Two-Step Sustained-Release PLGA/Hyaluronic Acid Gel Formulation for Intra-articular Administration

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In the development of drugs for intra-articular administration, sustained-release formulations are desirable because it is difficult to maintain the effect of conventional injections due to immediate drug leakage from the joint cavity. In this study, a sustained-release poly(lactic-co-glycolic acid) (PLGA) microsphere formulation for intra-articular administration containing indocyanine green (ICG) as a model drug was prepared to follow its fate after intra-articular administration in rats with a real-time in-vivo imaging system. ICG administered as an aqueous solution leaked from the joint cavity in a short time and was excreted outside the body within 1–3d. However, ICG in the sustained-release formulation was retained in the joint cavity and released for 2 weeks. Next, a sustained-release formulation containing PLGA microspheres in a hyaluronic acid (HA) gel formulation was prepared. After gradual release in two stages, we could achieve sustained release for a longer period. It is considered that a combination formulation of PLGA microspheres and HA gel can significantly improve the sustained release of a drug administered into the knee joint.

Key words poly(lactic-co-glycolic acid) (PLGA); sustained release; intra-articular injection; hyaluronic acid

As people age, they assume the same postures for longer periods of time, and the painfulness of joints such as the elbow, knee, and fingers will increase. The main causes of knee pain that plague many elderly people are osteoarthritis and rheumatoid arthritis, which is caused by joint inflammation. The cartilage of joints, which absorbs the impact of the knee or hip joint is deformed by aging and intensive exercise, causing inflammation and pain. As symptoms progress, cartilage and cartilage and causes inflammation and pain. The causes of joint pain are various, and the mechanism of onset has yet to be clearly elucidated. However, the mechanism of pain in joints has been established as inflammation.2

Current treatments include an oral formulation of methotrexate and systemic drug therapy, such as the subcutaneous injection of adalimumab to control joint inflammation. Methotrexate inhibits nucleic acid synthesis by inhibiting dihydrofolate reductase and alleviates joint pain by activating the immune system.5,6 Adalimumab is abundantly released into the joint and binds to tumor necrosis factor-α (TNF-α), which induces inflammation. It inhibits the binding of TNF-α to the receptor and the transmission of inflammatory signals. In addition, adalimumab binds to cells that produce TNF-α and promotes their apoptosis.7,8 Although systemic drug therapy may delay the progression of the disease, side effects such as myelosuppression and severe infection are problematic.3,9

The reduction of inflammation by the local administration of steroids such as prednisolone farnesylate and intra-articular injection of hyaluronic acid (HA) are also performed. HA is a substance originally present in the joint space, showing viscosity and elasticity, and it lubricates the articulation. It is also a component of the articular cartilage and meniscus and functions to reduce the burden on joints.11–13 Examples of joint pain relievers using HA include Cybisco®, Alz®, and Svenil®.

In parallel with the investigation of the cause of various types of inflammation, drugs promoting true remission are being developed. In order to suppress systemic side effects for effective drug-based treatment, it is desirable to inject the drug directly into the joint.14–19 In our previous studies, we confirmed that the residence time of the solution administered to the rat knee joint cavity was extremely short.20 Dynamics of drugs administered to the knee joints were monitored using indocyanine green (ICG), a fluorescent substance clinically used as a liver/circulatory function test agent. The fluorescence intensity of ICG injected into the knee joint cavity peaked at 1 h, and almost no ICG remained at 24 h. It is considered that the drug injected into the synovium will rapidly move into the blood. In order to maintain a high enough concentration of analgesic agent within the knee joint cavity to suppress inflammation, the release rate of the drug from the formulation to the synovial fluid needs to exceed it rate of disappearance from the fluid until the next injection. Therefore, we prepared HA-based sustained-release gel preparation. The drug contained in the sustained release gel preparation remained in the joint space and was released for 1 week after administration in rats. However, as a future medicine, is desirable a longer-term controlled release preparation.

In this study, a fine particle formulation using poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer, was studied as a long-term sustained release preparation. Biodegradable polymers such as PLGA are being studied as important materials in the fields of tissue engineering and drug delivery science. PLGA has been more extensively studied...
than any other biodegradable polymer, and it is degraded into harmless compounds by hydrolysis of the ester backbone in the body. At the same time, a combination preparation of PLGA microspheres dispersed in the HA gel sustained-release base material discussed above was also examined. It is thought that the HA gel has an effect to limit the initial burst of a drug.

Each ICG-containing preparation was prepared and administered to the knee joint cavity of rats. The fate of the drug was observed using a real-time in-vivo imaging system (IVIS) and the amount of drug residue was determined by extraction. Drug release from the PLGA microsphere formulation in the HA gel was suppressed and two-step sustained release, i.e., from the PLGA microspheres to the HA gel layer and from the gel layer to the joint cavity could be expected. Also, alleviation of the feeling of a foreign body in the articular cavity can be expected using the fine particle formulation.

MATERIALS AND METHODS

**Materials** As a biodegradable polymer and gel base, we used PLGA, lactic:glycolic=1:1, molecular weight: 18000–24000 kDa, EVONIC, Tokyo, Japan and HA FCH-200 molecular weight: 1800–2200 kDa, Kikkoman Bio Chemifa Co., Ltd., Tokyo, Japan, respectively. We used ICG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a fluorescent substance, water for injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) as a dissolution liquid, and isoflurane (DS Pharma Animal Health Co., Ltd., Osaka, Japan) as an anesthetic. We used a 25-µL micro syringe (HAMILTON Co., Reno, Nevada, U.S.A.) equipped with a needle of 26G×1/inner diameter 0.45×13 mm (TERUMO Co., Tokyo, Japan) for administration. The fluorescence of ICG administered to rats was detected with a real-time in-vivo imaging system (IVIS®; IVIS-SPECTRUM, Caliper Life Sciences, Hopkinton, MA), as discussed above. ICG was dissolved in organic solvent to prepare a 0.75% HA gel. A membrane filter (0.1 µm, Omnipore™, Merck Millipore, Germany) was sandwiched between the donor and receptor cells and assembled. A stir bar was placed in the receptor cell. Then, 1 mL of purified water was injected into the receptor cell using a syringe. Samples were placed in the donor cell

**Displacement Test Using Sink-Type Diffusion Cell** Dissolution of ICG from the formulations was confirmed with a scanning electron microscope (SEM, JSM-6060, JEOL, Tokyo, Japan). Each sample was directly adhered to a sample stage with black double-sided tape. Platinum coating (JFC-1600, JEOL) was then carried out at 30 mA for 90 s. Then, we observed the samples with SEM. PLGA microspheres were suspended in water for injection and the particle size was measured using Zetasizer Nano ZS (Malvern, Kobe, Japan).

**Preparation of PLGA/HA Gel Combination Formulation** The ICG-containing PLGA microspheres were dispersed in water for injection to prepare a 20 mg/mL suspension. Next, 10 mg of HA powder was gradually added to 1.0 mL of the dispersion with stirring. After the total amount had been added, stirring was continued under a reduced pressure to produce a PLGA microsphere formulation dispersed in the HA gel.

**Comparison of Drug Release from PLGA Microsphere Formulation into Water and HA Gel** The PLGA microsphere formulation was placed in a tube and then water or 1% HA gel was added. After checking the fluorescence by IVIS, gentle rocking was applied. After 1 and 3 h, they were centrifuged and IVIS was used to confirm the dispersion of the drug.

**Dissolution Test Using Sink-Type Diffusion Cell** Dissolution of ICG from the formulations was confirmed with a scanning electron microscope (SEM, JSM-6060, JEOL, Tokyo, Japan). Each sample was directly adhered to a sample stage with black double-sided tape. Platinum coating (JFC-1600, JEOL) was then carried out at 30 mA for 90 s. Then, we observed the samples with SEM. PLGA microspheres were suspended in water for injection and the particle size was measured using Zetasizer Nano ZS (Malvern, Kobe, Japan). In addition, the ICG-containing PLGA microspheres were dissolved in dichloromethane and the absorbance was measured to determine the ratio of ICG contained in the PLGA microspheres.
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and capped. The receptor fluid was agitated in a constant-
temperature water bath at 37°C. The total amount of the
sample was recovered from the receptor cell at predetermined
intervals using a syringe and the same volume of purified
water was injected. The absorbance at 784 nm of the recovered
sample was measured with a spectrophotometer (UV-1800,
Shimadzu, Kyoto, Japan) to quantify ICG.

Comparison of Sustained Release of Formulation Based
on ICG Fluorescence Intensity of PLGA Microsphere For-
mulation and PLGA/HA Gel Formulation We injected
20 µL each of the 4 formulations containing 20 µg of ICG
(1.0 mg/mL) in the rat’s knee joint. After administration of
the formulations, fluorescence around the knee joint was con-
firmed with IVIS, immediately after administration and at 1,
3, 6, 24, 72, 168, 336, 504, and 672 h (4 weeks).

Confirmation of Residual Drug in Knee Joint 4 Weeks
after Administration of ICG Formulation Rats were sac-
rificed 4 weeks after administration of the ICG formulation
to the knee joint. Then the knee joint was removed and cross-
sectioned at the center. The cut surface was observed with the
naked eye and IVIS to confirm fluorescence.

Statistical Analysis Statistical comparisons were made
with a one-way ANOVA. Comparisons of means were per-
formed with the least significant difference test. The signifi-
cance level was set at p<0.05.

RESULTS

Microscopic Confirmation and Measurement of Particle
Diameter The particle surface and shape of the PLGA mi-
crospheres observed by SEM were smooth and spherical (Fig.
2). The median diameter D50 of the particles was 2.3±1.3 µm
(n=3, mean±standard deviation (S.D.)). The proportion of ICG
contained in PLGA microspheres was 18.02% (w/w).

In-Vitro Release of ICG from PLGA Microspheres into
Water and HA Gel Drug release from PLGA microspheres
into the medium was confirmed by a simple experiment in-
volving release into water and HA gel (Fig. 3). Although the
synovial fluid contains ions, proteins, and other components,
we employed water to screen the formulations in vitro. Fluor-
escence was immediately confirmed when water was added
to the PLGA microspheres (Fig. 3b). Fluorescence was con-
firmed in water after 1 h after the start of oscillation (Fig. 3d),
and intense fluorescence was confirmed after 3 h (Fig. 3f). On
the contrary, when HA gel was added, fluorescence could not
be confirmed for the first 1 h (Figs. 3a, c) and weak fluores-
cence of the entire gel was confirmed after 3 h (Fig. 3e). This
suggests that the release of the drug was sustained by the HA
gel.

Dissolution Test Using Sink-Type Diffusion Cell The
suspension of ICG-containing PLGA microspheres was eluted
more slowly than ICG/HA 0.75% gel formulation. Further-
more, by adding HA to the suspension of ICG-containing
PLGA microspheres to produce a 0.75% gel formulation,
dissolution could be slowed even further (Fig. 4). Compared
with the aqueous solution, the PLGA suspension and HA gel
preparation facilitated the sustained release of ICG. This sus-
tained release was promoted further using the PLGA/HA gel
formulation.

Release of ICG from Formulations Administered into
Rat Knee Joint The fluorescence intensity of ICG at the
knee after the administration of ICG aqueous solution, PLGA
microspheres, HA gel formulation, and PLGA/HA gel formu-
lation was visualized with IVIS. Fluorescence disappeared
24 h after solution administration. On the other hand, in
PLGA and PLGA/HA gel, fluorescence could be confirmed
for up to two weeks (Fig. 5). When the fluorescence intensity
was measured, the fluorescence disappeared after 1 week with
the aqueous solution, but the fluorescence level could be measured for up to 4 weeks with PLGA and PLGA/HA gel (Fig. 6). The half-lives of ICG administered as the aqueous solution, PLGA microspheres, HA gel formulation, and PLGA/HA gel formulation calculated from the semi-logarithm plot in Fig. 6 were 17±3, 61±16, 63±3, and 149±15 h, respectively, suggesting that the combination of PLGA and HA gel was significantly effective to sustain ICG release in vivo. The initial strong fluorescence of the PLGA suspension is considered to be the initial burst of ICG present on the particle surface. The initial burst was suppressed with the PLGA/HA gel formulation compared to PLGA microspheres and HA gel formulations (Fig. 6).

**Confirmation of Residual ICG in Knee Joint 4 Weeks after Administration of ICG Formulation** The joints were cross-sectioned and the cut surfaces were observed with the naked eye and IVIS one month after formulation administration (Fig. 7). Although the green color of ICG could not be confirmed in joints treated with solutions by the naked eye (Fig. 7a), it could be confirmed that ICG remained on the cut surface treated with PLGA microspheres or the PLGA/HA gel formulation (Figs. 7b, c). When these samples were observed by IVIS, the strongest fluorescence was confirmed with the PLGA/HA gel formulation (Fig. 7f).

**DISCUSSION**

In our previous studies, it was confirmed that the residence time of ICG solution administered into the rat knee joint cavity was markedly short. The fluorescence intensity of ICG injected into the knee joint cavity peaked at 1 h, and almost no ICG remained at 24 h. It is considered that a drug injected into the joint rapidly moves into the blood through the articular membrane. Therefore, in order to maintain a sufficient concentration of the drug to suppress inflammation in the knee joint cavity, a preparation capable of prolonged and sustained release is necessary.

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**Fig. 4. Amount of ICG Eluted from an Aqueous Solution (○), 0.75% HA Gel Formulation (□), PLGA Aqueous Suspension (□), and PLGA/0.75% HA Gel Formulation (○)**

Each value represents the mean±S.D. (n=3).

**Fig. 5. Optical Images of Fluorescence Derived from ICG Intra-Articularly Administered into the Rat Knee Joint as a Formulation**

(a) ICG solution, (b) HA GEL formulation, (c) PLGA formulation, and (d) PLGA/HA gel formulation. Observation was carried out for 1 week after administration except for ICG solution.
release exceeding the outflow rate is desired. Since HA has an affinity for cartilage and has been reported to protect against destruction,\textsuperscript{18,19,28} sustained release using HA gel formulations was examined in previous studies. The gel formulation was a convenient preparation, which allowed sustained release for one week. Therefore, it is considered to be a significant formulation warranting further investigation. However, at the clinical stage, a formulation that allows longer-term sustained release is desired to enhance QOL of patients. With the gel formulation, it was judged that prolonging the period of sustained release was more difficult due to problems such as viscosity increase, and so combination with other preparations was examined.

We started with a study based on the proven PLGA microsphere formulation used in experiments on the knee joint. Studies using PLGA microspheres prepared by the emulsion solvent diffusion method have been conducted for a long time. ICG was included in this preparation and the monitoring of sustained release was carried out.

We studied two-step sustained release preparation using PLGA and HA gel formulation. The particle size of PLGA microspheres that have been clinically applied as subcutaneous injectables is around 20 µm. When the particle size is large, the drug content is high and the possibility of prolonged, sus-
tained release can be expected. However, there is a possibility that this will restrict the range of motion of the knee joint, causing a foreign-body sensation on bending and stretching. On the other hand, if the particle size is reduced, there is a possibility that the drug release rate will increase due to an increase in the specific surface area and the sustained-release property will decrease. Moreover, it is likely that the particle size will become small enough to pass through the articular membrane and flow out, precluding sustained release. To minimize patients discomfort but also promote sustained release, we aimed to prepare particles with a median diameter D50 of 1 µm in the present study.

The PLGA microsphere size and ICG content were investigated. By adjusting the rotation number of the homogenizer, a PLGA microsphere formulation with a median diameter D50 of 1 µm and an ICG content of about 20% was prepared. Even if this is mixed with water for injection, drug release can be sufficiently sustained for about 2 weeks to 1 month.

Also, by dispersing this in the HA sustained release gel that was previously examined, a preparation aiming at a more gradual sustained release was produced. At the time of studying the HA gel formulation, it was prepared at 5%. However, the viscosity was too high to disperse PLGA microspheres in it. We found that a 0.75−1% HA gel was suitable for the homogeneous dispersion and administration of PLGA. The viscosity of the 1.0% HA gel measured by a viscometer (Tuning Fork Vibro Viscometer, SV-1A, A&D Co., Ltd., Tokyo, Japan) was 3.9 × 10² mPa·s. Even under these conditions, sustained-release for several days was confirmed, and it was considered to be sufficient to suppress release from PLGA.

ICG as a fluorescent agent has been widely used for assessing liver function and hepatic blood flow in clinical fields. 29) The fluorescence intensity at the liver of the rats administered HA gel formulation was peaked at 6h, slower than the rats administered ICG solution (3h), suggesting sustained release of ICG from the gel (Fig. 5). This experiment confirmed that the PLGA microspheres comprise a preparation capable of sustained release for a longer period. As discussed in our previous report, 20) the fluorescence intensity of ICG was maximized at 30 µg/mL. The increase in the fluorescence intensity (Fig. 6) was due to ICG release at the injection site, where the concentration of ICG was higher than 30 µg/mL. The strong fluorescence at the initial stage of administration of the PLGA microsphere formulation is considered to be due to the initial burst of ICG located on the surface of the microspheres caused by the entry of water into the knee joint. This initial burst of ICG was not observed in the in-vitro dissolution study. We suggest that ICG is released from PLGA when rats move. In addition, the in-vitro study separated the gel and water by a membrane, which would suppress the entry of water into the gel. By creating a PLGA microsphere formulation with such characteristics dispersed in the HA gel, this initial burst could be reduced to some extent. The half-life of ICG from the PLGA/HA gel formulation was significantly longer than those of HA gel formulation and PLGA formulation. It is thought that by utilizing the property of two-step sustained release from microspheres to gel and gel to joint cavity, it is possible to realize slower release. Viscosity and drug particles can be visually observed even one month after injection, and the residual amount was also increased based on the fluorescence intensity of the cut surface. It was shown that the PLGA microspheres are suitable for long-term sustained release and, furthermore, administration using HA gel as a matrix results in a longer-lasting sustained release formulation, can also reduce the burden on a patient’s joints.

In this study, we developed a long-term sustained-release formulation that can reduce the burden on a patient’s knee by combining PLGA microspheres and HA gel. Because the scope of research on PLGA formulations is wide, we consider that we can develop an even more effective knee joint sustained release formulation by optimizing the composition and combination ratio with HA gel.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

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