Topical Therapies for Rheumatoid Arthritis by Gel Ointments containing Indomethacin Nanoparticles in Adjuvant-Induced Arthritis Rat

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Abstract: Indomethacin (IMC), a nonsteroidal anti-inflammatory drug, has been used in the treatment of rheumatoid arthritis (RA), although its clinical use has been limited by its systemic side effects that include gastrointestinal lesions. Therefore, the development of IMC formulations that do not cause gastrointestinal lesions is highly anticipated. In this study, we designed new topical formulations containing IMC solid nanoparticles (IMCnano gel ointment), and investigated their pharmacokinetics. In addition, we demonstrate the preventive effects of this topical application of IMC nanoparticles on inflammation in adjuvant-induced arthritis rat (AA rat). The IMCnano gel ointment was prepared using Bead Smash 12 (a bead mill) and additives including 2-hydroxypropyl-β-cyclodextrin, methylcellulose and Carbopol 934; the mean particle size of the IMC nanoparticles was 173 ± 91 nm (means ± S.D.). The application of the IMCnano gel ointment attenuated the increase in paw edema of the hind feet of AA rats in comparison with AA rats treated with gel ointment containing IMC microparticles (IMCmicro gel ointment, particle diameter 17.1 ± 11.6 μm, means ± S.D). In addition, the accumulation of IMC from the IMCnano gel ointment in skin tissue was significantly large than for the IMCmicro gel ointment; however, the plasma IMC concentrations were similar for the IMCmicro and IMCnano gel ointments. Our findings suggest that the dermal application of nanoparticles may enable a medication to be applied without high-systemic drug levels, which could provide efficient and effective therapy that spares patients from unwanted side effects. A formulation of a topical drug delivery system using IMC nanoparticles may provide a delivery option for the clinical treatment of RA.

Key words: nanoparticle, indomethacin, gel ointment, topical drug delivery, adjuvant-induced arthritis

1 INTRODUCTION

Rheumatoid arthritis (RA) is a complex chronic inflammatory disease dependent on multiple interacting environmental and genetic factors, which make it difficult to understand its pathogenesis and thereby to find effective therapies1. In treatments for RA, the focus is on the reduction of pain, inflammation and joint damage. The principal pharmacological agents are nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs, glucocorticoids and specific inhibitors of the mediator response2. Indomethacin (IMC), a NSAID, is widely used in the management of patients affected by dermatitis and RA3. Oral therapy with IMC is very effective, but its clinical use is often limited by its significant side effects4–9) that include irritation and ulceration of the gastrointestinal mucosa. Also, it requires frequent dosing10. In addition, it is known that RA patients taking IMC are more susceptible to IMC-induced gastrointestinal lesions in comparison with other patients4–8,11). Due to these problems, several studies are aimed at the development of an efficient means to allow for topical IMC administration in order to increase local soft-tissue and joint concentrations by reducing the systemic distribution so as to limit the harmful side-effects12,13).

Dermal application offers many advantages over conventional oral delivery for medications such as allowing smooth and continuous drug delivery and reduced the maximum plasma concentration of the drug (Cmax). Dermal systems improve the tolerability profile and permit the achievement of relatively high local drug concentrations without systemic side effects. However, the amount of drug that can be administered transdermally is quite low since transdermal delivery is severely limited by the inability of a large majority of drugs to cross the skin at therapeutic rates due
to the barrier imposed by the outer stratum corneum layer of the skin. Accordingly, considerable research efforts are focused on drug permeation, and various enhancing methods have been tried: iontophoresis and electroporation\textsuperscript{14, 15}, phonophoresis\textsuperscript{16, 17}, chemical methods and absorption enhancers\textsuperscript{18, 19}. Recently, strategies using micro/ nanoparticles have been developed and investigated. Nanoparticles can be engineered to carry drug payloads, image contrast agents, or gene therapeutics for diagnosing and treating disease, with cancer being a primary focus, and research into nanomedicines has increased enormously during the past years\textsuperscript{20–28}. The size of a particle influences its functionality in terms of uptake, residence in circulation, adherence, degradation, as well as clearance\textsuperscript{19–30}. The fate of particles inside the body has been reported as follows\textsuperscript{31}: ≥2 μm, trapped inside liver cells; ≥300 - 400 nm, captured by macrophages and excreted; ≥200 nm, filtered in the spleen; ≤100 nm, escape from blood vessels through the endothelial lining. Thus, size governs the movement of nanoparticles inside tissues. We also reported that a dermal application using these nanoparticles enhanced drug permeability through the skin\textsuperscript{30}. It is expected that drug systems using nanoparticles may provide an alternative strategy for increasing drug permeation\textsuperscript{36–39}, and it is possible that a topical drug delivery system using nanoparticles may lead to an expansion of their usage as therapy for RA patients.

Here, we have designed new topical formulations containing IMC solid nanoparticles, and investigated their pharmacokinetics. In addition, we demonstrate the preventive effects of these IMC nanoparticle formulations on inflammation in adjuvant-induced arthritis (AA) rats.

2 EXPERIMENTAL

2.1 Animals and reagents

Male Wistar rats, aged 7 weeks, and Dark Agouti (DA) rats, aged 6 to 13 weeks, were housed under standard conditions (12 h/d fluorescent light (07:00-19:00), 25°C room temperature), and allowed free access to a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water. 2-Hydroxypropyl-β-cyclodextrin (HPβCD, average molar substitution 0.6; average MW, 1380) was purchased from Nikon Shokuhin Kako Co., Ltd. (Tokyo, Japan). Low-substituted methylcellulose (MC, METOLOSE SM-4, average viscosity, 4 Pa·s at 20°C) was provided by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Carboxypolymethylene (Carbopol\textsuperscript{®} 934) was obtained from Serva (Heidelberg, Germany). Conventional IMC (solid, IMC microparticles, 17.1 ± 11.6 μm, means ± S.D.) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

2.2 Preparation of gel ointment containing IMC nanoparticles

Carbopol\textsuperscript{®} 934 was added to distilled water, allowed to swell for 1 h at room temperature, and neutralized with 5% ammonia water (gel base). IMC nanoparticles were prepared using zirconia beads and Bead Smash 12 (a bead mill, Wakenyaku Co. Ltd, Kyoto, Japan)\textsuperscript{35, 40–42}. Zirconia beads (diameter: 2 mm) were added to the IMC microparticles with MC, and the mixtures were crushed with the Bead Smash 12 for 30 sec (3,000 rpm, 4°C). The mixtures were then dispersed in saline with 5% HPβCD, and crushed again with the Bead Smash 12 (5,500 rpm, 30 sec × 15 times, 4°C) in the presence of zirconia beads (diameter: 0.1 mm). The particle size of milled IMC was 0.173 ± 0.091 μm (means ± S.D.), and the solubility of IMC in saline with and without 5% HPβCD was 0.003% and 0.012%, respectively (the inclusion complex by 5% HPβCD was 0.009%). After milling, the IMC nanoparticles containing HPβCD and MC were added to the Carbopol\textsuperscript{®} 934 gel base, and stirred until the mixture became uniform (IMCnano gel ointment). The gel ointment containing IMC microparticles was prepared by adding IMC microparticles, HPβCD and MC into the Carbopol\textsuperscript{®} 934 gel base (IMCmicro gel ointment). The formulation of the gel ointment containing IMC is as follows: 1% IMC, 5% HPβCD, 0.5% MC, 3% Carbopol\textsuperscript{®} 934, w/w%. The dispersity of the nanoparticles in the ointment base was confirmed as follows: 1% IMCmicro and IMCnano gel ointments were divided into 10 parts, and the IMC concentration in each part was measured by HPLC (IMCmicro, 0.99 ± 0.11; IMCnano, 1.05 ± 0.06, %, means ± S.E., n = 10). Figure 1 shows the particle size of the 1% IMCmicro and IMCnano gel ointments. The particle size was measured using a nanoparticle size analyzer SALD-T100 (Shimadzu Corp., Kyoto, Japan; refractive index 1.60-0.10i). The IMCmicro and IMCnano gel ointments were stable for one month after preparation (mean particle size: IMCmicro, 17.8 ± 12.6; IMCnano 0.191 ± 0.105 μm, means ± S.D.), and no decrease in IMC concentration in the IMCmicro or IMCnano gel ointments was observed during the one month.

2.3 Release of IMC from IMC gel ointments

An experiment was carried out using 100 and 450 nm pore size membrane filters (MF\textsuperscript{TM}_MEMBRANE FILLER, Merck Millipore, Tokyo, Japan) and a Franz diffusion cell\textsuperscript{36, 40}. The donor side of the membrane filter was soaked in buffer (0.85% NaCl-10 mM phosphate buffer, pH 7.4) for 12 h at 4°C to equilibrate the membrane. Then 0.3 g IMC gel ointment was spread uniformly over the membrane, which was then mounted in the Franz diffusion cell (reservoir volume 12.2 ml, 1.6 cm i.d. O-ring flange), and occluded with aluminium foil. The diffusion cells were thermostated in a water bath at 37°C for 24 h. One hundred microliter aliquots of sample solution were withdrawn from the reservoir chamber at the indicated time intervals and replaced.
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with the same volume of buffer. Ten microliters of filtrate was added to 100 μl methanol containing 0.1 μg propyl p-hydroxybenzoate (internal standard), and the mixture was filtered through a Chromatodisk 4A (pore size 0.45 μm, Kurabo Industries Ltd., Osaka, Japan). The filtered solution (10 μl) was injected into an Inertsil® ODS-3 (3 μm, column size: 2.1 mm × 50 mm) column on a Shimadzu LC-20AT system equipped with a column oven CTO-20A (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of acetonitrile/50 mM acetic acid (40/60, v/v) at a flow rate of 0.25 mL/min; the column temperature was 35°C, and the wavelength for detection was 254 nm.

2.4 In vitro skin penetration of IMC micro and IMC nano gel ointments

The in vitro skin penetration experiment was carried out using the Franz diffusion cell. On the day before the experiment, the hair on the abdominal area of 7-week-old Wistar rats was carefully removed with an electric clipper and electric razor. The following day, pieces (3 × 3 cm area) of full-thickness abdominal skin were excised from the rats, and the adherent fat and other visceral debris were removed from the undersurface. The dermal side of the full-thickness skin was soaked in buffer (NaCl-10 mM phosphate buffer, pH 7.4) for 12 h at 4°C to equilibrate the skin. Then 0.3 g IMC gel ointment was uniformly spread over the stratum corneum of the skin, which was then mounted in a Franz diffusion cell (reservoir volume 12.2 ml, 1.6 cm i.d. O-ring flange), and occluded with aluminum foil. The diffusion cells were thermostated in a water bath at 37°C for 30 h. The IMC concentrations in the samples were determined by the HPLC method described above. The data obtained were analyzed using the following formula (Eqs. 1-3):

\[
t_{lag} = \frac{\delta^2}{6D}
\]

Eq. 1

\[
J_c = \frac{K_m \cdot D \cdot C_{IMC}}{\delta} = K_p \cdot C_{IMC}
\]

Eq. 2

\[
Q_t = J_c \cdot A \cdot (t - t_{lag})
\]

Eq. 3

where \( J_c \) is the IMC penetration rate, \( K_m \) is the skin/preparation partition coefficient, \( D \) is the diffusion constant within the skin, \( t_{lag} \) is the lag time, \( \delta \) is thickness of the skin (0.071 cm, average for 5 rats), \( Q_t \) is the total amount of IMC appearing in the reservoir solution at time \( t \), and \( A \) is the effective area of skin (2 cm²); \( J_c \) and \( t_{lag} \) were estimated by fitting each penetration profile to Eq. 3. The penetration coefficient through the skin, \( K_p \), is given by \( J_c/C_{IMC} \). A non-linear least-squares computer program (MULTI) was used to fit the data.
employed for the calculation.\(^{45}\) 

2.5 In vivo percutaneous absorption of IMC\(_{\text{micro}}\) and IMC\(_{\text{nano}}\) gel ointments

On the day before the experiment, the hair on the abdominal area of 7 week-old Wistar rats was carefully removed with an electric clipper and electric razor, and a canula filled with 30 \(\mu\)g/ml heparin (silicone tubing; I.D., 0.5 mm, O.D., 1.0 mm) was inserted into the right jugular vein under isoflurane anesthesia. 0.3 g of IMC gel ointment was fixed on the shaved abdominal skin with an adhesive and immediately occluded with adhesive tape. At 24 h after the start of the experiment, the skin surface to which the gel ointment was applied was washed with saline, and the IMC gel ointments were reapplied. Venous blood (200 \(\mu\)l) was collected at 0-96 h after the application of IMC gel ointment from the jugular vein through the canula. The blood was centrifuged at 3,000 rpm for 20 min at 4\(^\circ\)C, and the plasma obtained was stored at \(-80^\circ\)C until IMC analysis. The IMC concentrations in the samples were determined by the HPLC method described above. The IMC concentration in the plasma after a single injection of 0.3 ml of IMC solution (200 \(\mu\)g/kg) into the femoral vein was analyzed according to Eq. 4\(^{46}\):

\[
C_{\text{IMC}} = C_0 \cdot e^{-k_a \cdot t}
\]

Eq. 4

where \(C_{\text{IMC}}\) is IMC concentration in the plasma, \(C_0\) is the initial concentration of IMC in the plasma (2.68 \(\pm\) 0.13 \(\mu\)g/ml), \(k_a\) is the elimination rate constant for IMC from the plasma. The \(k_a\) and distribution volume (\(V_a\)) data obtained from 5 experiments were 0.05 \(\pm\) 0.07 h\(^{-1}\) and 52.1 \(\pm\) 1.98 ml/kg, respectively.

The absorption of IMC after a single administration of IMC gel ointment was calculated as the apparent absorption rate constant (\(k_a\), h\(^{-1}\)) according to Eq. 5\(^{46}\):

\[
C_{\text{IMC}} = \frac{k_a \cdot F \cdot D}{V_a (k_a - k_s)} \left( e^{-k_a \cdot t} + \frac{e^{-k_a \cdot t}}{1 + e^{-N \cdot k_a \cdot t}} \right)
\]

Eq. 5

where \(C_{\text{IMC}}\) is the IMC concentration in the plasma, \(D\) is the dose of IMC administered (1% gel ointments, 0.3 g), \(k_s\) is the absorption rate constant, \(t\) is time (0-24 h) after IMC administration, \(t_{\text{lag}}\) is lag time (h), \(V_a\) is the distribution volume, \(F\) is the fraction of IMC absorbed. In addition, the plasma IMC concentration data after repeated administration of IMC gel ointments (0.3 g/day, interval 24 h) were estimated by Eq. 6\(^{46}\):

\[
C_{\text{IMC}} = \frac{k_a \cdot F \cdot D}{V_a (k_a - k_s)} \left[ \left( \frac{1 - e^{-N \cdot k_a \cdot t}}{1 - e^{-k_a \cdot t}} \right) e^{-k_a \cdot t} - \left( \frac{1 - e^{-N \cdot k_a \cdot t}}{1 - e^{-k_a \cdot t}} \right) e^{-k_a \cdot t} \right]
\]

Eq. 6

where \(t\) is the interval after IMC administration (24 h), \(N\) is the frequency of IMC administration. A nonlinear least-squares computer program (MULTI) was employed for these calculation (Eqs. 4-6).

The area under the IMC concentration-time curve (AUC\(_{0-24h}\)) was analyzed according to the following equation (Eq. 7):

\[
AUC_{0-24h} = \int_0^{24} C_{\text{IMC}} dt
\]

Eq. 7

Briefly, AUC\(_{0-24h}\) was determined according to the trapezoidal rule up to 24 h, which was the time of the final IMC concentration measurement.

2.6 Accumulation of IMC\(_{\text{micro}}\) and IMC\(_{\text{nano}}\) gel ointments in skin tissue

On the day before the experiment, the hair on the abdominal area of 7 week-old Wistar rats was carefully removed with an electric clipper and electric razor. The following day, 0.3 g of IMC gel ointment was fixed on the shaved abdominal skin with an adhesive and immediately occluded with adhesive tape. At 24 h after the start of the experiment, the skin surface to which the gel ointment was applied was washed with saline, and the IMC gel ointments were reapplied. The gel ointment on the skin surface was wiped off with an Elleair Prowipe Soft Micro Wiper S220 (Prowipe, DAIO PAPER CORPORATION, Tokyo, Japan) soaked in saline, and pieces (2 cm\(^2\)) of abdominal skin to which the IMC gel ointments were applied were excised at 0-24 h after application. The adherent fat and other visceral debris were removed from the undersurface, and the skin samples obtained were stored at \(-80^\circ\)C until IMC analysis. The frozen skin was homogenized in methanol using a Physocotan homogenizer (MICROTEC CO., LTD., Chiba, Japan). The homogenates were centrifuged at 15,000 rpm for 20 min at 4\(^\circ\)C, and the supernatants were used for the measurement of IMC. The IMC concentrations in the samples were determined by the HPLC method described above.

2.7 Application of IMC gel ointments to AA rats

Arthritis was induced by the injection of 50 \(\mu\)l of adjuvant, a suspension of 10 mg/ml heat-killed Mycobacterium butyricum (Difco, Detroit, MI) in Bayol F oil, into the plantar region of the right hind foot and tail of DA rats. The control group received 50 \(\mu\)l of Bayol F oil alone. The rats were housed to prevent them from licking, and 0.3 g of IMC gel ointment was applied to the right foot daily (9:00). When reapplying the gel ointment, the old gel ointment on the skin surface was first wiped off with saline on a Prowipe. The application of IMC gel ointments was started after adjuvant injection. In this study, inflammation during the development of AA was assessed by measuring paw volume (paw edema), a parameter of inflammation, by plethysmometry. In this paper, the inflammatory scores are represented as AUC (the area under the paw volume-time curve, AUC\(_{0-42d}\)). The values of AUC\(_{0-42d}\) were calculated according to the following equation (Eq. 8):

\[
AUC_{0-42d} = \int_0^{42} V dt
\]

Eq. 8

where \(V\) and \(t\) are the paw volume and the number of days.
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2.8 Statistical analysis
All values are represented as mean ± standard error of the mean (S.E.). Unpaired Student’s or Aspin-Welch’s t-tests were used to determine statistical difference, and multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison. P values less than 0.05 were considered significant.

3 RESULTS AND DISCUSSION
3.1 Percutaneous Penetration of IMC Released from IMC_dro and IMC_nano Gel Ointments
First, we prepared IMC nanoparticles. Additives are very important in the preparation of high quality nanomedicines since they affect the environment around the surface of the particles. In a previous study, we found that the addition of HPβCD and MC was suitable for the preparation of nanoparticles using mill methods [45, 46, 47, 48, 49]. HPβCD is a cyclic oligosaccharide with a hydrophilic outer surface and a lipophilic cavity in its center. It is capable of forming inclusion complexes with many lipophilic drugs by taking up the drug molecule, or part of it, into the lipophilic cavity [46, 47]. Moreover, HPβCD enhances the stability of drug dispersions containing nanoparticles by attaching to the surface of the nanoparticles [45, 46, 47]. MC is a water-soluble substance with a high degree of purity, uniformity and transparency. MC solutions are neutral, odorless, and tasteless, and are also stable over a wide pH range. We have reported that MC is needed to prepare drug nanoparticles by the bead mill method [45, 46, 47]. Therefore, the IMC nanoparticles were prepared using additives (HPβCD and MC) and Bead Smash 12, which resulted in the preparation of high quality IMC nanoparticles (particle size, 173 ± 91 nm, means ± S.D.). The data indicate that the milled IMC nanoparticles are homogeneous with a narrow particle size distribution. Clinically, ointments, creams and gels are used in formulations for dermal application, and gels are particularly used pharmaceutically as lubricants as well as carriers for many drugs to provide a local effect and percutaneous absorption [48, 49]. Therefore, a gel ointment containing IMC nanoparticles was prepared using Carbopol 934, which has excellent thickening, emulsifying and gelling properties, allowing the uniform incorporation of the IMC nanoparticles (Fig. 1). The IMC_dro and IMC_nano gel ointments were stable for one month after preparation (mean particle size: IMC_dro, 17.8 ± 12.6; IMC_nano, 0.191 ± 0.105, μm, means ± S.D.), with no decrease in IMC concentration in either ointment observed during the one month. These results show that the procedures developed in this study are suitable for the preparation of gel ointments containing IMC nanoparticles.

Next, the drug release and skin penetration of IMC_dro and IMC_nano gel ointments was demonstrated. Figure 2 shows the penetration profiles of IMC through a membrane filter after the application of IMC_dro and IMC_nano gel ointments. Although the IMC release from the IMC_nano gel ointment was similar to that from the IMC_dro gel ointment in experiments using 100 nm pore size membranes, the IMC penetration profile of the IMC_nano gel ointment through a 450 nm pore size membrane was significantly higher than that of the IMC_dro gel ointment, and the release from the IMC_dro gel ointment was 38.9% of that from the IMC_nano gel ointment 24 h after the application of 1% IMC gel ointment. This result suggests that the IMC emitted from the IMC_nano gel ointment remains in the state of IMC nanoparticles.

Figure 3 shows the penetration profiles of IMC through rat skin after the application of IMC_dro and IMC_nano gel ointments, and Fig. 4 shows the absorption profiles of IMC

Fig. 2 Release of IMC from Gel Ointments containing IMC Nanoparticles through 100 nm (A) and 450 nm (B) Pore Size Membrane.
0.3 g of IMC gel ointment containing micro (open circles) or nanoparticles (closed circles) was applied to 100 nm (A) and 450 nm (B) pore size membranes for 0-24 h. The data represent the means ± S.E. of 6 independent experiments. *p < 0.05 vs. IMC_dro gel ointment for each category.
through rat skin following the applications of IMC micro and IMC nano gel ointments. Table 1 and 2 summarize the pharmacokinetic parameters calculated from the in vitro skin penetration data (Table 1) and the in vivo percutaneous absorption data (Table 2). In the in vitro rat skin penetration experiments, the amounts of IMC that penetrated the skin increased linearly in the case of both IMC gel ointments up to 30 h after the application of the gel ointment (Fig. 3). Therefore, we used data collected in the 0 - 30 h range to calculate the pharmacokinetic parameters (Table 1). The amount of penetrated IMC, the penetration rate ($J_c$), penetration coefficient through the skin ($K_p$) and skin/preparation partition coefficient ($K_m$) values of the IMC nano gel ointment were all significantly higher than those of the IMC micro gel ointment. The diffusion constant within the skin ($D$) and lag time ($t_{lag}$) for the IMC micro and IMC nano gel ointments showed no significant difference. However, the absorption profiles of IMC through the skin following the applications of IMC micro and IMC nano gel ointments were similar (Fig. 4A and Table 2), and the IMC plasma concentration following the repetitive administration of the IMC micro or IMC nano gel ointments reached a plateau at 48-96 h with a value of 1.4 - 3.0 μg/ml (concentration at steady state, $C_{ss}$, Fig. 4). On the other hand, the IMC concentrations in the skin tissue of rats receiving the IMC nano gel ointment were significantly higher than those of rats receiving the IMC micro gel ointment (Fig. 5). We show that the supply of IMC from the IMC nano gel ointment was higher than that from IMC micro gel ointment in this study (Figs. 2 and 3). Therefore, the greater amount of IMC from the IMC nano gel ointment may be related to the IMC concentrations in the skin tissue, and the IMC in skin may show only a limited shift to the blood.

These results show that the characteristics of the IMC micro and IMC nano gel ointments in skin differ. In addition, we demonstrated the plasma concentration and accumulation in the skin following the application of a commercially available IMC gel ointment (IDOMETHINE KOWA gel 1%). The plasma concentration following the repetitive administration of IMC nano gel ointment was lower than that following the commercially available ointment ($C_{ss}$, 10.3 - 22.9 μg/ml).
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Table 1 Pharmacokinetic Parameters for the In Vitro Skin Penetration of IMC Released from 1% IMC_{micro} and IMC_{nano} Gel Ointments.

<table>
<thead>
<tr>
<th>Ointment</th>
<th>$J_c$ (µmol/cm²/h)</th>
<th>$K_p$ ($\times 10^{-4}$ cm/h)</th>
<th>$K_m$</th>
<th>$t_{lag}$ (h)</th>
<th>$D$ ($\times 10^{-4}$ cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMC_{micro} gel</td>
<td>9.02 ± 0.40</td>
<td>0.90 ± 0.39</td>
<td>0.14 ± 0.06</td>
<td>1.89 ± 0.05</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>IMC_{nano} gel</td>
<td>24.8 ± 0.47*</td>
<td>2.49 ± 0.40*</td>
<td>0.37 ± 0.05*</td>
<td>1.80 ± 0.06</td>
<td>0.47 ± 0.02</td>
</tr>
</tbody>
</table>

The experiments were carried out using a Franz diffusion cell; the pharmacokinetic parameters were calculated according to Eqs. 1-3. IMC_{micro} gel: gel ointment containing IMC microparticles, IMC_{nano} gel: gel ointment containing IMC nanoparticles. The data represent the means ± S.E. of 6 rat skins. *p < 0.05 vs. IMC_{micro} gel ointment for each category.

Table 2 Pharmacokinetic Parameters for the In Vivo Percutaneous Absorption of IMC Released from 1% IMC_{micro} and IMC_{nano} Gel Ointments.

<table>
<thead>
<tr>
<th>Ointment</th>
<th>$k_a$ (h⁻¹)</th>
<th>$t_{lag}$ (h)</th>
<th>$F$</th>
<th>$AUC_{0-24}$ (nmol·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMC_{micro} gel</td>
<td>0.39 ± 0.03</td>
<td>0.09 ± 0.01</td>
<td>0.71 ± 0.18</td>
<td>41.3 ± 4.1</td>
</tr>
<tr>
<td>IMC_{nano} gel</td>
<td>0.31 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>0.73 ± 0.20</td>
<td>41.5 ± 3.9</td>
</tr>
</tbody>
</table>

IMC gel ointment was applied to the abdominal skin; the pharmacokinetic parameters were calculated according to Eqs. 4, 5 and 7. IMC_{micro} gel: gel ointment containing IMC microparticles, IMC_{nano} gel: gel ointment containing IMC nanoparticles. The data represent the means ± S.E. of 6 rats.

3.2 Therapeutic Effects of IMC_{micro} and IMC_{nano} Gel Ointments on Paw Edema in AA Rats

In studies to develop new topical formulations containing IMC solid nanoparticles for treating RA, the selection of the experimental animal is very important. The AA rat is an animal model in which arthritis is induced by the injection of an adjuvant. Inflammatory pain during the development of AA is assessed by measuring paw edema. Paw edema in AA rats is known to involve two inflammatory processes, primary and secondary inflammation. Primary inflammation starts from the day following the injection of adjuvant into the right hind foot. Secondary inflammation is observed from 7 days after adjuvant injection, and reaches a maximum in both feet 14 days after adjuvant injection into the right and/or left hind foot. It is noteworthy that changes in the biological characteristics of AA rats correspond to those that occur in human RA.

Therefore, AA rats provide a useful model for use in studies to evaluate transdermal therapeutic systems in RA. Figure 6 and Table 3 show the preventive effects of IMC_{micro} and IMC_{nano} gel ointments on paw edema in AA rats. Paw edema in the right hind foot injected with adjuvant appeared on the day following injection, and reached a maximum after 14 days (Fig. 6A). On the other hand, paw edema of the left hind foot, which was not injected with adjuvant, was not observed during the first 7 days after adjuvant injection, but clearly increased from 10 days, and reached a maximum at 14 days (Fig. 6B). The application of commercially available ointment was significantly prevented the increase in paw edema of the right and left hind feet of AA (right hind feet 29.8 ± 0.85, left hind feet 24.8 ± 0.68, ml).
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means ± S.E. n = 6 rats. This result showed that the increase in plasma IMC concentration caused the suppression of paw edema in both of right and left hind feet. Although, paw edema of the right hind feet of AA rats to which the IMCmicro gel ointment was applied tended to be less than, no significant difference was found in comparison with AA rats treated with gel ointment containing no IMC (control gel ointment). In contrast to the results in AA rats treated with the IMCmicro gel ointment, paw edema of the right hind feet of AA rats to which the IMCnano gel ointment was less in the days following adjuvant injection, and the AUC0-42d values were significantly lower than those of AA rats treated with control gel ointment or the IMCmicro gel ointment (Table 3). On the other hand, the paw edema of the left hind feet of AA rats to which the IMCmicro or IMCnano gel ointment was applied did not differ significantly from that of rats receiving the control gel ointment. These data suggest that the achievement of relatively high local IMC concentrations in the case of the IMCnano gel ointment resulted in effective therapy.

Further studies are needed to elucidate the precise mechanism for the skin penetration of gel ointments containing IMC nanoparticles. In addition, it is important to clarify a suitable formulation and particle size for a dermal application for RA using IMC particles. Therefore, we are now investigating the therapeutic effects of a topical drug delivery system using IMC particles of various sizes on inflammation and pharmacokinetics in AA rats.

4 CONCLUSIONS
IMC, [1-{4-chlorobenzoyl}-5-methoxy-2-methylindol-3-yl]acetic acid, molecular weight 357.8, pKa 4.5, has been used as therapy for RA patients. However, gastrointestinal lesions are the most common side effect in patients taking IMC, and RA patients taking IMC are more susceptible to IMC-induced gastrointestinal lesions in comparison with other patients. Therefore, the development of IMC formulations that do not cause gastrointestinal lesions is highly anticipated. In this study, we have developed a new topical drug delivery system that includes IMC nanoparticles using Bead Smash 12 and additives including HPβCD, MC and Carbopol 934. The accumulation of IMC from the gel ointment in skin tissue and the therapeutic effect on inflammation for the IMCnano gel ointment were significantly greater than those for the IMCmicro gel ointment; however, the plasma IMC concentrations were similar between the IMCmicro and IMCnano gel ointments. Our findings suggest that a dermal application using nanoparticles may enable a medication to be applied without leading to high systemic levels, providing efficient and effective therapy that spares

Table 3 Preventive Effects of 1% IMCmicro and IMCnano Gel Ointments on Paw Edema in AA Rats.

<table>
<thead>
<tr>
<th>Ointment</th>
<th>AUC0-42d (ml·day)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
</tr>
<tr>
<td>Vehicle</td>
<td>48.9 ± 1.9</td>
</tr>
<tr>
<td>IMCmicro</td>
<td>45.4 ± 2.1</td>
</tr>
<tr>
<td>IMCnano</td>
<td>32.5 ± 1.7∗</td>
</tr>
</tbody>
</table>

The values for AUC0-42d were calculated according to Eq. 8.
Vehicle: gel ointment without IMC treated AA rat, IMCmicro gel: gel ointment containing IMC microparticles treated AA rat, IMCnano gel: gel ointment containing IMC nanoparticles treated AA rat. The data represent the means ± S.E. of 6 independent rats. ∗p < 0.05 vs. Vehicle for each category; ∗∗p < 0.05 vs. IMCmicro gel for each category.

Fig. 6 Changes in Paw Edema of the Right (A) and Left (B) Hind Feet of AA Rats Treated with IMCmicro and IMCnano Gel Ointments. The applications of 0.3 g of gel ointment containing no IMC (control, open circles), or IMC gel ointment containing micro- (closed circles) or nanoparticles (closed triangles) was applied to the right foot once a day (9:00). The data are presented as means ± S.E. of 6 independent rats. ∗p < 0.05 vs. IMCmicro gel ointment for each category.
patients from unwanted side effects. A formulation of a topical drug delivery system using IMC nanoparticles may provide a delivery option for the clinical treatment of RA.

References


