Effect of Solid Nanoparticle of Indomethacin on Therapy for Rheumatoid Arthritis in Adjuvant-Induced Arthritis Rat

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Received November 25, 2013; accepted April 21, 2014

We designed new oral formulations containing indomethacin (IMC) solid nanoparticles, and investigate their usefulness by evaluating bioavailability and gastrointestinal lesions. The IMC solid nanoparticles were prepared using methylcellulose (MC), 2-hydroxypropyl-β-cyclodextrin (HPβCD), and the bead mill method, and high quality dispersions containing 1.0% IMC nanoparticles were prepared (IMCnano, particle size: 76±58 nm, mean±S.D.). The fate of serum IMC and the induction of paw edema in adjuvant-induced arthritis (AA) rats receiving low-doses IMCnano (0.4 mg/kg) were similar to those following the administration of a therapeutic dose of conventional IMC prepared with MC and HPβCD (conventional IMC, 2 mg/kg), and the bioavailability in 0.4 mg/kg IMCnano was 5.3-fold higher in comparison with that in 2 mg/kg conventional IMC. IMC-induced gastrointestinal lesions in AA rats administered IMCnano (8 mg/kg), in consideration of bioavailability, were significantly less than for conventional IMC (40 mg/kg). On the other hand, the toxicity caused by conventional IMC and IMCnano was similar in Caco-2 cells. It is possible that the oral administration of IMC solid nanoparticles will show increased effectiveness in treating RA without causing IMC-induced gastrointestinal lesions, since the bioavailability is higher than that of conventional IMC. An oral drug delivery system using drug nanoparticles may expand the usage of NSAIDs for therapy in the inflammatory field.

Key words nanoparticle; indomethacin; bioavailability; gastrointestinal lesion; adjuvant-induced arthritis

Arthritis is a chronic disease that affects several parts of the joints including the cartilage, synovium, tendons and muscles. Rheumatoid arthritis (RA) is a specific type of arthritis, and is a complex chronic inflammatory disease dependent on multiple interacting environmental and genetic factors, making it difficult to understand its pathogenesis and thereby to find effective therapies.1 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 antirheumatic drugs, glucocorticoids and specific inhibitors of the anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs, glucocorticoids and specific inhibitors of the mediators response.2 It is well known, however, that these drugs have significant side effects.3–8 Gastroenteropathy is the most common side effect among patients taking NSAIDs, and RA patients taking NSAIDs are more susceptible to NSAIDs-induced gastrointestinal lesions in comparison with other patients.3–11 NSAIDs-induced gastric lesions are believed to involve the depletion of endogenous prostaglandins (PG) via the inhibition of cyclooxygenase, and an overproduction of nitric oxide (NO) via inducible NO synthase (iNOS)3,5,10,11. On the other hand, a number of elements, such as bacterial flora, neutrophils and iNOS, in addition to the depletion of endogenous PGs are involved in the pathogenesis of intestinal ulceration induced by NSAIDs12–14 and the presence of enterobacteria is essential for NSAID-induced ulceration to occur in the small intestine.11,14 Kato et al. reported that the overproduction of NO plays a key pathogenic role in both the gastric and small intestinal ulcerogenic response to indomethacin (IMC) in RA model rats, and these gastrointestinal lesions increased in number with dose of oral administration.15–17 Therefore, a decrease in amount administered orally by improving bioavailability may suppress the development of both of gastric and small intestinal ulcers in response to NSAIDs in RA patients.

Recently, strategies using micro/nanoparticles have been developed and investigated, and it was reported that these strategies increase bioavailability and usefulness of drug.16–18 Implants fabricated using the biodegradable polymer PLGA [poly(3-lactide-co-glycolide)] with mean particle diameters of 50–200 nm have been widely utilized as carriers for bioactive molecules and present a possible solution to limitations surrounding ocular drug penetration.19–22 In addition, Sha et al.19 developed a newly designed oral nanotherapeutic using redox nanoparticles (RNPO) with the potential to scavenge reactive oxygen species including nitroxide radicals for the treatment of inflammation in the gastrointestinal tract. RNPO is a core–shell-type polymeric micelle prepared by the self-assembly of methoxy-poly(ethylene glycol)-b-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethyl-styrene]. It is expected that drug systems using nanoparticles may provide an alternative strategy for increasing drug bioavailability.19–22 On the other hand, we have also developed methods to prepare tranilast solid nanoparticles by a bead mill method, and showed that these solid nanoparticles enhance drug bioavailability.22 It is possible that decreasing the amount of orally administered NSAIDs by improving their bioavailability may lead to an expansion of their usage as therapy for RA patients.

Here, we have designed new oral formulations containing IMC solid nanoparticles, and investigated their usefulness by evaluating the drug bioavailability and gastrointestinal ulcerogenic responses.

MATERIALS AND METHODS

Animals and Materials Male Dark Agouti (DA) rats, aged 6 to 13 weeks, were housed under standard conditions (12h/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. All procedures were
performed in accordance with the Kinki University Faculty of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals. 2-Hydroxypropyl-β-cyclodextrin (HP/β-CD, average molar substitution, 0.6; average molecular weight (MW), 1380) was purchased from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). Low-substituted methylcellulose (MC, METOLOSE SM-4, average viscosity, 4Pa·s at 20°C) was provide from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Conventional IMC (solid, IMC microparticles, 17.2±12.8 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.

Preparation of IMC Solid Nanoparticles IMC solid nanoparticles were prepared using zirconia beads and Bead Smash 12 (a bead mill, Wakenyaku Co., Ltd., Kyoto, Japan). Zirconia beads (diameter: 2 mm) were added to the IMC microparticles with or without MC, and the mixtures were crushed with the Bead Smash 12 for 30s (3000 rpm, 4°C). The mixtures were then dispersed in saline with or without 5% HP/β-CD, and crushed again with the Bead Smash 12 (5500 rpm, 30 s×15 times, 4°C) in the presence of zirconia beads (diameter: 0.1 mm). The compositions of the dispersions containing IMC are shown in Table 1. The pH was 6.5 for both dispersions containing IMC micro- or nanoparticles. The particle size was measured using a nanoparticle size analyzer SALD-7100 (Shimadzu Corp., Kyoto, Japan; refractive index 1.60-0.10i). The data are presented as means±S.D.

Stability of Drug Dispersions in IMC Formulations Three milliliters of dispersions containing IMC as described in Table 1 were incubated in 5 mL test tubes in the dark at 20°C for 14d, after which 50 µL sample solutions were withdrawn of samples at 30s intervals (total height of liquid before withdrawn of samples is 4 cm). The IMC concentrations in the samples were determined by the following HPLC method. Fifty microliters was added to 100 µL sample solutions were with -

### Table 1. Changes in IMC Particle Size in Formulations of Particle Dispersions Containing IMC 14d after Treatment with a Bead Mill

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Content (w/v%)</th>
<th>Treatment</th>
<th>Particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IMC microparticles</td>
<td>MC</td>
<td>HP/β-CD</td>
</tr>
<tr>
<td>IMC&lt;sub&gt;micro&lt;/sub&gt;</td>
<td>1.0</td>
<td>0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Milled-IMC</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Milled-IMC&lt;sub&gt;MC&lt;/sub&gt;</td>
<td>1.0</td>
<td>0.5</td>
<td>—</td>
</tr>
<tr>
<td>IMC&lt;sub&gt;nano&lt;/sub&gt;</td>
<td>1.0</td>
<td>0.5</td>
<td>5.0</td>
</tr>
</tbody>
</table>

IMC particle sizes and standard deviations of dispersions containing IMC as described in Table 1 were determined by a nanoparticle size analyzer SALD-7100 (refractive index 1.60-0.10i). The data are presented as means±S.D.
(2.24±0.27 h⁻¹) and β (0.35±0.033 h⁻¹) show the elimination rate constants in the first and second-phases (mean±S.E., n=5), and A (8.40±2.14 µg/mL) and B (4.35±1.39 µg/mL) are the IMC concentrations in the α- and β-phases, respectively.

The serum IMC concentration data after the oral administration of 0.3 mL of IMC micro or IMC nano (2 mg/kg) were analyzed by Eq. 4 (the each parameters were fixed with the value provided from the Eq. 3):

\[
C_{IMC} = \frac{A}{k_a - \alpha} e^{-\alpha t} + \frac{B}{k_a - \beta} e^{-\beta t} = \frac{A}{k_a - \alpha} + \frac{B}{k_a - \beta} e^{k_a \alpha - k_a \beta t} \quad (4)
\]

where \(C_{IMC}\) is the IMC concentration in the serum, \(k_a\) is the absorption rate constant. A nonlinear least-squares computer program (MULTI) was employed for the calculation.\(^{25}\)

The area under the first moment curve (AUMC) and absolute bioavailability (BA) were calculated according to the following equations (Eqs. 5–8):

\[
\text{AUC} = \int_0^{6\text{h}} C_{IMC} dt + \frac{C_{IMC,at\text{6h}}}{\beta} \quad (5)
\]

\[
AUMC = \int_0^{6\text{h}} C_{IMC} \cdot \alpha dt \quad (6)
\]

\[
\text{MRT} = \frac{AUMC}{\text{AUC}} \quad (7)
\]

\[
\text{BA} = \frac{\text{AUC}_{p.o.}}{\text{Dose}_{p.o.}} / \frac{\text{AUC}_{i.v.}}{\text{Dose}_{i.v.}} \times 100 \quad (8)
\]

Briefly, AUC was determined according to the trapezoidal rule up to 6 h, which is the time of the final IMC concentration measurement. The \(AUC_{p.o.}\) and \(AUC_{i.v.}\) are AUC after oral and intravenous administrations, respectively.

**Assay of the IMC Absorption Using in Situ Loop Technique**

Fourteen days after adjuvant injection, AA were fasted overnight, and anesthetized with isoflurane. A small midline incision allowed the gentle exposure of a 4–5 cm target portion of intestine. This target small intestine, selected because of its suitable vasculature to collect venous blood, was washed gently and enough with saline solution. In addition, the bile duct was tied. An 8 cm length of silicon tubing (o.d.: 2.0 mm, i.d.: 1.0 mm, TERUMO Corp., Tokyo, Japan) was inserted into one end of the intestine and tied securely with an appropriate size of polyethylene tubing (Hibiki Co., Tokyo, Japan), and all venous blood was collected in a micro tube.\(^{26,27}\) This blood was centrifuged at 10000 rpm for 30 min at 4°C, and the IMC concentration in the serum obtained was determined by the HPLC method described above.

**Evaluation of Gastric Mucosal Lesions**

The AA rats were fasted for 18 h before experiments, but had free access to water. The rats were administered IMC\(_{micro}\) or IMC\(_{nano}\) (8 or 40 mg/kg) orally, and killed under deep ether anesthesia 6h later.\(^{3,6,7}\) The stomachs were excised, washed and fixed in 10% formalin solution, and the area of gastric glandular muscus lesions was observed in digital photographs and quantified with Image J (NIH). Lesion area is expressed as a percentage of the total area of glandular stomach except the fundus.

**Evaluation of Small Intestinal Ulcerogenic Lesions**

AA rats were administered IMC\(_{micro}\) or IMC\(_{nano}\) (8 or 40 mg/kg) orally, and killed under deep ether anesthesia 24 h later.\(^{3,5}\) The small intestines were excised, washed and fixed in 10% formalin solution, and the area of the intestinal ulcerogenic lesions was observed in digital photographs and Image J. The lesion area is expressed as a percentage of the total area of small intestine.

**RNA Preparation**

The gastric mucosa and small intestine (epithelium) were removed, after which the lenses and retinas were isolated, and the samples were snap-frozen in liquid nitrogen. Total RNA was prepared from each individual lens and retina by the acid guanidium thiocyanate–phenol–chloroform extraction method using Trizol reagent (Life Technologies Inc., Rockville, U.S.A.). The purity and concentrations of RNA were determined spectrophotometrically. The OD\(_{260}\)/OD\(_{280}\) values of all RNA used were greater than 1.8, which indicates low protein contamination and high purity RNA.

**Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

The RT reaction was performed using an RNA PCR Kit (AMV Ver 3.0, Takara Bio Inc., Shiga, Japan). One microgram of total RNA was mixed with 3 µL of 10 mM Tris–HCl buffer (pH 8.3) containing 5 mM MgCl\(_2\) and 50 mM KCl. The following components were then added to give a final volume of 10 µL: 1 unit/µL RNAse inhibitor, 10 mM deoxynucleotide triphosphate, 2.5 units/µL reverse transcriptase, and 0.125 µM oligo dT-adaptor primer. The RT reaction was performed at 42°C for 15 min, followed by 5 min at 95°C. PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer’s instructions (Roche Diagnostics Applied Science, Mannheim, Germany). Briefly, 2 µL of cDNA was mixed with 2 µL of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart Taq DNA Polymerase, reaction buffer, MgCl\(_2\), SYBR Green I dye, and deoxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 µL containing specific primers for iNOS or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 10 pmol each). The following primers were used: 5'-GGGAGAATTTTTTCGACACC3'-3' and 5'-CCATGCTAAATTTGACTTGCA-3' for iNOS (GenBank accession No. NM_012611); and 5'-ACGGACGCACTAGCCATATTGTGACTTGCA-3' and 5'-CGCCTCTGGAAATGTTGAT-3' for GAPDH (GenBank accession No. NM_017008). The PCR conditions were 95°C for 10 min, 50 cycles of 95°C for 10 s (denaturing), 60°C for 10 s (annealing), and 72°C for 5 s (extension). The quantities of PCR products were measured fluorometrically in a real-time manner using a LightCycler DY 400 (Roche Diagnostics Applied Science, Mannheim, Germany). After completion of the PCR reactions, dissociation curves of the PCR products were generated using the LightCycler Software Version 4.0 program to detect nonspecific amplification, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and iNOS were used to calculate the levels...
of mRNA expression in the rats.

**Measurement of Protein** Protein levels in the gastric mucosa and small intestine (epithelium) of rats were determined according to the method of Bradford using a Bio-Rad Protein Assay Kit (BIO-RAD, CA, U.S.A.) with bovine serum albumin as the standard.

**Measurement of NO Levels** The gastric mucosa and small intestine (epithelium) were excised from rats and stored at −80°C until use. Tissue samples were homogenized in saline on ice, and centrifuged at 10000 rpm for 15 min at 4°C. The resultant supernatants were used for the measurement of NO levels. A concentric microdialysis probe (A-1-20-05, 5 mm in length; Eicom, Kyoto, Japan) was placed in the supernatant and perfused with Ringer’s solution (140 mM NaCl, 4 mM KCl, 1.26 mM CaCl₂, and 1.15 mM MgCl₂, pH 7.4) at a constant flow rate of 2 µL/min using a micro syringe pump (ESP-64, Eicom). NO₂⁻ and NO₃⁻ in the supernatant were separated on a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6×50 mm, Eicom); NO₃⁻ was reduced to NO₂⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). NO₂⁻ was mixed with Griess reagent to form a purple azo dye in a reaction coil, and placed in a column oven set at 35°C. The absorbance of the color product dye at 540 nm was determined on a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase consisted of 10% methanol containing 0.15 M NaCl–NH₄Cl and 0.5 g/L Na₄-EDTA delivered by a pump at a rate of 0.33 mL/min. The Griess reagent, 1.25% HCl containing 0.25 g/L N-napthylethylenediamine, was delivered at a rate of 0.1 mL/min. In this paper, the amounts of NO reflect the level of the NO₂⁻ metabolite, which is produced from NO.

**Measurement of IMC Concentration in the Mucosal Membrane of Stomach and Small Intestine Following Oral**

![Fig. 1. Cumulative Size Distribution and Frequency of IMC Dispersions with or without MC, HPβCD and Bead Mill Treatment](image)

The compositions of the IMC dispersions are shown in Table 1. Particle size was determined using a nanoparticle size analyzer SALD-7100 (refractive index 1.60–0.10i). (A) Cumulative distribution and frequency of IMC microparticles, (B) cumulative distribution and frequency of Milled-IMC, (C) cumulative distribution and frequency of Milled-IMC MC⁺, (D) cumulative distribution and frequency of IMCnano.
Administration  The AA rats were fasted for 18 h before the experiments, but had free access to water. The rats were administered IMC_mic or IMC_nano (40 mg/kg) orally, and killed under deep isoflurane anesthesia 3, 6 and 12 h later. The stomachs, duodenum (lower 2 cm of the stomach), jejunum (40% of the upper part of small intestine, 28.1 ± 3.9 cm), and ileum (60% of the lower part of small intestine, 40.1 ± 7.2 cm) were washed by saline, and these mucosal membrane were excised (mean ± S.E., n = 25). These samples were homogenized in methanol on ice, and centrifuged at 10000 rpm for 15 min at 4°C. IMC in the supernatant was analyzed by the HPLC method described above.

Stimulation Studies with Caco-2 Cells  The Caco-2 cell line was used in this study. Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, Tokyo, Japan) containing 10% (v/v) heat-inactivated fetal bovine serum (GIBCO), 1% non-essential amino acid solution (GIBCO), 1% L-glutamine and 10 μg/mL streptomycin and 1000 U/mL penicillin (GIBCO). Caco-2 cells (1.5 × 10^4 cells) were seeded in 96-well microplates (IWAKI, Chiba, Japan). At 1 d after seeding, the medium was changed to medium containing 0–1% IMC_mic or IMC_nano and the cells were incubated for 24 h. In this study, we showed the concentration range (0–0.16%) where cytotoxicity was seen (Fig. 6). Following stimulation, culture medium containing TetraColor One (SEIKAGAKU Co., Tokyo, Japan) was added, the absorbance at 490 nm was measured, and cell viability was calculated according to the manufacturer’s instructions as represented by Eq. 9:

\[
\text{cell viability} (\%) = \frac{\text{Abs}_{\text{treatment}}}{\text{Abs}_{\text{non-treatment}}} \times 100 \quad (9)
\]

Statistical Analysis  All values are presented as mean ± standard deviation (S.D.) or standard error of the mean (S.E.). Unpaired Student’s t-test was used to evaluate statistical differences, and multiple groups were evaluated by one-way ANOVA followed by Dunnett’s multiple comparison. The p values less than 0.05 were considered significant.

RESULTS

Preparation of Dispersions Containing IMC Nanoparticles  Figure 1 shows the particle size distribution of dispersions containing IMC as described in Table 1. The mean particle size using the IMC treated bead mill method was 15.8 ± 14.3 μm (Milled-IMC, means ± S.D.), with the particle size being on the micrometer order (Fig. 1B). On the other hand, IMC particles obtained by the addition of MC and HPβCD, and the stability of drug dispersions in IMC formulation were enhanced by the combination of added MC and the bead mill method. Furthermore, the addition of HPβCD further enhanced the stability of drug dispersion in IMC_nano, and no precipitation was observed 14 d after preparation. Although the particle size of Milled-IMC NC+ increased to 0.56 ± 0.28 μm from 0.19 ± 0.11 μm, there was no change in the particle size of IMC_nano between 0 and 14 d after preparation (Table 1).

Pharmacokinetics in the Oral Administration of Dispersion Containing IMC Nanoparticles  Figure 3 shows the serum IMC concentrations following the oral administration of IMC_mic and IMC_nano at a low dose (0.4 mg/kg) or therapeutic dose (2 mg/kg) in AA rats 14 d after adjuvant injection. Table 2 summarizes the pharmacokinetic parameters calculated from the in vivo intestinal penetration data. The serum IMC levels, absorption rate constant (ka), AUC and AUMC in AA rats administered 2 mg/kg IMC_mic were all significantly higher than those in in rats administered 2 mg/kg IMC_mic, and the T_max in the case of IMC_nano was faster than that in the case of IMC_mic. On the other hand, there were no significant differences in the pharmacokinetic parameters (C_max, AUC, AUMC and MRT) between the administration of low dose IMC_nano (0.4 mg/kg) and therapeutic dose IMC_mic (2 mg/kg). The bioavailability in the case of 0.4 mg/kg IMC_mic was 5.3-fold higher than that in the case of 2 mg/kg IMC_mic.

Gastric and Small Intestinal Ulcerogenic Response to IMC in AA Rats  Figure 4 shows the changes in gastrointestinal lesions and NO in AA rats administered IMC_mic and IMC_nano. The oral administration of IMC_mic or IMC_nano (40 mg/kg) caused hemorrhagic lesions in both the gastric...
mucosa and small intestine of AA rats 14 d after adjuvant injection, with the lesion score in AA rats administered 40 mg/kg IMC nano tending to be higher than that in AA rats administered 40 mg/kg IMC micro. Following the administration of 8 mg/kg IMC nano, a dose given in consideration of the increased bioavailability in the case of the IMC nano preparation, the lesion areas were significantly less than in the case of 40 mg/kg IMC micro. In addition, the iNOS mRNA and NO levels in the gastric mucosa and small intestines of AA rats administered 8 mg/kg IMC nano were also clearly lower than in the case of 40 mg/kg IMC micro administration (iNOS mRNA, gastric mucosa, 8 mg/kg IMC nano 0.46 ± 0.19, 40 mg/kg IMC micro 1.87 ± 0.28, small intestine, 8 mg/kg IMC nano 1.01 ± 0.29, 40 mg/kg IMC micro 4.94 ± 0.42, iNOS/GAPDH, means ± S.E., n = 3–5). Figure 5 shows the amounts of IMC in the mucosal membrane of stomach and small intestine following the oral administration of IMC micro and IMC nano 14 d after adjuvant injection. IMC was detected in the mucosal membrane of stomach and small intestine following the administration of IMC micro and IMC nano for 12 h.

Table 2. Pharmacokinetic Parameters for Plasma IMC Concentration after Oral Administration of IMC micro and IMC nano

<table>
<thead>
<tr>
<th>Rp.</th>
<th>( k_a \times 10^3 ) (h(^{-1}))</th>
<th>( T_{max} ) (h)</th>
<th>( C_{max} ) (nmol/mL)</th>
<th>( AUC ) (nmol·h(^{-1})·L(^{-1}))</th>
<th>( AUMC ) (nmol·h(^2)·L(^{-1}))</th>
<th>MRT (h)</th>
<th>BA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMC micro (2 mg/kg)</td>
<td>0.38 ± 0.11</td>
<td>0.92 ± 0.09</td>
<td>5.29 ± 0.13</td>
<td>19.11 ± 1.93</td>
<td>40.08 ± 3.47</td>
<td>2.29 ± 0.08</td>
<td>14.8 ± 4.7</td>
</tr>
<tr>
<td>IMC nano (0.4 mg/kg)</td>
<td>1.21 ± 0.49*</td>
<td>0.59 ± 0.19*</td>
<td>5.37 ± 0.49</td>
<td>21.67 ± 2.40</td>
<td>48.43 ± 4.96</td>
<td>2.34 ± 0.16</td>
<td>78.8 ± 9.3*</td>
</tr>
<tr>
<td>IMC nano (2 mg/kg)</td>
<td>1.35 ± 0.56*</td>
<td>0.53 ± 0.31*</td>
<td>28.5 ± 4.60*</td>
<td>121.8 ± 24.3*</td>
<td>321.5 ± 63.8*</td>
<td>2.46 ± 0.24</td>
<td>83.4 ± 15.5*</td>
</tr>
</tbody>
</table>

Dispersions containing IMC as described in Table 1 were administered orally to rats. Parameters were calculated according to Eqs. 4–8 (see Materials and Methods). α 2.24 ± 0.27 h\(^{-1}\), β 0.35 ± 0.033 h\(^{-1}\), A 8.40 ± 2.14 μg/mL, B 4.35 ± 1.39 μg/mL. IMC micro, IMC micro-administered AA rats; IMC nano, IMC nano-administered AA rats. The data are presented as means ± S.E. of 6–7 independent rats. * \( p < 0.05 \) vs. 2 mg/kg IMC micro. ** \( p < 0.05 \) vs. 0.4 mg/kg IMC nano.
and the amounts in the mucosal membrane of stomach and small intestine were similar between IMC micro and IMC nano administration. The amount of IMC in mucosal membrane of the stomach reached a maximum 3 h after administration, and then decreased with time. On the other hand, the amounts of IMC in the mucosal membrane of small intestine remained constant during the period 3–12 h after administration. Figure 6 shows the changes in the viability of Caco-2 cells following treatment with IMC micro and IMC nano. The viability of Caco-2 cells decreased with increasing IMC concentration, with the viabilities of Caco-2 cells treated with IMC micro and IMC nano being similar. The viabilities of Caco-2 cells treated with 0.04% IMC micro and IMC nano were approximately 32.7% and 35.6%, respectively.

Preventive Effects of the Oral Administration of IMC micro and IMC nano on Paw Edema in AA Rats

Fig. 5. Changes in IMC Amount in Mucosal Membrane of Stomach (A), Duodenum (B), Jejunum (C) and Ileum (D) Following the Oral Administration of IMC micro or IMC nano

- IMC micro, IMC micro-administered AA rats. IMC nano, IMC nano-administered AA rats. AA rats were fasted for 18 h before the experiments, but had free access to water. The rats were administered IMC micro or IMC nano (40 mg/kg) orally, and killed under deep ether anesthesia 3, 6 or 12 h later. The data are presented as means±S.E. of 5 independent rats.

Fig. 6. Effects of IMC micro and IMC nano on the Viability of Caco-2 Cells

Caco-2 cells were incubated in medium containing IMC micro or IMC nano for 24 h. Cell viability was calculated using TetraColor One according to Eq. 9 in Materials and Methods. The data are presented as means±S.E. of 5–10 experiments.

Fig. 7. Changes in Paw Edema of the Right (A) and Left (B) Hind Feet of AA Rats Administered IMC micro or IMC nano

- IMC micro, a low dose (0.4 mg/kg) or therapeutic dose (2 mg/kg), was administered orally to rats once a day starting on the day following adjuvant injection. Treatments were continued for 42 d after adjuvant injection. The data are presented as means±S.E. of 5–10 in dependent rats. *p<0.05 vs. vehicle-administered rats.
Table 3. Effects of IMCnano on Paw Edema in AA Rats

<table>
<thead>
<tr>
<th></th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>47.87±1.77</td>
<td>31.56±1.13</td>
</tr>
<tr>
<td>IMCnano (0.4 mg/kg)</td>
<td>43.99±1.83</td>
<td>27.10±0.94</td>
</tr>
<tr>
<td>IMCnano (2 mg/kg)</td>
<td>22.13±1.81</td>
<td>17.80±1.05</td>
</tr>
<tr>
<td>IMCnano (0.4 mg/kg)</td>
<td>21.62±1.76</td>
<td>16.97±0.99</td>
</tr>
</tbody>
</table>

Dispersions containing IMC as described in Table 1 were administered orally to AA rats once a day starting on the day following adjuvant injection. Parameters (AUCedema) were calculated according to Eq. 2 (see Materials and Methods). IMCnano-administered AA rats; IMCnano-IMCnano-administered AA rats. The data are presented as means±S.E. of 5 independent rats. *p<0.05 vs. vehicle. **p<0.05 vs. 0.4 mg/kg IMC micro.

7 shows the changes in paw edema in the right (A) and left (B) hind feet of AA rats administered IMCmicro or IMCnano. IMCmicro or IMCnano at a low dose (0.4 mg/kg) or therapeutic dose (2 mg/kg) was administered orally to rats once a day starting on the day following adjuvant injection, and Table 3 shows the AUCedema values. Paw edema in the right hind foot, into which adjuvant was injected, appeared on the day following injection, and reached a maximum 14 d after injection. On the other hand, paw edema in the left hind foot, which was not injected with adjuvant, was not observed during the first 7 d after adjuvant injection, but clearly increased from 10 d. Fourteen days after adjuvant injection, paw edema in the right and left hind feet of AA rats administered 0.4 mg/kg IMCmicro was significantly less than in AA rats administered vehicle, and paw edema in the right and left hind feet of AA rats administered 2 mg/kg IMCmicro was significantly less than in rats receiving 0.4 mg/kg IMCmicro. The AUCedema values in the right and left hind feet of AA rats administered 2 mg/kg IMCmicro were 46.2% and 56.4% those of AA rats administered vehicle, respectively. Furthermore, paw edema in the right and left hind feet of AA rats administered 0.4 mg/kg IMCnano was significantly less than in AA rats receiving vehicle; paw edema and AUCedema values for AA rats receiving 0.4 mg/kg IMCnano were similar to those for AA rats receiving 2 mg/kg IMCmicro.

DISCUSSION

IMC, [(4-chlorobenzoyl)-5-methoxy-2-methyleneIMCol-3-yl] acetic acid, molecular weight 357.8, pH 4.5, is practically insoluble in water, but has been used as a therapy for RA patients. Gastrointestinal lesions are the most common side effect in patients taking IMC, and RA patients taking IMC are more susceptible to IMC-induced gastric lesions in comparison with other patients.1-7 Therefore, the development of IMC formulations that do not cause gastrointestinal lesions is highly anticipated. In this study, we designed new oral formulations containing IMC solid nanoparticles, and investigated their usefulness by evaluating drug bioavailability and the toxicity (gastric and small intestinal ulcerogenic responses to IMC).

First, we attempted to mill IMC microparticles further by the bead mill method, but the mean particle size remained on the micro order (Milled-IMC, Fig. 1B); therefore, new innovations to regulate IMC particles were required, so we searched for useful additives to aid the performance of the bead mill. MC, a derivative of cellulose, is a water-soluble substance with a high degree of purity, uniformity and transparency. MC molecules link to water molecules by intermolecular hydrogen bonds to form a cage-like structure, and such MC solutions are neutral, odorless and tasteless. MC solutions are also stable over the pH range,20 and it is not necessary to remove the gel following the complete release of a drug.20 MC is highly biocompatible21-33 and is used to prepare drug formulations. The gel strength depends on the degree of substitution and the molecular weight.24,35 The particle size of IMC was decreased using a combination of MC addition and the bead mill method with the obtained IMC particles having a mean particle size of 190±110 nm (Milled-IMC+MC, mean±S.D.).

Next, the stability of dispersions containing IMC was demonstrated. The IMCmicro preparation precipitated 4 h after preparation. The precipitation was related the big particle size (weight) of IMCmicro. The stability of drug dispersions in IMC formulation (Milled-IMC+MC) was improved by the combination of MC addition and the bead mill method; however, precipitation was still observed 2 d after preparation (Fig. 2, Table 1). Mori et al.36 reported that adsorption to the surface of cyclodextrin decreases the cohesion of nanoparticulate solids, and we previously reported that the addition of HP/CD to the mixture is suitable for the preparation of nanoparticles using mill methods.25,26 Therefore, we attempted to prepare an IMC dispersion containing 0.5% MC and 5% HP/CD using the bead mill method. HP/CD was found to enhance the stability of the IMC dispersion (IMCnano), and no precipitation was observed 14 d after preparation (Table 1). In addition, there was no difference in the particle size of IMCnano between 0 and 14 d after preparation, and the addition of HP/CD was also found to enhance the recovery percentage of IMC by the bead mill method (Milled-IMC+MC 73.2±4.1%, IMCnano 93.5±3.3%, means±S.E., n=5). The size of HP/CD (approximately 0.8 nm) is smaller than IMCnano. Therefore, the HP/CD attach the surface of IMC nanoparticles, and may enhance the stability.

In this study, we investigated whether the IMC in IMCnano and IMCnano penetrated the membrane of small intestine by using the in vivo study (Fig. 3). The serum IMC level, absorption rate constant (kα), AUC and AUMC for AA rats administered 2 mg/kg IMCnano (therapeutic dose) were significantly higher than those for rats administered 2 mg/kg IMCmicro, and the tmax for IMCnano was faster than that for IMCmicro (Table 2). In addition, the AUC of serum IMC following the administration of low dose IMCnano (0.4 mg/kg) was similar to that following the administration of therapeutic dose IMCmicro (2 mg/kg), and the bioavailability for 0.4 mg/kg IMCnano was 5.3-fold higher than that of 2 mg/kg IMCmicro (Table 2). Furthermore, the particle size of IMCnano was 85±67 nm (means±S.E.) in the pH 1.2 buffer based on Japanese Pharmacopoeia (JP) test protocols. These results show that the amount of IMC administered as RA therapy can be reduced by using IMCnano. It is important to clarify the mechanism for the BA enhancement by nanoparticles. The solubility of IMCnano may be increased in the in vivo, since the specific surface in nanoparticles is larger than that in microparticles. Moreover, we reported that the HP/CD was increased the absorption via membrane.37 Taking these findings together, we hypothesize that high solubility of IMCnano caused the enhancement of BA.

In studies to identify the mechanisms of NSAIDs-induced gastrointestinal lesions in RA, the selection of an 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 volume (paw edema)\(^{38,39}\). Paw edema in AA rats is known to involve two inflammatory stages, primary and secondary inflammation. The primary inflammation starts from the day following the injection of adjuvant into the right hind foot. Secondary inflammation is observed from 7d after adjuvant injection, and inflammation reaches a maximum 14d after adjuvant injection into the right and/or left hind foot.\(^{3,5}\) In addition, it has been reported that gastrointestinal lesions induced by conventional NSAIDs, such as indomethacin, naproxen and aspirin, are significantly aggravated in AA rats as compared with normal rats. It is noteworthy that changes in the biological characteristics of AA rats correspond to those that occur in human RA\(^{3,5,6,38,39}\). Therefore, AA rats may provide a useful model for studies on the mechanisms of NSAIDs-induced gastrointestinal lesions in RA. It is known that the severity of arthritis in Wistar rats is moderate and occurs with an incidence of only 50–60%. On the other, the severity of arthritis in DA and Lewis rats is much higher than in Wistar rats, and occurs with an incidence of 100%.\(^{3,5,7}\) Therefore, in this study we investigated the effect of orally administered IMC solid nanoparticles on drug bioavailability and the gastric and small intestinal ulcerogenic responses to NSAIDs using IMC-administered DA rats injected with adjuvant.

The oral administration of IMC\(_{micro}\) or IMC\(_{nano}\) (40 mg/kg) resulted in the formation of hemorrhagic lesions in both the gastric mucosa and small intestine of AA rats 14d after adjuvant injection, and the lesion score at AA rats administered 40 mg/kg IMC\(_{nano}\) tended to be higher than that of rats administered 40 mg/kg IMC\(_{micro}\). When the difference in bioavailability was taken into account, and the dose of IMC\(_{nano}\) was reduced to 8 mg/kg, the lesion area in rats administered IMC\(_{nano}\) was significantly less in rats administered 40 mg/kg IMC\(_{micro}\) (Fig. 4), while the fates of serum IMC were similar (8 mg/kg IMC\(_{nano}\), \(AUC\), 453.0±54.7 nmol/mL·h, mean±S.E. \(n=5\); 40 mg/kg IMC\(_{micro}\) : \(AUC\), 441.9±44.1 nmol/mL·h, mean±S.E. \(n=5\)). In addition, the \(in\ space\) loop experiment, the intestinal penetration in IMC\(_{nano}\) increased with time; however, there was little permeation in the case of IMC\(_{micro}\) found (the IMC levels 40 min after the injection of IMC micro were only 1.3% those of the levels following the injection of IMC nano, \(n=5\)). Also, the iNOS mRNA and NO levels in the gastric mucosa and small intestine of AA rats administered 8 mg/kg IMC\(_{nano}\) were clearly lower than in rats administered 40 mg/kg IMC\(_{micro}\). It is known that the overproduction of NO plays a key pathogenic role in both the gastric and small intestinal ulcerogenic responses to IMC in RA patients.\(^{3,5–7}\) Furthermore, we showed that the amounts of IMC in the mucosal membrane of stomach and small intestine of AA rats following the oral administration of IMC\(_{micro}\) and IMC\(_{nano}\) 14d after adjuvant injection was similar (Fig. 5), and the cell stimulation by IMC\(_{micro}\) and IMC\(_{nano}\) were similar in the \(in\ space\) study (Fig. 6). These results show that the high IMC concentration in blood and intensity of cell stimulation relate the gastrointestinal toxicity by the oral administration of IMC\(_{micro}\) and IMC\(_{nano}\). We conclude that treatment with low doses of IMC\(_{nano}\) may enable RA therapy without gastric and small intestinal ulcerogenic responses to NSAIDs. Moreover, paw edema of the right hind feet of AA rats receiving oral administration of low dose IMC\(_{nano}\) (0.4 mg/kg) was significantly less in comparison with AA rats who received vehicle, and the paw edema and \(AUC\) values of AA rats receiving low doses IMC\(_{nano}\) (0.4 mg/kg) was similar to that of AA rats receiving therapeutic dose IMC\(_{micro}\) (2 mg/kg). These results show that the oral administration of IMC\(_{nano}\) for RA treatment can be kept at a low dose, since the drug bioavailability is higher than that of conventional IMC. In addition, the oral administration of IMC\(_{nano}\) provides therapy for RA with fewer IMC-induced gastrointestinal lesions.

Further studies are needed to elucidate the precise mechanism for the gastrointestinal toxicity of IMC\(_{nano}\) by hematoxylin–eosin (HE) staining. In addition, it is important to clarify the drug solubility of the IMC formulation and structure of the delivery system. Therefore, we are now investigating the structure of the delivery system for IMC nanoparticles by scanning electron microscopy.

In the present study, we attempted to establish a new method for the preparation of drug solid nanoparticles, and succeeded in preparing a high quality dispersion containing IMC nanoparticles (particle size, 76±58 nm, mean±S.D.). It is possible that the oral administration of IMC nanoparticles will provide increased effectiveness in treating RA without the toxicity (gastric and small intestinal ulcerogenic responses to NSAIDs), and that an oral drug delivery system using drug nanoparticles may expand the usage of NSAIDs for therapy in the inflammatory field.

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