) as a solvent at a flow rate of 3 mL/min using a pump. The eluted solution was fractionated, and the eluted compounds were assessed by thin-layer chromatography (TLC), which was performed using pre-coated DC Kieselgel 60 F_{254} plates (No. 1.05715.0001; Merck). The spots on TLC were detected by UV light at 254 nm. The fractions [*Rf*=0.08 with chloroform–methanol (90 : 1, v/v)] were collected, and the solvent was evaporated. The yield of Tr-GP was 400 mg. ^{1}H-NMR (DMSO-d_6), δ (ppm): 7.20–7.42 (m, 16H, Tr-G Tr-H, PD

C1-H), 6.15–6.18 (d, 1H, PD C2-H), 5.92 (s, 1H, PD C4-H), 5.02–5.05 (d, 1H, PD C21-H), 4.72–4.75 (d, 1H, PD C21-H′), 4.29 (s, 1H, PD C11-H), 2.97–2.99 (m, 2H, Tr-G C-H_2). EI-MS *m/z*: 659 ($M^{+\cdot}$).

GP was obtained by detritylation of Tr-GP using aqueous acetic acid. Namely, 75% (v/v) aqueous acetic acid (10 mL) was added to Tr-GP (300 mg) and heated at 75°C to dissolve Tr-GP completely. Immediately after the white powder started to precipitate, the mixture was cooled on the ice and kept for 30 min. After the white precipitate was removed by the filtration, the solvent of the filtrate was evaporated. The residue was dissolved in several milliliters of a mixture of chloroform and methanol (15 : 1, v/v) and underwent column chromatography using a silica gel column in the same manner as stated above except that the mixture of chloroform and methanol (15 : 1, v/v) was used as the elution solvent. Each fraction was assessed by TLC as above. The fractions in which *Rf* was 0.23 [chloroform–methanol (15 : 1, v/v)] were collected, and the solvent was evaporated. The yield of GP was 60 mg. ^{1}H-NMR (DMSO-d_6), δ (ppm): 7.31–7.33 (d, 1H, PD C1-H), 6.15–6.17 (d, 1H, PD C2-H), 5.92 (s, 1H, PD C4-H), 4.97–5.00 (d, 1H, PD C21-H), 4.84–4.87 (d, 1H, PD C21-H′), 4.27–4.38 (m, 3H, glycine C–H_2, PD C11-H). MALDI-TOF-MS *m/z* : 418.54 ($[M+H]^+$).

CS and GP were conjugated by carbodiimide coupling to prepare CS-GP conjugate. CS (120 mg) was dissolved in 10 mL water, and 2.5 mL THF containing 30 mg GP was added. WSC (500 mg) and NHS (300 mg) were added to the solution, and the resultant mixture was stirred at room temperature for 4.5 h. The solution was chromatographed with a Sephadex G50 gel column [2.8 cm (inner diameter)×19 cm (length)] using 0.1 M NaCl aqueous solution as the elution solvent. The eluted solution was fractionated at 10 mL each. The high MW fractions were collected, and the mixture was dialyzed against water at 4°C. The final solution was lyophilized. The amount of the product (CS-GP) was 120 mg.

Measurement of PD Content in CS-GP PD, CS and CS-GP were dissolved in a mixture of phosphate-buffered saline (PBS) at pH 7.4 and methanol (7 : 3, v/v) at concentrations of 12.5, 622 and 528 μg/mL, respectively. The PD content of CS-GP was calculated by comparing the net absorbance at 246 nm of the conjugated PD, obtained from UV absorption profiles of CS and CS-GP, with the absorbance at 246 nm of PD alone.

In addition, CS-GP (1.2 mg) was dissolved in 0.4 mL water, and PBS (1.2 mL) and methanol (0.533 mL) were added. To this solution, 0.1 M NaOH aqueous solution was added at the ratio of 1 : 1 (v/v). The resultant solution was incubated at 45°C under horizontal shaking at 60 rpm. Aliquot samples (50 μL) were withdrawn at 10, 20, 25 min after the start of incubation. Immediately after sampling, 0.1 M acetate buffer of pH 4 (150 μL) was added to stop the hydrolysis. After the addition of 150 μL of the mobile phase of high performance liquid chromatography (HPLC), the resultant solution was analyzed by HPLC for the regenerated PD. The content of PD in CS-GP was estimated from the maximal amount of regenerated PD.

***In Vitro* Conversion Examination for GP in Various pH Media** GP was dissolved in a mixture of methanol and aqueous buffer (1 : 3, v/v) to obtain solutions at a concentration of 60 μg/ml. As to the aqueous buffers, 0.1 M acetate buffer of pH 4, 1/15 M phosphate buffer at pH 6 and 8, and PBS (pH 7.4) were used. Each solution (0.5 mL) was incubated at 37°C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (50 μL) were withdrawn. To this sample, 0.1 M acetate buffer of pH 4 (150 μL) was added to stop the hydrolysis of GP. After the addition of 150 μL of the HPLC mobile phase, the resultant solution was analyzed by HPLC for both GP and PD.

***In Vitro* Conversion Examination of CS-GP in Various pH Media** After CS-GP (1.2 mg) was dissolved in 0.4 mL water, aqueous buffer (1.2 mL) and methanol (0.533 mL) were added. Regarding the aqueous buffers, the same buffers as stated above in the GP incubation were used. Each solution (0.5 mL) was incubated at 37°C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (50 μL) were withdrawn. These samples were treated in the same manner as stated in the incubation of GP, and analyzed similarly by HPLC.

In Vitro* Conversion Examination of GP and CS-GP in PBS–Plasma Mixture** A blood sample was taken from Wistar rats *via* the jugular vein using a heparinized syringe. Plasma was obtained by centrifugation of the blood and subsequent collection of the supernatant. A mixture of PBS and the plasma (10 : 3, v/v) was used as the incubation medium. GP and CS-GP were dissolved in the medium at concentrations of 7.7 and 230 μg/mL, respectively. Each solution (1.95 mL) was incubated at 37°C under horizontal shaking at 60 rpm. At appropriate time points, aliquot samples (100 μL) were withdrawn. To each sample (100 μL), saturated NaCl aqueous solution (100 μL) and 6% (v/v) phosphoric acid aqueous solution (100 μL) were added. Then, 4 mL of the mixture of *t*-butyl-methyl ether and *n*-pentane (3 : 2, v/v) was added, and the solution was shaken vigorously. After centrifugation of the mixture at 1400×g*** for 10 min, 3 mL of the resultant supernatant was taken and evaporated to dryness under nitrogen gas. To the residue, 100 μL of the HPLC mobile phase was added, and 20 μL of the resultant solution was injected on the HPLC column to analyze the concentration of PD.

Preliminary Anti-inflammatory Examination Using Rats with Adjuvant-Induced Arthritis CS-GP was examined in order to identify the pharmacological potential. Namely, *in vivo* potential was investigated based on the extent of the anti-inflammatory effect and its duration using Lewis rats with adjuvant-induced arthritis. First, arthritis was induced as follows. Heat-killed *Mycobacterium tuberculosis* M37Ra (20 mg) was suspended in 4 mL liquid paraffin. The suspension (100 μL) was injected intracutaneously into the pad of the right hind paw of each rat. CS-GP saline solution (0.14–0.18 mL) was then injected intravenously *via* the jugular vein at 2 mg PD eq/kg on the day when the paw swelling reached the plateau. That is, CS-GP was administered 16 and 17 d after injection of the adjuvant; that is, total dose=2×2 mg PD eq/kg. On appropriate days after adjuvant injection, non-treated and CS-GP-injected rats were weighed and the volume of both hind paws investigated, in which the volume of the hind paw was measured by immersing it into water and reading the buoyant force.

In addition, for the preliminary study of the PD therapeutic potential, PD was investigated for the suppressive effect on the paw volume. Namely, PD solution in 30% (w/v) PEG400 solution in saline was injected intravenously *via* the jugular vein at 2.5 mg PD eq/kg on the day when the paw swelling

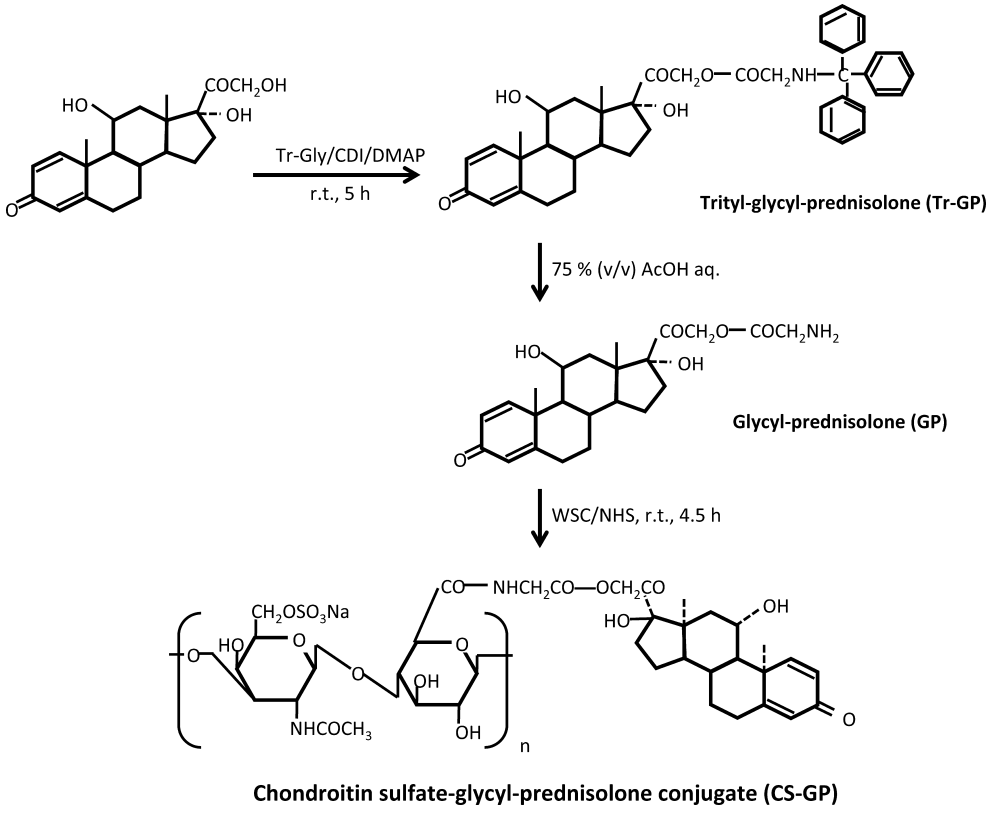


Fig. 1. Synthetic Procedures and Chemical Structures of Tr-GP, GP and CS-GP

reached a maximal level. That is, PD was administered 15 and 16 d after injection of the adjuvant; that is, total dose= 2.5×2 mg PD eq/kg/mL. On appropriate days after adjuvant injection, non-treated and CS-GP-injected rats were weighed and the volume of both hind paws investigated in a similar manner as above.

HPLC Assay As the HPLC apparatus, a Shimadzu LC-6AD pump equipped with a Shimadzu SPD-10AV VP UV-VIS detector and a Shimadzu C-R7A Chromatopac was used. An YMC Pack ODS-AM column (6 mm inner diameter×150 mm length; YMC Co., Ltd., Kyoto, Japan) was used as the analytical column. The detector was set at a wavelength of 246 nm, and a 26% (v/v) 2-propanol aqueous solution containing 0.1% (v/v) trifluoroacetic acid was used as the mobile phase. The HPLC assay was conducted at room temperature. The injection volume was set at 20 μL. The concentration of GP and PD was determined by the absolute calibration curve method. For samples obtained by extraction with organic solvent, only PD was analyzed by the absolute calibration method using standard samples treated in the same manner as the tested samples.

Statistical Analysis For statistical analysis, the unpaired *t*-test was used, and significant difference was set as $p<0.05$.

Results and Discussion

Chemical Characteristics of GP and CS-GP A flow chart for the preparation of CS-GP is illustrated in Fig. 1. For Tr-GP, it was observed that the chemical shifts of protons at the C21 of PD (4.04–4.09, 4.47–4.51 ppm) changed to those of 4.72–4.75 and 5.02–5.05 ppm. This change in the chemical shifts of C21-H_2 to a low field indicated ester formation between the carboxy group of Tr-G and the hydroxyl group at the C21 position of PD. Furthermore, the signals in the high field of 0–3 ppm were observed to be derived from protons derived from PD (data not shown). In addition, the binding ratio between Tr-G and PD was calculated to be 1 : 1 (mol/mol) from their signal integrated intensities. Mass spectra also supported the structure of Tr-GP. Furthermore, ^{13}C-NMR spectra DEPT135°, DEPT90°, heteronuclear multiple bond connectivity (HMBC) and heteronuclear multiple quantum coherence (HMQC) (data not shown) supported the chemical structure of Tr-GP in Fig. 1.

GP was obtained by acidic hydrolysis of Tr-GP. In GP, the ester formation at the C21 position of PD was confirmed from the ^{1}H-NMR spectra, and the signals at 0–3 ppm was observed to be derived from PD (data not shown). Also, the binding ratio between glycine and PD was confirmed to be 1 : 1 (mol/mol) from the signal integrated intensities. ^{13}C-NMR spectra indicated that the compound contained 23 carbons, and DEPT135° revealed two methylene carbons at 66.36 and 50.81 ppm, which were obviously derived from C21 and glycine methylene carbon, respectively. In addition, HMQC showed that C21 was coupled with the protons at 4.97–5.00 and 4.84–4.87 ppm and that the glycine methylene carbon was directly combined to the protons at 4.27–4.38 ppm. Mass spectra also supported the structure of GP. From these data, GP was identified as a glycine ester of PD at the C21 position as shown in Fig. 1.

CS-GP was prepared by carbodiimide coupling using WSC and NHS. The ^{1}H-NMR spectrum showed that CS-GP was formed by the combination of GP and CS. GP had a similar absorption curve in shape to that of PD, with the maximal wavelength at 246–247 nm in the mixture of PBS and methanol (7 : 3, v/v) (data not shown). The UV absorption profiles

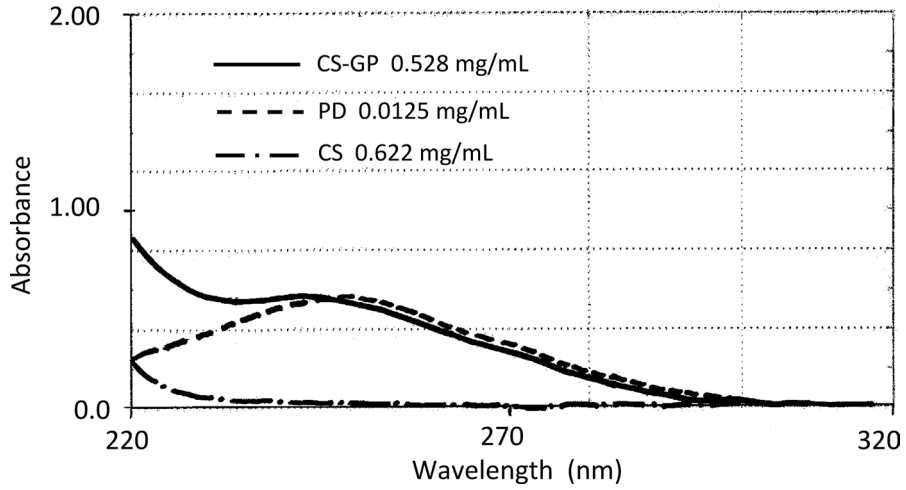


Fig. 2. UV Absorption Spectra of PD, CS and CS-GP

The mixture of PBS and methanol (7 : 3, v/v) was used as a solvent.

were compared among PD, CS-GP and CS (Fig. 2) for the drug content estimation. The PD content in CS-GP was calculated to be 2.24% (w/w) from the net absorbance of the conjugated PD at 246 nm. The results by HPLC analysis after the alkaline hydrolysis of CS-GP gave a near content, which was approximately 80% of the value estimated by UV absorbance. Furthermore, the content calculated by the UV absorbance was also consistent with that estimated from integrated intensities of the proton signals in the ^{1}H-NMR spectrum of CS-GP. Finally, the UV absorption measurement was employed to determine the PD content of CS-GP because it was the simplest and clearest approach. The MW distribution of CS-Na was checked using the ultrafilter (Amicon Ultracel®, MW cutoff: 30000, 50000, 100000) produced by Millipore Corp. (Billerica, U.S.A.). The filtrate eluted with the ultrafilter was measured spectrophotometrically at 246 nm. As a result, 80%, 70% and 14% of CS-GP was eluted with the ultrafilters with MW cutoff of 100000, 50000 and 30000, respectively, which suggested that MW of CS-GP was mainly distributed at 30000–50000. This supported that the original MW of CS (MW 30000–80000), reported by the supplier, was preserved well in CS-GP.

Hydrolytic Conversion of GP at Different pH Media As it took some time for the powder of PD and GP to completely dissolve in the aqueous buffer alone, methanol was added at the degree not to influence the pH of the buffer, which allowed the rapid dissolution of the whole powder of GP and PD. In HPLC analysis, the retention times of GP and PD were 7.6–7.7 and 10.2–10.3 min, respectively (Fig. 3); therefore, GP and PD could be determined simultaneously. The degradation profiles of GP were obtained as shown in Fig. 4a. The stability of GP fell with the increase of pH of the media. At the same time, PD was released by the degradation of GP. The detected PD amount displayed a good mass balance with the degraded GP amount at acidic pH, but the mass balance became lower at weakly alkaline conditions, particularly at pH 8. Although GP was decomposed almost completely at pH 8 by 1 h incubation, the appearing PD was only 11.4 and 12.8% at 1 and 4 h, respectively (Fig. 4b). Based on these observations, the conversion process was suggested as shown by the scheme in Table 1. The conversion rate was analyzed according that scheme. First, the stability of PD was checked independently using the same incubation media. PD was stable at pH 4 and 6, but degraded slowly at pH 7.4 and somewhat fast at pH 8 (Fig. 5). As the remaining amount did not change greatly at these pH values, it was difficult to decide whether the degradation follow the pseudo-zero order or pseudo-first order kinetics. In the previous paper,[18] PD was found to be degraded at pH 9 obviously in the mono-exponential decline manner (remaining percentage: 78.6% at 7 h, 40.5% at 24 h, 19.1% at 48 h). Therefore, from the analogy to the stability in the weakly basic pH, the degradation profile of PD ($P_{PD}(t)$) was considered to follow the pseudo-first order kinetics in the present study. Namely, it could be expressed by Eq. 1. Also, the conversion of GP was analyzed with the pseudo-first order kinetics according to the scheme in Table 1. The equations for the remaining GP ($G(t)$) and appearing PD ($P_{GP}(t)$) were expressed as shown in Eqs. 2 and 3.

$$P_{PD}(t) = P_{PD}(0) \times \exp(-h_0 t) \tag{1}$$

$$G(t) = G(0) \times \exp(-(h_1 + h_2)t) \tag{2}$$

$$P_{GP}(t) = G(0) \times h_1 \times \frac{\exp(-(h_1 + h_2)t) - \exp(-h_0 t)}{h_0 - h_1 - h_2} \tag{3}$$

The analysis was conducted by fitting the equation to the plots represented by the mean observed values. The h_0 values were calculated by fitting Eq. 1 to the observed profiles in Fig. 5. The h_1 and h_2 values were obtained by fitting Eqs. 2 and 3 to the observed profiles in Figs. 4a, b, respectively, with the simultaneous fitting method using the MULTI program,[38] in which the h_0 values were fixed to the calculated values obtained by Eq. 1. The obtained values for the parameters are shown in Table 1. When the individual profile of each incubation sample, not being the mean profile, was processed in a similar manner, minus values were obtained in some cases, in particular, the stability parameter of PD itself. Furthermore, the variation was fairly large in some case, especially, the stability parameter of PD itself. Considering these points, the fitting was conducted to the observed mean profile, expressed

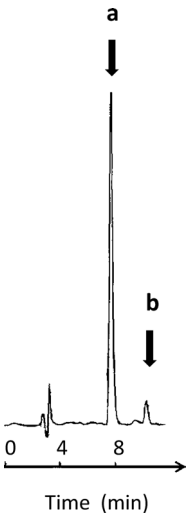


Fig. 3. HPLC Chromatogram in Conversion Studies of GP at 1 h after the Incubation at pH 4 and 37°C

Peak a: GP, peak b: PD.

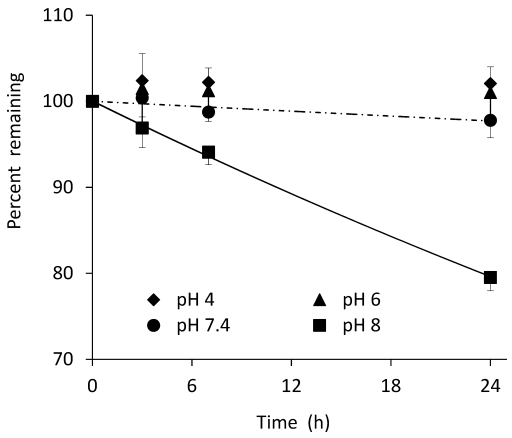


Fig. 5. Decomposition Profiles of PD in Different pH Media at 37°C

Each result is expressed as the mean±S.D. (n=3). Dashed-dotted and solid lines are calculated curves for the observed profiles at pH 7.4 and 8, respectively.

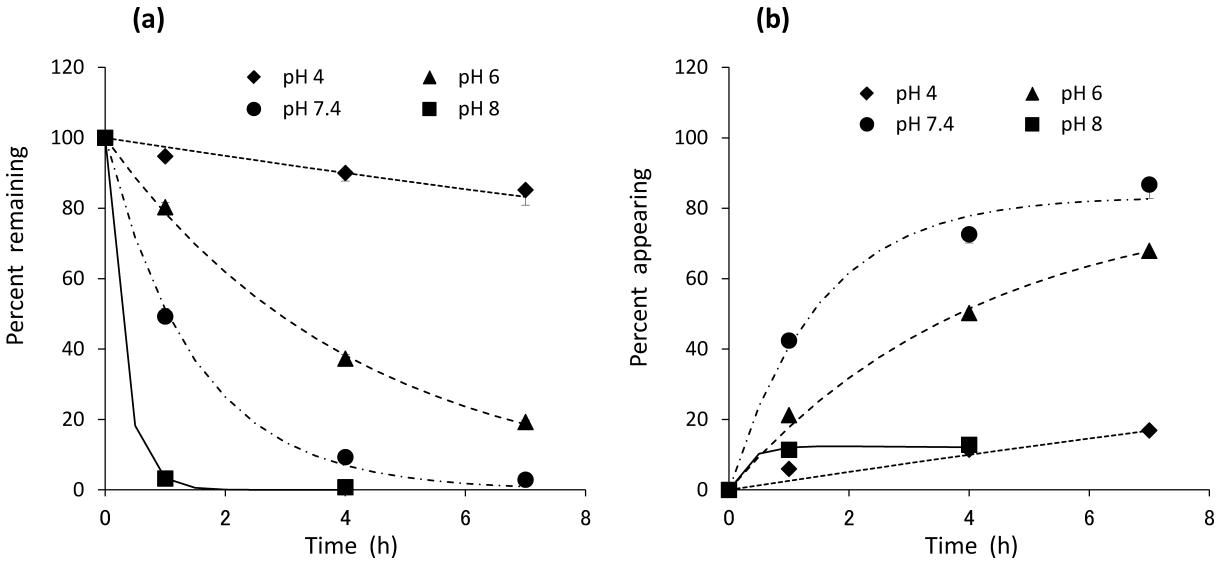
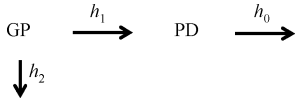


Fig. 4. Conversion Profiles of GP in Different pH Media at 37°C

(a) Remaining GP (%), (b) appearing PD (%). Each result is expressed as the mean±S.D. (n=3). The dotted, broken, dashed-dotted and solid lines are calculated curves for the observed profiles (mean values) at pH 4, 6, 7.4 and 8, respectively.

Table 1. *In Vitro* Conversion Rate Constants for PD and GP in Different pH Media at 37°C



pH	Rate constant (h^{-1})			Half life (h)	
	h_0	h_1	h_2	PD	GP
4	0.0	0.0263	0.0	—	26.4
6	0.0	0.200	0.0400	—	2.89
7.4	0.000967	0.559	0.108	716	1.04
8	0.00979	0.429	2.98	70.8	0.203

For analysis, the mean values in Figs. 4 and 5 were used.

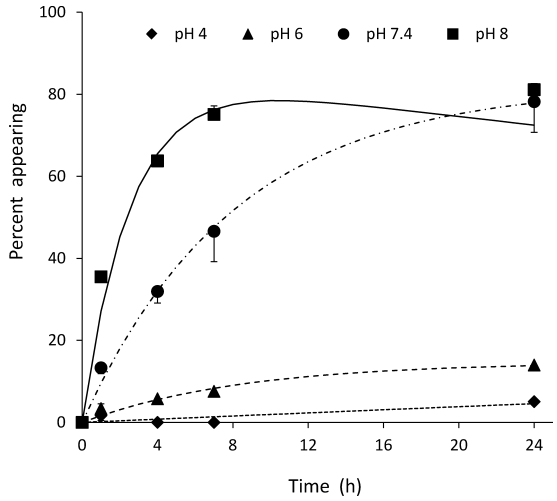


Fig. 6. Conversion Profiles of CS-GP to PD in Different pH Media at 37°C

Each result is expressed as the mean±S.D. (n=3). Dotted, broken, dashed-dotted and solid lines are calculated curves for the observed profiles at pH 4, 6, 7.4 and 8, respectively.

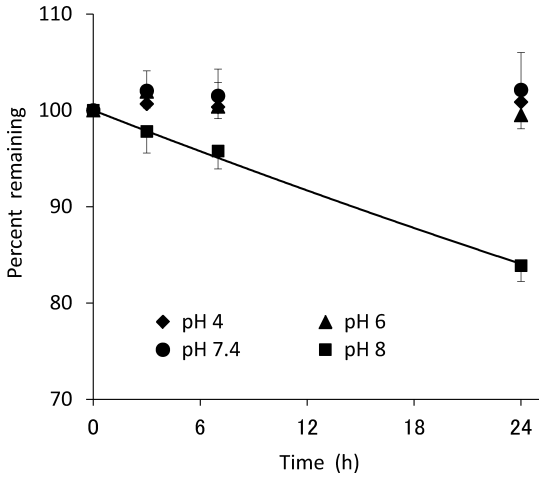
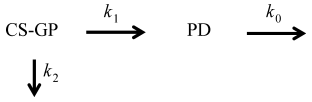


Fig. 7. Decomposition Profiles of PD in Different pH Media at 37°C

Each result is expressed as the mean±S.D. (n=3). The solid line is the calculated curve for the observed profiles at pH 8.

Table 2. *In Vitro* Conversion Rate Constants for PD and CS-GP in Different pH Media at 37°C



pH	Rate constant (h^{-1})			Half life (h)	
	k_0	k_1	k_2	PD	CS-GP
4	0.0	0.00195	0.0	—	355
6	0.0	0.0175	0.102	—	5.80
7.4	0.0	0.102	0.0222	—	5.58
8	0.00724	0.328	0.0596	95.7	1.79

For analysis, the mean values in Figs. 6 and 7 were used.

in percentage. The half-lives of GP and PD were calculated with $\ln 2/(h_1+h_2)$ and $\ln 2/h_0$. The calculated curves were superimposed with various lines in Figs. 4 and 5. The calculated profiles were well-fitted to the observed ones, supporting the conversion of GP followed the pseudo-first order kinetics. The peculiarity of GP conversion was observed at pH 8. Namely, GP degraded quickly at pH 8, but PD appeared to a slight extent, indicating abnormality in mass balance. In fact, many peaks, considered to exhibit various degradation products, were observed in the HPLC at pH 8. The glycine moiety appeared to promote the decomposition of GP at the parts other than the ester bond, though the detailed mechanism was unknown. The calculated conversion profiles were well-fitted to the observed ones, even at pH 8. The large h_2 values at pH 8 explained less conversion of GP to PD.

Hydrolytic Conversion of CS-GP at Different pH Media The conversion analysis for CS-GP was performed in a way similar to that in the above GP conversion except that a mixture of water, aqueous buffer and methanol (1 : 3 : 1.33, v/v) was used as a solvent. GP and PD existing during the incubation period were investigated by HPLC. As a result, no GP was detected in any incubation. The generated PD was obtained as shown in Fig. 6. The release rate of PD became faster with the increase in pH. Overall, the conversion to PD was much slower in CS-GP than GP. In particular, the release pattern at pH 8 was crucially different between CS-GP and GP. CS-GP released PD gradually and much more effectively at pH 8 than GP. The difference in release patterns was considered to be due to the structural difference. That is, the amino group of GP was presumed to make the compound more unstable because of its high reactivity, while, in CS-GP, the GP part appeared to be more stable due to the poor reactivity of the amide form. As the composition of the incubation media is slightly different from that in GP, the PD stability was checked. As a result, the degradation rate was found to be similar to but a little different from that in the media used for GP incubation (Fig. 7). No difference in pH was observed between CS-GP medium and GP medium. Therefore, the small difference in the composition between media might cause the slight difference in the stability of PD. The conversion scheme was set as shown in Table 2. The degradation profile of PD in the used media ($P_{PD2}(t)$) was analyzed by the pseudo-first order kinetics as state above, and was expressed with Eq. 4. When the conversion of CS-GP was analyzed with the pseudo-first order kinetics based on the scheme in Table 2, the equation for the appearing PD ($P_{CS\text{-}GP}(t)$) was expressed as shown in Eq. 5.

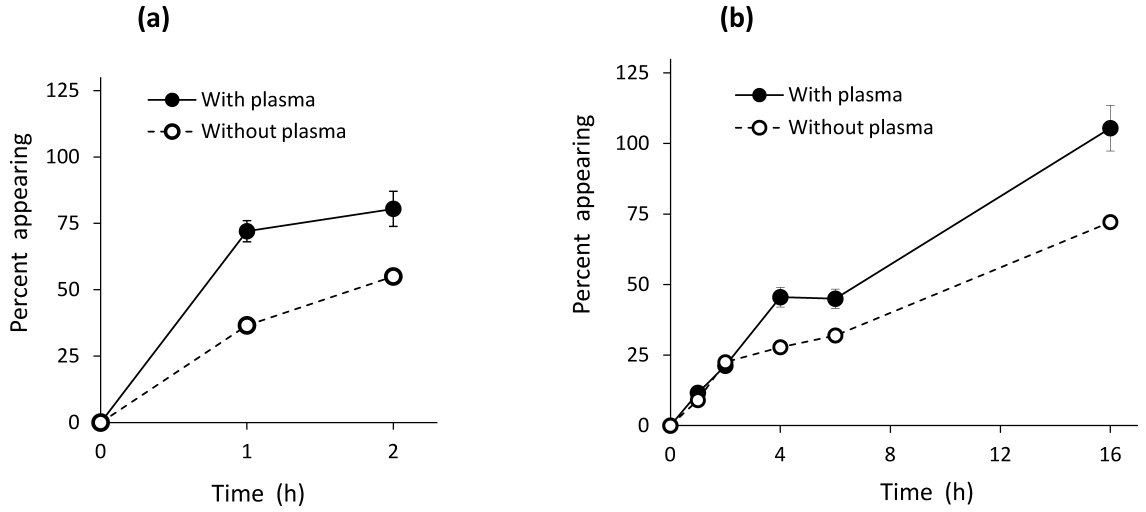


Fig. 8. Release Profiles of PD from GP (a) and CS-GP (b) in PBS with or without Rat Plasma at 37°C

With plasma: the results, obtained using the PBS–rat plasma (10 : 3, v/v) mixture as a medium, are expressed as the mean±S.D. (n=3). Without plasma: the results, obtained using PBS as a medium, are expressed by one experiment.

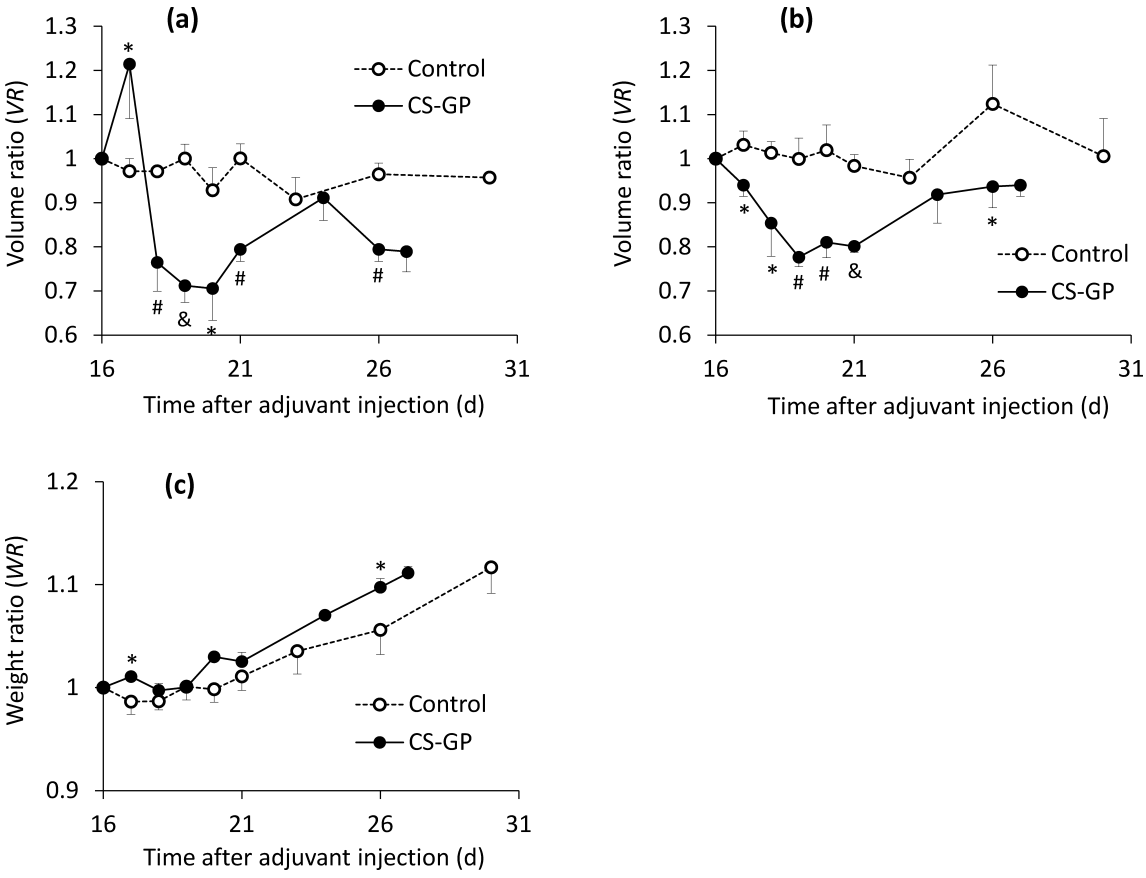


Fig. 9. Effect of CS-GP against Adjuvant-Induced Arthritis in Rats

CS-GP was administered intravenously consecutively on the 16th and 17th day (total dose: 2×2 mg PD eq/kg). (a) Right hind paw (injected paw); (b) left hind paw (opposite paw); (c) change in body weight. Each result is expressed as the mean±S.E. (n=3). $^{*}p<0.05$, $^{\#}p<0.01$, and $^{\&}p<0.001$ *vs.* control.

$$P_{PD2}(t) = P_{PD2}(0) \times \exp(-k_0 t) \quad (4)$$

$$P_{CS\text{-}GP}(t) = PT_{CS\text{-}GP} \times k_1 \times \frac{\exp(-(k_1+k_2)t) - \exp(-k_0 t)}{k_0 - k_1 - k_2} \quad (5)$$

in which $PT_{CS\text{-}GP}$ was an initial content of PD in the CS-GP. The rate constant and half-life were determined in the same manner as for GP. The parameter values were calculated as shown in Table 2. The calculated curves were superimposed with various lines in Figs. 6 and 7. The k_1 value was smaller than the h_1 value at each pH. The k_2 value was much smaller than the h_2 value at pH 7.4 and 8, which indicated that the degradation rate and manner should be very different between GP and CS-GP at these pH conditions. These results suggest-

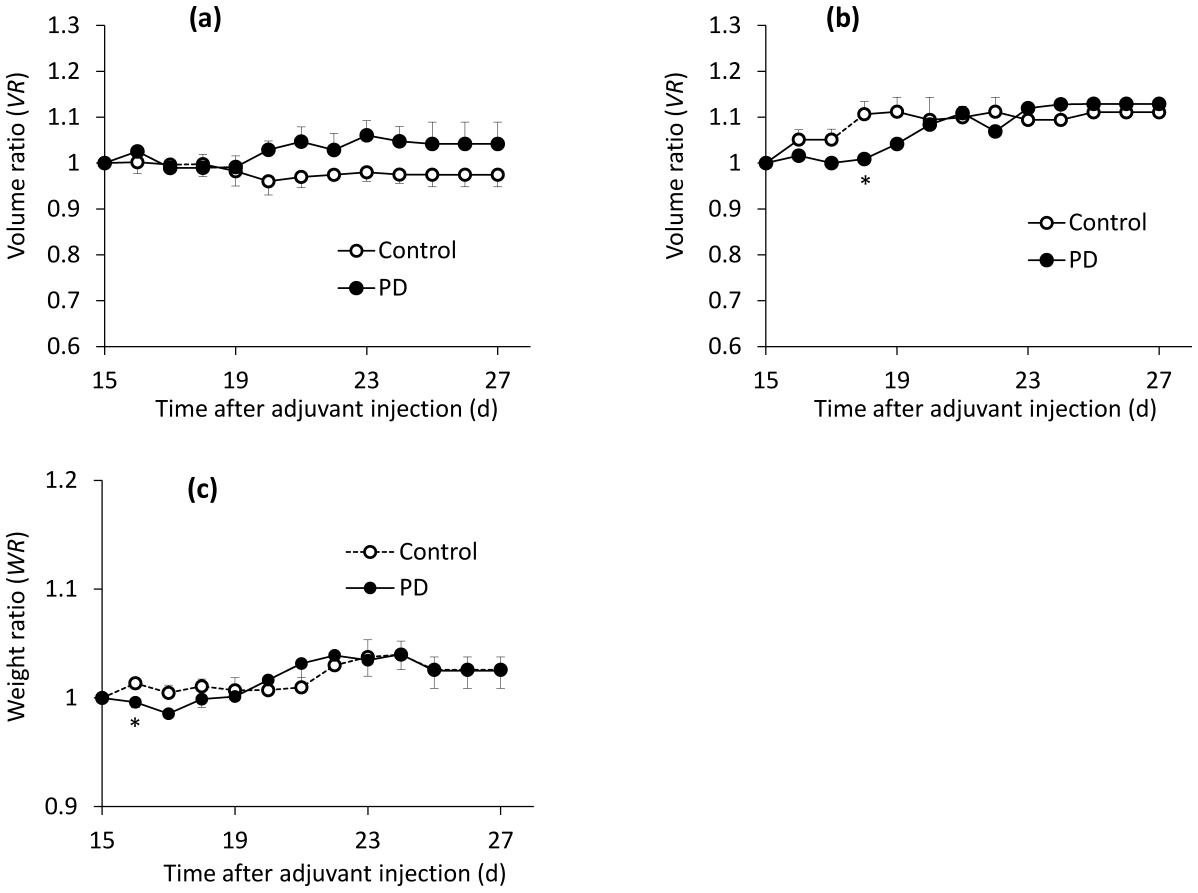


Fig. 10. Effect of PD against Adjuvant-Induced Arthritis in Rats

PD was administered intravenously consecutively on the 15th and 16th day (total dose: 2.5×2 mg PD eq/kg). (a) Right hind paw (injected paw); (b) left hind paw (opposite paw); (c) change in body weight. Each result is expressed as the mean±S.E. (n=4). *p<0.05. *vs.* control.

ed that CS-GP should exhibit a gradual and effective release of PD at physiological pH, but the release rate of PD would become lower at the inflammatory pH, which is known to be weakly acidic, pH 6–7.4.[35]

Release of PD from GP and CS-GP in the PBS–Plasma Mixture In order to elucidate more clearly the stability under the physiological conditions, effect of rat plasma on the release rate of PD was examined for GP and CS-GP. The release profiles are shown in Fig. 8. The release rate was accelerated in GP to a fair extent by the addition of rat plasma. Namely, more than 75% of PD was released within 2 h, while approximately 50% of PD was released without plasma. As to CS-GP, the release rate of PD was increased to a small extent by the addition of rat plasma; a slow release was maintained, and the release ratio was less than 50% at 6 h even by the addition of rat plasma.

The activity of carboxy esterase is known to be fairly high in rat plasma.[39] The hydrolysis of GP was accelerated appreciably by the addition of rat plasma. On the other hand, the release rate from CS-GP was accelerated to a small extent. Probably, in CS-GP, the ester bond might be insusceptible to the enzymatic hydrolysis due to the bulky CS backbone. The results suggested that CS-GP should function as a prodrug showing slow release of PD in the systemic or physiological conditions, which was adequate because the conjugate should be stable before delivered to the target site.

Those findings for the stability and drug release of CS-GP suggested that PD should be released gradually systemically (approximately pH 7.4) and the diseased part (approximately pH 6–7.4[35]). According to the targeting of macromolecular conjugates to the inflammatory sites such as tumor tissues based on the high permeability of neovascular vessels, the conjugates with several tens of thousands of MW are known to be distributed well to the target site within a fairly short period (several h).[28,36] Therefore, CS-GP, being moderate in size (30000–50000), is expected to be distributed fairly fast to the inflammatory diseased site such as rheumatoid arthritis. Although CS-GP was not stable completely in the systemic circulation conditions, it was considered to be delivered to the inflammatory site and to release drug gradually, leading to the promotion of effectiveness.

Preliminary Studies for Anti-inflammatory Effect of CS-GP In this study, CS-GP was examined for its pharmacological effectiveness *in vivo* for a preliminary evaluation. The volumes of both hind paws were measured on the adequate time (d), and the first dosing was conducted on the day when the paw volume reached plateau, and the second dosing was performed on the next day. Higaki *et al.* evaluated the drug potential by comparing the inflammatory volumes between after and immediately before treatment.[16] Therefore, the drug pharmacological potential was evaluated in a similar concept. Namely, the drug effect was investigated from the volume ratio (VR) obtained by the comparison between the paw volume after treatment ($V(t)$) and that immediately before

the first dosing (V(1st dosing)) as follows.

$$\text{volume ratio } (VR) = V(t) / V(\text{1st dosing}) \quad (6)$$

in which t was the time (d) after injection of the adjuvant.

In addition, the change in body weight was checked in order to check the animal conditions including toxic side effect. Namely, the weight ratio (WR) was calculated in the following equation.

$$\text{weight ratio } (WR) = W(t) / W(\text{1st dosing}) \quad (7)$$

in which $W(t)$ is the body weight t d after injection of the adjuvant and W(1st dosing) is that just before the first drug administration.

One day after injection of the adjuvant, swelling and redness appeared in the adjuvant-injected hind paw (right paw), while no such change was observed in the opposite hind paw (left paw); however, from 10 d after injection of the adjuvant, disseminated arthritis was observed in both hind paws. For both hind paws, the paw volume reached a maximal level (plateau) 15 or 16 d after injection of the adjuvant. The *VR* value was observed to decrease to a fair extent in both hind paws after the dosing of CS-GP (2 mg PD eq/kg×2 d), while it was maintained at almost the constant level around the value of 1.0 for the control (Fig. 9). When the *VR* values were compared between CS-GP and the control, they were significantly lower with CS-GP. This demonstrated that CS-GP was pharmacologically effective against the adjuvant-induced arthritis in rats. In addition, the comparison of the *VR* values between PD (2.5 mg/kg×2 d) and control was performed separately. As shown in Fig. 10, PD exhibited no suppression of the paw volume, and the *VR* values were hardly different between PD and the control. This suggested that PD should not be effective against the present arthritis model. As to the change in body weight, the *WR* value increased a little in CS-GP (Fig. 9), while it was not changed with PD (Fig. 10). Furthermore, the change in body weight was not different between PD and control (Fig. 10), which suggested that the intravenous (i.v.) dosing of PD should not lead to the increase in the body weight. van den Hoven *et al.* reported that liposomal glucocorticoids exhibited improved therapeutic effect and that the body weight regain was observed in conjunction with the improvement.[14] They considered the remission of inflammation to be the main reason for the body weight recovery.[14] Furthermore, it was stated that the body weight loss was induced by the arthritis itself and the toxic side effect of glucocorticoids.[14] In the treatment with CS-GP (2 mg PD eq/kg×2), the change in body weight was not marked. Probably, the slight weight increase in CS-GP was due to the remission of the inflammation.

The present *in vivo* studies were performed as a preliminary study to get the rough outline of the pharmacological potential of CS-GP. That is, PD was administered at a little greater dose. In addition, the *in vivo* present results of CS-GP and PD were obtained separately at the different periods. In the *in vivo* study of CS-GP, the plateau of the right paw swelling tended to retard a little, which was recognized from the fact that the *VR* value increased on 17th day. Therefore, the 1st dosing time was conducted on the 16th day for CS-GP, while that was done on the 15th day for PD. The suppressive effect against the arthritis based on the paw volume ratio indicated that CS-GP appeared to be more potent than PD alone (Figs. 9, 10). Probably, the design concept, stated at i)–iv) in the introduction, appeared to be completed to a fair extent. However, the present *in vivo* results were preliminarily obtained. For more precise and detailed evaluation of the CS-GP efficacy, further refined dosing studies have to be conducted, including time schedules and check of the contribution of CS. In the near future, the effectiveness of CS-GP will be elucidated more exactly.

Conclusion

The conjugate of PD with CS, named CS-GP, was prepared using glycine as a linker. Synthesis of GP was performed by the two-step method. CS-GP was prepared by coupling between CS and GP with water-soluble carbodiimide. Overall, GP was less stable than CS-GP. For GP, PD released well in the buffer at pH 6–7.4, but not at pH 8 due to rapid composition other than conversion to PD. On the other hand, PD was released more slowly in CS-GP. CS-GP released PD efficiently at a moderate rate at physiological pH. All the conversion profiles could be analyzed with pseudo-first order kinetics models. The calculated rate constants supported the slower release of PD from CS-GP. The addition of rat plasma accelerated the release rate of PD from CS-GP to a small extent, suggesting that PD should be released gradually under the physiological conditions. The pharmacological tests using rats with adjuvant-induced arthritis revealed that CS-GP should have a good anti-inflammatory potential, while PD hardly exhibited effectiveness. At the same time, CS-GP did not display toxic side effects related with the body weight loss. Thus, it was elucidated that CS-GP behaved as a macromolecular prodrug of PD and showed good effectiveness against arthritis. The more detailed evaluation of the efficacy for CS-GP will be reported in the next paper.

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