Percutaneous Application of a Cell Permeable Form of Methotrexate Improved Collagen Induced Arthritis without Systemic Adverse Effects

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Directed by Professor **Soo-Kon Lee**

A Dissertation submitted to
the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Sang-Won Lee

December 2009

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December 2009

ACKNOWLEDMENTS

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ABSTRACT

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Methotrexate (MTX) is the mainstay for the treatment of rheumatoid arthritis, however, it may induce serious complications. To maintain the therapeutic efficacy and reduce the complication of MTX, we generated a cell permeable form of MTX by conjugating the novel protein transduction domain (Hph-1-MTX) to MTX. Skin-penetration efficiency of Hph-1-MTX was proved using Franz cell, and strong fluorescence of percutaneous Hph-1-MTX labeled with FITC was detected around joints. Percutaneous Hph-1-MTX significantly improved symptoms and preserved joint-structures in CIA mice in a dose-dependent manner, which was also revealed by 3D-CT scan. Percutaneous Hph-1-MTX markedly decreased the expression of inflammatory cytokines in inflamed joints as well as their serum concentration without side effects. Thus, a cell permeable form of Hph-1-MTX can be a new therapeutic modality in rheumatoid arthritis patients with several and refractory joints affected and those in whom serious complications may be expected due to systemic MTX administration.

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I. INTRODUCTION

Rheumatoid arthritis (RA) is the paradigm of a systemic autoimmune disease characterized by the inflammation in multiple joints. The hallmark of RA is the symmetrical synovial proliferation and the tenderness of multiple joints, particularly the small joints of the hands and feet[1]. Joint inflammation is primarily induced by the overproduction of inflammatory cytokines: tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which are centrally involved in the pathogenesis of RA. These cytokines could mediate the long-term cartilage degradation and bone erosion, resulting in pain and joint dysfunction, which are irreversible in patients with RA inadequately treated[2-4]. Thus, the early and aggressive intervention of RA is recently suggested to prevent the joint destruction[5].

Among various disease modifying anti-rheumatic drugs (DMARDS), methotrexate (MTX) is the most widely used oral medication for the treatment of RA, due to its relatively rapid onset of efficacy[6-8]. Furthermore, MTX could slow the rate of the joint destruction through their crucial inhibitory effects on the cascade of events initiated by inflammatory cytokines and subsequent joint-destructive enzymes[9-11]. However, the long-term administration of MTX may induce serious adverse effects, such as infection, hepatitis and bone marrow suppression, despite its potent efficacy[12-14]. Recently, a new therapeutic modality of

biological agents, such as anti-TNF-α blockade, anti-IL-1β receptor antagonist and anti-CD20 monoclonal antibody, has been developed and commonly used[15,16]. Since these agents are targeting key molecules or their receptors involved in the inflammatory process of RA, their therapeutic efficiency is extremely potent and rapid. However, these agents also exhibited serious and fatal adverse effects such as infection and malignancy[17-20], and left the limitation that a combined treatment with MTX is more efficient, suggesting that MTX still remains a mainstay of the treatment of MTX.

From the clinical point of view, increasing the dose of MTX in RA patients who have the refractory joint inflammation, especially confined to several small joints or continuing MTX in those who have systemic diseases where the use of MTX is inappropriate, may provoke the unwanted adverse effects. Thus, in these cases, the necessity of the local application of MTX has been raised, but there was no effective delivery method to apply MTX locally in joints where the repetitive intra-articular injection is unavailable, till now.

In out previous studies, we already identified a novel cell and tissue-permeable protein transduction domain (PTD), YARVRRRGPRR, from the human transcriptional factor Hph-1, constructed a fusion protein consisting of Hph-1 and the cytoplasmic domain of cytotoxic T lymphocyte antigen (CTLA)-4, and elucidated its effectiveness in a mouse model of asthma and RA through the intranasal and percutaneous application, respectively[21]. In this study, to lessen the systemic toxicity of MTX and improve its therapeutic effect on the affected joints, we generated a cell permeable form of MTX, a fusion between Hhp-1-PTD to MTX (Hph-1-MTX). Effective transcutaneous delivery of Hph-1-MTX was confirmed in *in vivo* and *ex vivo* using Franz cells. When percutaneous Hph-1-MTX was applied to mice with collagen induced arthritis (CIA), the severity of arthritis and the joint damages were significantly improved, and the level of inflammatory cytokines in the

affected joints as well as serum was markedly reduced in a dose-dependent manner. In the preclinical studies of Hph-1-MTX, the toxicity was not detected in any major organs, and Hph-1-MTX was mainly distributed in the application site, and the stable tyrosine-conjugated MTX was detected in the joints as a metabolite after transcutaneous delivery. These results demonstrate that a cell permeable Hph-1-MTX can be a new therapeutics for the treatment of patients with RA, especially those with the refractory joint inflammation or other systemic disease where the use of MTX is not recommended, via topical application of MTX.

II. MATERIALS AND METHODS

Purification and generation of a cell permeable form of MTX

Hph-1-MTX consists of Hph-1, 6-amino cupric acid (ACA) and MTX. First of all, Hph-1 (YARVRRRGPRR-OH) was synthesized using solid phase techniques and commercially available fluorenylmethoxycarbonl (Fmoc) amino acids (Nova biochem) on a Applied Biosystems 433 peptide synthesizer, and 6-amino cupric acid and methotrexate were coupled on the N-terminal of Hph-1 sequentially. The Hph-1-MTX conjugate were cleaved from the resin using 96% trifluoroacetic acid, 2% triisopropyl silane and 2% phenol for 12hrs. The longer reaction times were necessary to completely remove the 2,2,4,6,7-Pentamethyldihydrobenzofurane-5-sulfonyl (PbfO-protecting groups) for the arginine. The conjugates subsequently were filtered from the resin, precipitated using diethyl ether, purified using high-performance liquid chromatography reverse-phase columns (Waters 2487, Waters) and characterized using Maldi-tof (Ultarflex III, Bruker Daltonik).

For the Cy5.5 labeling in the Hph-1-MTX, N-hydroxy succinimide (NHS) Cy5.5 (GE healthcare) was coupled in N-methyl pyrolidone (NMP) solution for 5 hrs on the side chain of lysine which was added at N-terminal end of Hph-1. The cleavage, purification and characterization had same processes of Hph-1-MTX synthesis.

Franz cell experiment

We conducted an experiment using a 9mm unjacketed Franz cell with a flat flange joint and clear glass[22]. Dorsal skin of hairless mouse was used as membrane. The donor chamber was filled with 10 mg Hph-1-MTX or MTX in 0.4 ml of 0.5 M Tris buffer (pH 10.8) and the receptor chamber was filled with 4.6 ml of Dulbecco's phosphate-buffered saline, then incubated at 37°C with stirring. We collected Hph-1 residues (tyrosine-alanine) conjugated with MTX and MTX from receptor chamber at 5, 10, 15, 20 and 25 hours after initiation.

Fluorescence microscopic analysis of transcutaneous penetration of Hph-1-MTX

We conducted an experiment using DBA/1 mice to elucidate the transcutaneous penetration of Hph-1-MTX labeled with fluorescein isothiocyanate (FITC). We applied 1% Hph-1-MTX labeled with FITC on the skin of mice and obtained joint tissue at 3, 6 and 24 hours after application. FITC fluorescence was detected using the confocal microscopy.

Induction of collagen induced arthritis

All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea. Forty-nine male DBA/1 mice at 8 weeks of age (SLC, Shizoka, Japan) were evenly divided into seven groups as followings: group 1 = controls; group 2 = untreated; group 3 = transdermal MTX-treated; group 4 = intraperitoneal MTX-treated; group 5 = transdermal 0.1% Hph-1-MTX-treated; group 6 = transdermal 0.5% Hph-1-MTX-treated; group 7 = transdermal 1% Hph-1-MTX-treated. All mice except controls were given an intra-dermal injection of 100 μg of bovine type II collagen emulsified in complete Freund's adjuvant (Arthrogen-CIA, Redmond, WA) (1:1, w/v) at the base of the tail. Two weeks later, the mice were given a booster intra-dermal injection of 100 μg bovine type II collagen in incomplete Freund's adjuvant (DIFCO, Detroit, MI) (1:1, v/v)[23]. The control mice were treated with Freund's adjuvant without bovine type II collagen.

Treatment protocol for collagen-induced arthritis

Treatment began four weeks after the primary immunization (following full development of arthritis). The period of treatment was 5 weeks. Hph-1-MTX was mixed with sterile ointment at concentrations of 0.1%, 0.5% and 1% (mass/mass), respectively, and applied on the skin of

CIA mice in percutaneous Hph-1-MTX-treated groups in a total volume of 200 μL, twice per week. Intact MTX was also mixed with ointment at a concentration of 1% (mass/mass) and administered percutaneously to CIA mice in percutaneous MTX-treated group in same volume twice per week. Thirty-five mg/kg of MTX was intrapenitoneally injected to CIA mice in intraperitoneal MTX-treated group twice per week. All mice except intraperitoneal MTX-treated mice received intraperitoneal PBS injections twice per week. Sterile ointment without MTX or Hph-1-MTX in the same volume was applied percutaneously to controls, untreated CIA mice and intraperitoneal MTX-treated CIA mice, twice per week.

Assessment of the arthritis severity

Mice were observed twice per week for 65 days after the primary collagen injection. The arthritis score was evaluated by visual inspection. All four legs in the mice were evaluated and scored from 0 to 4 according to the following scale: 0 = no signs of arthritis, 1= swelling and/or redness of the paw or 1 digit, 2 = 2 joints involved, 3 = more than 2 joints involved, and 4 = severe arthritis of the entire paw and all digits[24]. Paw thickness was measured with a Vernier caliper. The arthritis scoring and paw thickness measurement were performed by two blinded independent observers.

Histopathologic and immunohistochemical examination

Mice were anesthetized and sacrificed on day 65, and paws and knee joints were removed for histopathologic examination after routine fixation, decalcification and paraffin embedding of the tissue. Tissue sections were prepared and stained with Hematoxylin and Eosin (H&E). Sections were sequentially incubated with specific antibodies directed against murine TNF-α (Hycult biotechnology, Uden, Netherlands), IL-1β, IL-6, IL-10, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (SantaCruz Biotechnology, Santa Cruz, CA, USA) followed

by the appropriate secondary antibodies (ISU Abxis, Seoul, Korea). All tissue samples were counterstained with Hematoxylin.

After immunohistochemical staining, the expression of the different markers in the synovial tissue of paw and knee joints was scored semi-quantitatively on a four-point scale independently and blindly by 2 individual pathologists, and the average of their scores were calculated. A score of 0 represented minimal expression, a score of 1 represented mild expression, and a score of 2 represented moderated expression, whereas a score of 3 represented abundant expression of a marker. Minor differences between the observers were resolved by mutual agreement.

Measurement of serum level of TNF-α, IL-1β, IL-6 and interferon (IFN)-γ

Twenty-eight male DBA/1 mice at 8 weeks of age (SLC, Shizoka, Japan) were evenly divided into 4 groups as followings: group 1 = controls; group 2 = untreated; group 3 = percutaneous 0.1% Hph-1-MTX-treated; group 4 = percutaneous 1% Hph-1-MTX-treated. The induction of arthritis, the treatment schedules and the sacrifice were all done according the same protocol mentioned above. The TNF-α (Hycult biotechnology, Uden, Netherlands), IL-1β, IL-6 and IFN-γ (SantaCruz Biotechnology, Santa Cruz, CA, USA) concentrations in the experimental CIA mice serum were measured by the sandwich ELISA method according to the manufacturer's instructions. Murine recombinant TNF-α, IL-1β, IL-6 and IFN-γ diluted in medium was used as a calibration standard and a standard curve was generated by plotting the OD versus the log of the concentration.

Micro-computed tomography (CT) imaging

We conducted an additional experiment using 35 mice for micro-CT imaging and evenly divided them into 5 groups (controls, untreated CIA mice, and CIA mice treated with percutaneous 0.1%, 0.5% and 1% Hph-

1-MTX) according to the same method mentioned above. Mice were euthanized and their legs were excised and fixed in 4% formalin for 2 days. The paws obtained from experimental mice were scanned and reconstructed into the three-dimensional structure with micro-CT (Skyscan 1076, SKYSCAN, Antwerpen, Belgium) with a voxelsized of 18μm. The X-ray tube voltage was 60kV and the current was 170μA with a 0.5 mm thickness of aluminum filter. The exposure time was 1180ms. The X-ray projections were obtained at 0.5^o intervals with a scanning angular rotation of 360°. The reconstructed data set was segmented with an automated thresholding algorithm[25]. The projection images are reconstructed into three-dimensional image using the software program (NRECON (version 1.5.1), CT-Analyzer^{TM} (version 1.7, Skyscan, Belgium). Parameters measured and calculated were as below; 1) bone surface (BS) was calculated by the Marching Cubes method to triangulate the surface of the bone[26], 2) bone volume (BV) was calculated using polyhedrons corresponding to the enclosed volume of the triangulated surface[27], 3) Tissue volume (TV), a volume of the whole examined sample and the normalized index, was measured and bone volume fraction BV/TV enabled the comparison of samples of different size, 4) the specific bone surface to volume ratio was given by bone surface density (BS/BV), 5) the mean thickness of the trabeculae, Tb.Th, was obtained by filling maximum size of spheres in the structure with the distance transformation[28], and then the average thickness of all bone voxels was calculated to give Tb.Th.

Toxicity of transdermal Hph-1-MTX in CIA mice

We applied 5mg/kg of Hph-1-MTX conjugated with radioactive ^{14}C on the skin of mice once and sacrificed the experimental mice at 1, 4, 24, 48 and 120 hours after application. We obtained the skin from application site, intestine, liver and kidney of mice and detected the concentration of radioactivity by radioactivity detector. The concentrations were presented

as ng.eq/gram of Hph-1-MTX. We also obtained blood from controls, 0.1% and 1% percutaneous Hph-1-MTX-treated CIA mice after sacrifice and examined white blood cell count, hemoglobin, platelet count, and the levels of aspartate aminotransferase, alanine aminotransferase, blood nitrogen and creatinine for liver and kidney toxicity.

Statistical analysis

All statistical analyses were conducted using SPSS package for Windows, version 11.5 (SPSS Inc., Chicago, IL). The representative values were the means of those obtained from each mouse in each group, and all values in the experimental groups were compared to controls and untreated CIA mice. All results and measurements are expressed as the mean ± standard deviation. Statistical comparisons of arthritis score, paw thickness, semiquantitative histopathologic and immunohistochemical examination, and laboratory findings between the 2 groups were evaluated by Mann-Whitney U test, and parameters in micro-CT imaging were compared using t-test. Correlations between parameters measured by micro-CT were calculated using Spearman's correlation coefficient. When we compared the values between untreated and each treated groups, we gave the asterisk (*) to the mean value of each treated group which had statistical significance (P-value < 0.05).

III. RESULTS

Generation of a cell permeable form of MTX and transcutaneous delivery kinetics of Hph-1-MTX

To generate a cell permeable form of MTX which can facilitate the transcutaneous delivery of MTX, we sequentially conjugated 6-amino cupric acid and methotrexate on the N-terminal of Hph-1 containing arginine-oligomers that have been reported to enable the conjugated peptide to penetrate through skin and adjacent tissues[29,30]. We inserted 6-amino cupric acid, a small and flexible spacer, between Hph-1-PTD and MTX to prevent Hph-1 residues (tyrosine and alanine) after the cleavage by proteinase during transcutaneous delivery from inhibiting MTX to bind to enzymatic site of dihydrofolate reductase (**Fig. 1a**). To examine the efficacy of transcutaneous delivery of Hph-1-MTX, we conducted an experiment using Franz cell[22]. We filled the donor chamber with 10mg Hph-1-MTX and MTX and measured their permeated amount in the receptor chamber at each 5 hours till 25 hours. The amount of both Hph-1-MTX and MTX increased in a time dependent manner, however, Hph-1-MTX was more efficient than MTX for the skin penetration (**Fig. 1b**). To test the potential of Hph-1-MTX for the penetration into the joint, we percutaneously applied Hph-1-MTX labeled with FITC on the skin of paws and legs of mice and detected FITC fluorescence after 3, 6 and 24 hours after application. To lessen the confounding background signals, we reduced the detection energy until no FITC fluorescence was detected in the joint specimen obtained from normal mice. Strong FITC fluorescence was detected around the joints as well as the periosteal and periarticular tissues after 24 hours (**Fig. 1c**). Taken together with these results, we prove that Hph-1 efficiently can enable MTX to penetrate through the skin as well as into the joint.
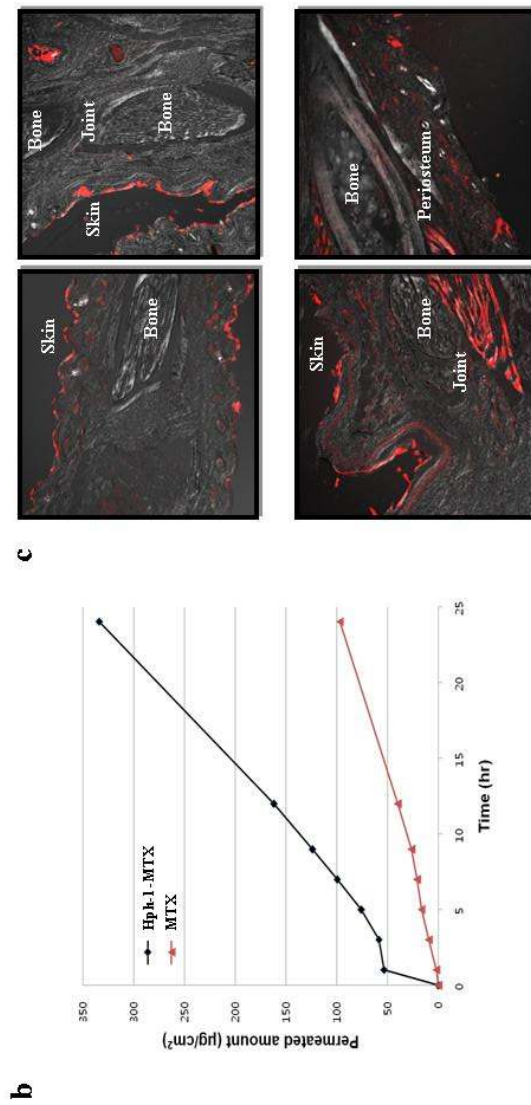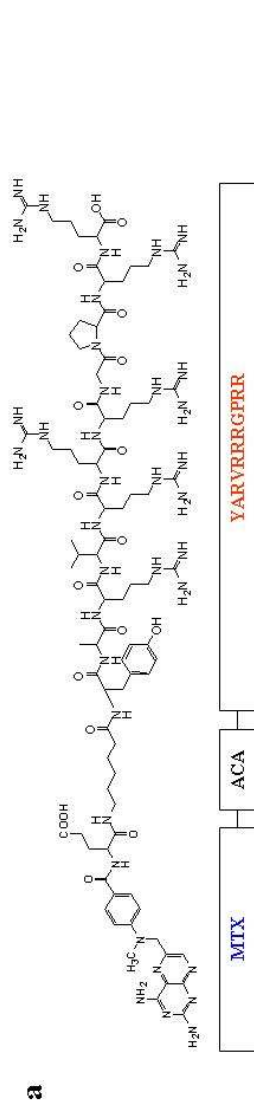
Fig. 1. The structure and transcutaneous delivery kinetics of Hph-1-MTX

(**a**) MTX was sequentially conjugated with 6-amino cupric acid and the N-terminal of Hph-1 containing arginine-oligomers. 6-amino cupric acid, a small but flexible spacer, was inserted to prevent Hph-1 residues after the cleavage from inhibiting MTX to bind to enzymatic site of dihydrofolate reductase. (ACA=6-amino cupric acid) (**b**) The amount of both Hph-1-MTX and MTX increased in a time dependent manner, however, Hph-1-MTX was more efficient than MTX for the skin penetration in Franz cell experiment. (**c**) Fluorescein isothiocyanate (FITC)-labeled Hph-1-MTX was applied on the skin of paws and legs of mice. Strong FITC fluorescence was detected around the joint as well as the periosteal and periarticular tissues of CIA mice (original magnification ×100).

Percutaneous application of Hph-1-MTX significantly decreased the severity of arthritis in CIA mice

To evaluate the *in vivo* therapeutic effectiveness of percutaneous Hph-1-MTX in RA animal model, we induced arthritis in DBA/1 mice, applied percutaneous Hph-1-MTX in difference doses, 0.1%, 0.5% and 1% in the paws and legs, and assessed the gross and histopathologic findings compared to control, untreated, intraperitoneal MTX-treated and percutaneous MTX-treated CIA mice. Control was defined as mice not injected with type II collagen. Percutaneous application of 0.1%, 0.5% and 1% Hph-1-MTX significantly decreased both mean arthritis score and paw thickness in comparison with untreated CIA mice, in a dose-dependent manner (**Fig. 2a and 2b**). The macroscopic evidences of arthritis such as erythema or swelling were definitely observed in untreated and percutaneous MTX-treated CIA mice. In contrast, the severity of arthritis in percutaneous Hph-1-MTX-treatd CIA mice was significantly lower than that in untreated or percutaneous MTX-treated CIA mice, similar that in intraperitoneal MTX-treated CIA mice (**Fig. 2c**). Histopathologic evaluation of paw and knee joint sections of untreated and percutaneous MTX-treated CIA mice revealed inflammatory cell infiltration, synovial hyperplasia and partial bone destruction (pannus). In contrast, in CIA mice applied by percutaneous Hph-1-MTX, the extent of inflammatory cell infiltration and bone destruction was significantly reduced compared to those in untreated and percutaneous MTX-treated CIA mice (**Fig. 2c**). The semi-quantitative analysis of histopathologic features such as infiltrate score and erosion score were drawn in **Fig. 2d**. Percutaneous application of Hph-1-MTX significantly decreased both scores, similar to intraperitoneal application MTX. With these results, we conclude that percutaneous Hph-1-MTX can quench the inflammation in the joints through skin and its adjacent tissues.

c

| Control | Untreated | MTX PC | MTX IP | Hph-1-MTX 0.1% | Hph-1-MTX 0.5% | Hph-1-MTX 1% |

d

16
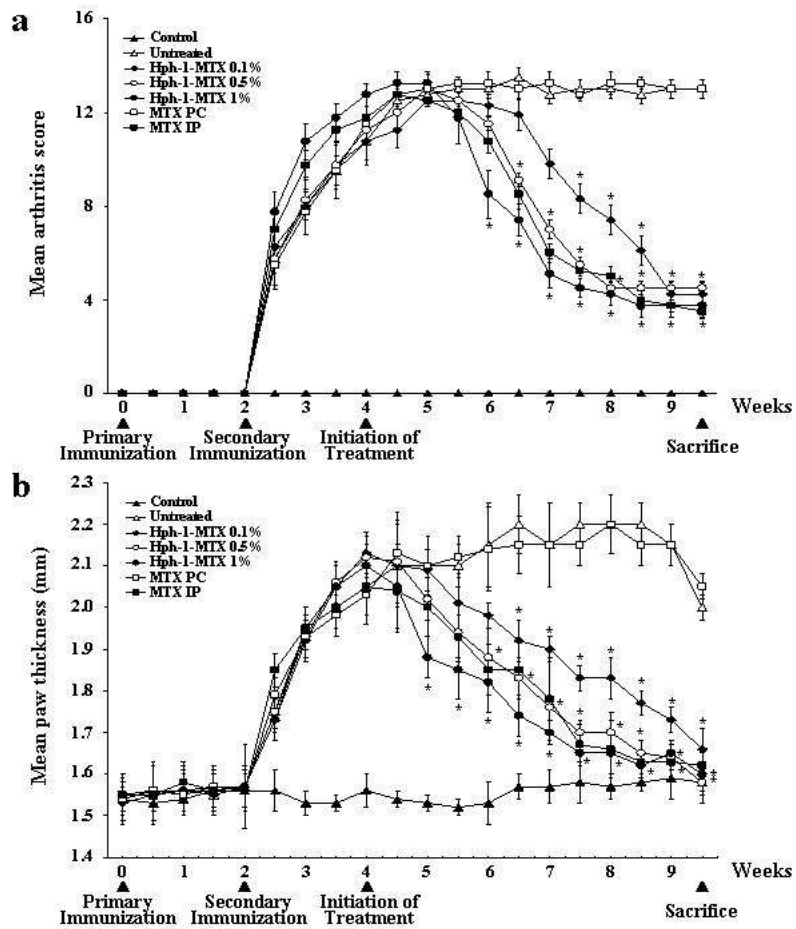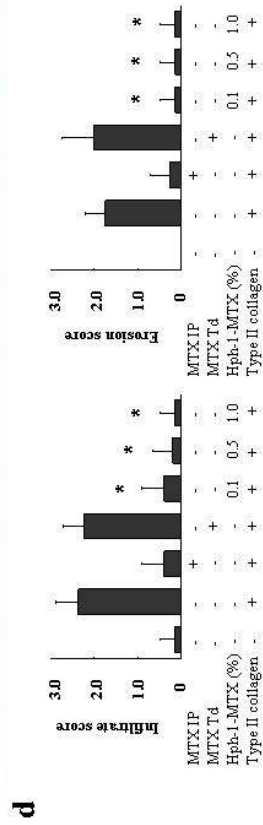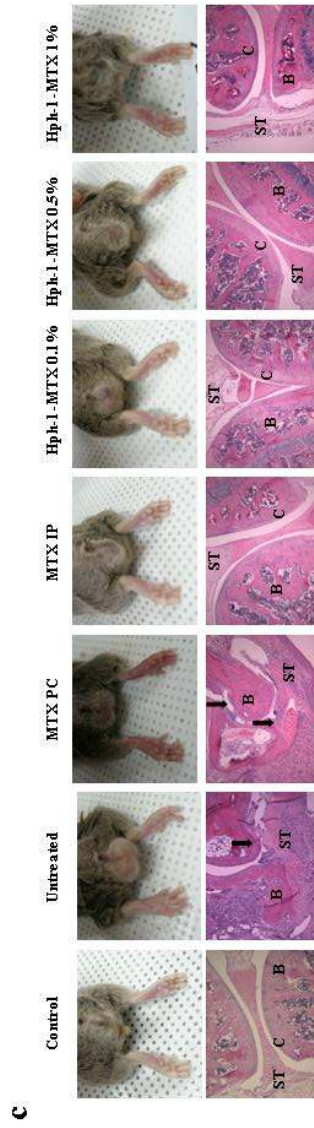
Fig. 2. Suppression of the severity of arthritis in CIA mice by Hph-1-MTX in a dose-dependent manner via topical application

0.1%, 0.5% or 1% Hph-1-MTX was topically applied on day 28 after primary immunization, twice per week and for five weeks. (**a**) The mean arthritis score. (**b**) The mean paw thickness. The macroscopic evidences of arthritis such as erythema or swelling were assessed. CIA mice treated with topical or intraperitoneal injected intact MTX were compared to topical Hph-1-MTX-treated CIA mice. (PC=percutaneous, IP=intraperitoneal). (**c**) Histologic findings of the joint section was also evaluated (original magnification ×100, Hematoxylin-Eosin). (B=bone, C=cartilage, and ST=synovial tissue). (**d**) The semi-quantitative analysis of histologic findings of the inflamed joints such as infiltrate score and erosion score. *$P < 0.05$ compared with untreated CIA mice.

The expression of cytokines was reduced in the inflamed joints and the serum of CIA mice treated with a cell permeable Hph-1-MTX

TNF-α, IL-1β, IL-6, iNOS and COX-2, inflammatory cytokines and joint-destructive enzymes, in the affected joints are the pivotal messengers in the pathophysiology of RA and their gene expression is closely related to the inflammatory activity of RA[2,3,10,11]. To examine whether Hph-1-MTX transcutaneously delivered into the joints of CIA mice can inhibit the expression of inflammatory cytokines and enzymes, we stained joints sections obtained from CIA mice with specific antibodies directly against those molecules followed by the appropriate secondary antibodies. The expression of inflammation-related genes in the synovial tissue of paw and knee joints was scored semi-quantitatively on a four-point scale independently and blindly by two individual pathologists, and the average of their scores were calculated. Immunohistochemical analysis of the paws and knee joint tissues obtained from untreated and percutaneous MTX-treated CIA mice exhibited markedly positive staining for TNF-α, IL-1β, or IL-6 which were localized primarily around the joint compared to controls showing negative staining. In contrast, few significant positive TNF-α, IL-1β, or IL-6 staining were observed in percutaneous Hph-1-MTX-treated CIA mice. Similarly, staining for iNOS and COX-2 were significantly positive only in untreated and percutaneous MTX-treated CIA mice, but were unlikely in percutaneous Hph-1-MTX-treated CIA mice (**Fig. 3a and 3b**). We also assessed the production of IL-10, anti-inflammatory cytokine, and found that its expression had a similar pattern of other inflammatory cytokines (**Fig. 3a and 3b**). In this study, we used anti-murine cytokines and enzymes rabbit or goat antibodies as primary antibodies. In order to evaluate the isotype irrelevant primary antibodies, we treated normal rabbit or goat sera containing various nonspecific antibodies and then added secondary antibodies in the joint sections obtained from controls. We could not find out the positive immunohistochemical staining in the

joint sections. We find that percutaneous application of Hph-1-MTX reduced the expression of inflammation-related genes as well as IL-10 gene in the inflamed joints of CIA mice. The serum concentration of inflammatory cytokines is known to be directly proportional to the extent of inflammation in joints of CIA mice[31,32]. To examine whether percutaneous application of Hph-1-MTX can also inhibit the secretion of inflammatory cytokines, we conducted an additional experiment using CIA mice treated with 0.1% and 1% percutaneous Hph-1-MTX, measured their serum concentration and compared those with untreated CIA mice. In comparison with the serum concentration of inflammatory cytokines in untreated CIA mice, percutaneous Hph-1-MTX significantly reduced the serum concentration of those cytokines as much as below; TNF-α (65% for 0.1% and 80% for 1%), IL-1β (57% for 0.1% and 74% for 1%), IL-6 (67% for 0.1% and 77% for 1%) and IFN-γ (50% for 0.1% and 73% for 1%), respectively (**Fig. 3c**). We also find that percutaneous application of Hph-1-MTX can decrease the secretion of inflammatory cytokines in CIA mice.

Control | Untreated | MTX PC | MTX IP | Hph-1-MTX 0.1% | Hph-1-MTX 0.5% | Hph-1-MTX 1%

TNF-α | IL-1β | IL-6 | IL-10 | iNOS | COX-2

a

b

TNF-α

MTX IP	-	+	-	-	-	-
MTX Td	-	-	+	-	-	-
Hph-1-MTX (%)	-	-	-	0.1	0.5	1.0
Type II collagen	-	+	+	+	+	+

IL-1β

MTX IP	-	+	-	-	-	-
MTX Td	-	-	+	-	-	-
Hph-1-MTX (%)	-	-	-	0.1	0.5	1.0
Type II collagen	-	+	+	+	+	+

IL-6

MTX IP	-	+	-	-	-	-
MTX Td	-	-	+	-	-	-
Hph-1-MTX (%)	-	-	-	0.1	0.5	1.0
Type II collagen	-	+	+	+	+	+

IL-10

MTX IP	-	+	-	-	-	-
MTX Td	-	-	+	-	-	-
Hph-1-MTX (%)	-	-	-	0.1	0.5	1.0
Type II collagen	-	+	+	+	+	+

COX-2

MTX IP	-	+	-	-	-	-
MTX Td	-	-	+	-	-	-
Hph-1-MTX (%)	-	-	-	0.1	0.5	1.0
Type II collagen	-	+	+	+	+	+

iNOS

MTX IP	-	+	-	-	-	-
MTX Td	-	-	+	-	-	-
Hph-1-MTX (%)	-	-	-	0.1	0.5	1.0
Type II collagen	-	+	+	+	+	+

c

TNF-α (pg/ml)

Hph-1-MTX (%)	-	0.1	1
Type II collagen	-	+	+

IL-1β (pg/ml)

Hph-1-MTX (%)	-	0.1	1
Type II collagen	-	+	+

IL-6 (pg/ml)

Hph-1-MTX (%)	-	0.1	1
Type II collagen	-	+	+

IFN γ (pg/ml)

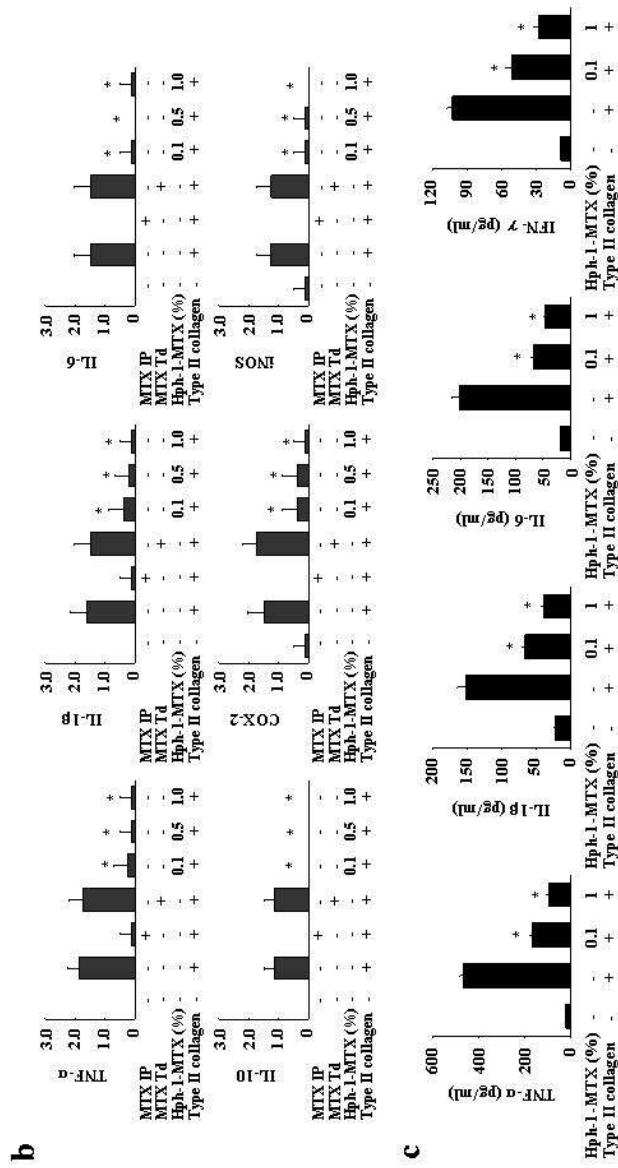| Hph-1-MTX (%)	-	0.1	1
Type II collagen	-	+	+

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Fig. 3. The reduced expression of inflammatory cytokines and joint-destructive enzymes in the affected joints and their serum concentration in CIA mice

0.1%, 0.5% or 1% Hph-1-MTX was topically applied twice per week and for five weeks. (**a**) Immunohistochemical staining for TNF-α, IL-1β, IL-6, IL-10, iNOS and COX-2 in the joint sections from CIA mice was done. (original magnification ×100). (PC=percutaneous, IP=intraperitoneal) (**b**) Semi-quantitative analysis of the degree of stain-positivity of immunohistochemical staining. (**c**) The serum concentrations of TNF-α, IL-1β, IL-6 and IFN-γ in CIA mice. 0.1%, or 1% Hph-1-MTX was topically applied in the same methods. *$P < 0.05$ compared with untreated CIA mice.

Micro- CT proved the efficiency of percutaneous application of Hph-1-MTX on arthritis in CIA mice

Since the analysis of histopathologic findings or the inflammatory cytokines gene expression and secretion in the inflamed joints has a limitation of semi-quantitative analysis and the common alterations of the bone in the inflamed joints in RA are bone erosion and periarticular osteopenia, we used performed 3-dimensional micro-CT to quantitatively investigate these alterations in control, untreated CIA mice and 0.1%, 0.5% and 1% percutaneous Hph-1-MTX-treated CIA mice. Severe bone destruction was observed in untreated CIA mouse and 0.1% percutaneous Hph-1-MTX-treated mice showed slightly progressed bone destruction in MTP joints, while 0.5% and 1% percutaneous Hph-1-MTX-treated mice exhibited relatively well-preserved bones of paws (**Fig. 4a**). We also analyzed four parameters of micro-CT analysis including BV, bone volume/tissue volume BV/TV, bone surface areas adjusted to BV, BS/BV and Tb,Th. Despite the range of the measurement of bone was already set, since there might be subtle differences in size of the samples, we measured not only BV, but also BV/TV that can compare the bone samples of different sizes. These parameters reflected the bone loss in the affected joints. 0.5% and 1% percutaneous Hph-1-MTX-treated mice showed well-preserved bone volume compared to untreated mice (**Fig. 4b**). BS/BV was also used to reflect the loss of bone surface due to erosion. Percutaneous Hph-1-MTX significantly reduced the extent of bone erosion in a dose dependent manner (**Fig. 4b**). Tb.Th was calculated to represent the extent of periarticular osteopenia induced by joint inflammation[33]. CIA mice treated with 0.5% and 1% percutaneous Hph-1-MTX exhibited significantly higher Tb.Th than untreated mice. (**Fig. 4b**). However, 0.1% percutaneous Hph-1-MTX-treated mice did not show any significant differences in 4 parameters compared with untreated mice. To clarify the validity of the parameters measured or calculated by micro-CT, we evaluated the correlation coefficiencies

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among 4 parameters. Tb,Th was significantly correlated with BV/TV as well as BS/BV (r^2=0.757 and r^2=-0.698, p<0.001, respectively), and BV/TV was also well correlated with BS/BV (r^2=-0.925, p<0.001) (**Fig. 4c**). These results suggest that percutaneous application of Hph-1-MTX can relatively preserve bone volume and quality of trabecular bone despite the joint inflammation.
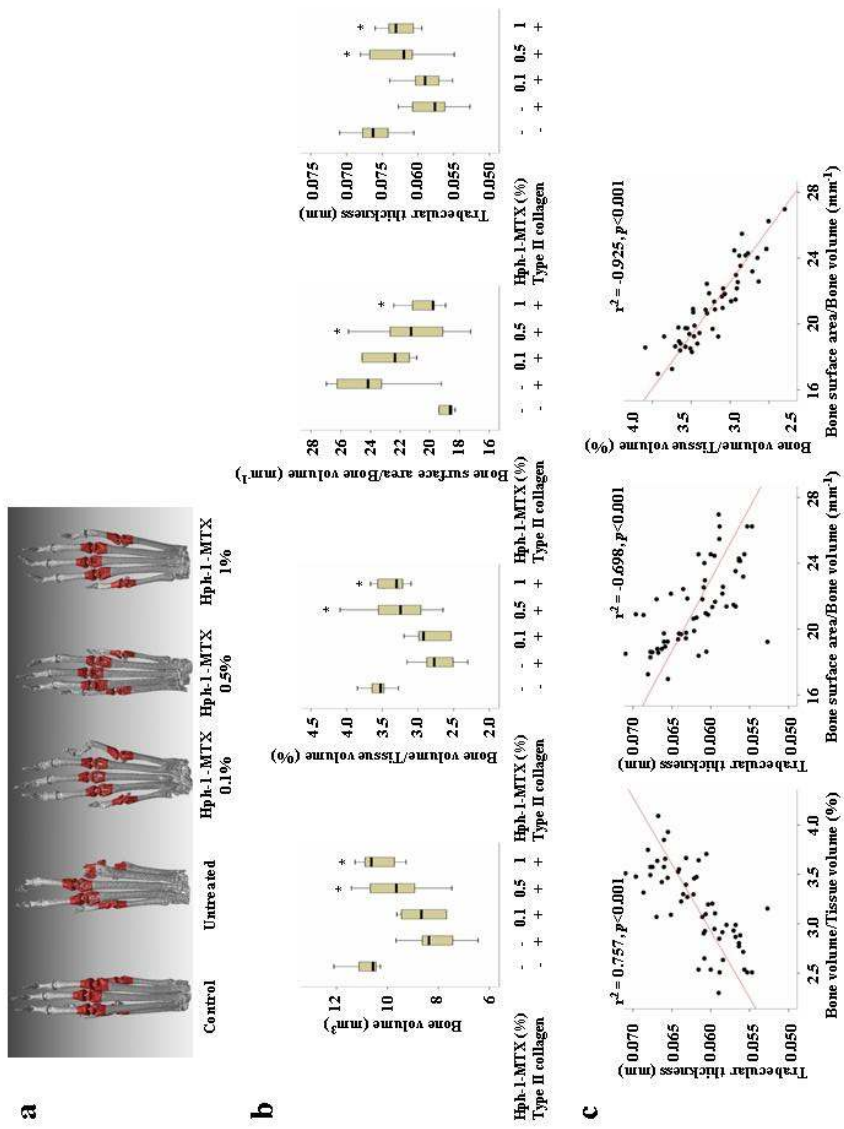
Fig. 4. The reduced bone destruction and juxta-articular bone loss by topical Hph-1-MTX via micro-CT scan. 0.1%, 0.5% or 1% Hph-1-MTX was topically applied in the same methods

(**a**) Three-dimensional reconstruction of micro-CT imaging of the paws of CIA mice. (**b**) The parameters of bone integrity and bone loss. (**c**) The correlation of the parameters obtained form micro-CT scan. *$P < 0.01$ compared with untreated CIA mice.

Toxicity of transcutaneous Hph-1-MTX in CIA mice

To examine the systemic distribution of percutaneous Hph-1-MTX, we applied 5mg/kg of Hph-1-MTX conjugated with radioactive ^{14}C on the skin of mice once and detected the concentration of radioactivity in application site (skin), intestine, kidney and liver according to time after application. High concentration of radioactivity was detected at application site during whole experimental period, while, radioactivity was detected high in other tissue during 24 and 48 hours but not detected at 120 hours after application. Furthermore other tissues exhibited weaker peak radioactivity by up to 20 fold than application site (**Fig. 5**). We conclude that most of percutaneous Hph-1-MTX is distributed at application site, moreover, its clearance in kidney and liver was rapid, suggesting that percutaneous Hph-1-MTX may not significantly affect the systemic circulating concentration of MTX.
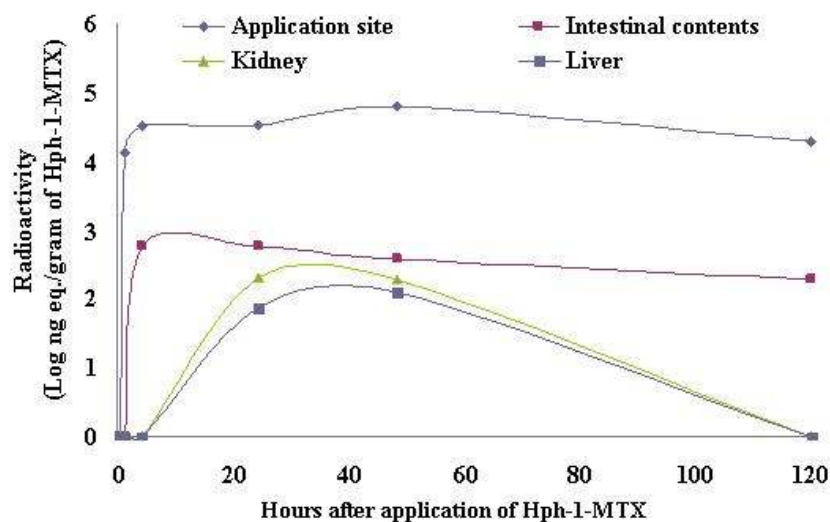
Fig. 5. The distribution of topical Hph-1-MTX in the major organs
5mg/kg of Hph-1-MTX conjugated with radioactive ^{14}C was applied on
the skin of mice once and detected the concentration of radioactivity in
application site (skin), intestine, kidney and liver according to time after
application.

To evaluate the clinical toxicity of percutaneous Hph-1-MTX, we also applied 0.1% and 1% Hph-1-MTX on the skin of paws and knees of CIA mice, counted blood cells and measured the serum concentration of parameters reflecting functions of liver and kidney. There were no differences in blood cell counts among controls and 0.1% and 1% percutaneous Hph-1-MTX-treated CIA mice. Deterioration of liver and kidney function was not observed in CIA mice treated with 0.1% and 1% percutaneous Hph-1-MTX (**Table 1**). With these results, we conclude that percutaneous application of Hph-1-MTX may not induce serious systemic complication.

Table 1. Laboratory findings in control and Hph-1-MTX treated mice

	Control	Hph-1-MTX 0.1%	Hph-1-MTX 1%
WBC ($/m^3$)	8420.1 ± 1024.2	8552.6 ± 973.0	8592.5 ± 668.5
Hb (mg/dL)	13.4 ± 0.5	12.6 ± 0.3	13.1 ± 0.5
PLT(x 1,000/m^3)	632.0 ± 54.8	665.0 ± 82.4	640.0 ± 90.2
BUN (mg/dL)	22.6 ± 1.3	23.7 ± 2.5	24.6 ± 2.2
Cr (mg/dL)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
AST (IU/L)	133.2 ± 34.2	129.5 ± 22.1	135.2 ± 29.8
ALT (IU/L)	72.4 ± 12.5	73.4 ± 15.2	73.6 ± 10.2

WBC, white blood cell; *Hb*, hemoglobin; *PLT*, platelet; *BUN*, blood urea nitrogen; *Cr*, creatinine; *AST*, aspartate transaminase; *ALT*, alanine transaminase.

IV. DISCUSSION

In this study, we first demonstrated that percutaneous application of MTX ameliorated the severity of arthritis in CIA mice using a new PTD of Hph-1. Hph-1-MTX not only induced histopathologic improvement of a marked reduction of inflammatory cells infiltration, synovial hyperplasia, and bone destruction, but also decreased the production and secretion of pro-inflammatory cytokines in the inflamed joint tissue. Particularly, a finding in immunohistochemical staining that the expression of TNF-α and IL-1β, critical cytokines in RA pathophysiology[2], was reduced following percutaneous application of Hph-1-MTX strongly supports a presumption of the direct effect of Hhp-1-MTX on joint synovial cells through the skin and its adjacent tissues. In addition to TNF-α and IL-1β, the expression of IL-6, another arthritis-inducing inflammatory cytokine[2-4], and joint-destructive enzyme, iNOS and COX-2[10,11], was significantly reduced in the inflamed joint sections of CIA mice, similar to those observed in intraperitoneal MTX-treated CIA mice. These results suggest that the efficiency of transcutaneous conveyance of MTX by Hph-1 may not fall behind that of intraperitoneal injection of MTX. Moreover, the dose-dependent reduction in the severity of arthritis by percutaneous application of Hph-1-MTX provided the additional evidence that the improvement was done by pharmacologic action of MTX.

There was a discrepancy in the co-expression of inflammatory cytokines as well as anti-inflammatory cytokine of IL-10 in the inflamed joint. IL-10 has been generally known to reduce the inflammation against those inflammatory cytokines in pathogenesis of RA[34-36]. However, considering there was a paradoxical positive correlation between histopathologic joint destruction and the expression of IL-10, the increase in the expression of IL-10 in the inflamed joint might be a secondary phenomenon to counteract the inflammation which is stimulated by the markedly expressed inflammatory cytokines including TNF-α, IL-1β and

IL-6[37].

Although two independent pathologists assessed the remedial efficiency of percutaneous application of Hph-1-MTX on arthritis by histopathologic findings and the degree of positive staining of cytokines and joint-destructive enzymes in immunohistochemical staining, there was left a potential limitation that these results were analyzed by a semi-quantitative method. In order to come over this limitation, we measured four parameters reflecting the bone destruction using micro-CT and three-dimensional reconstruction, relatively objective and quantitative tool[38-40]. The typical radiological findings in RA are marginal bone erosions and periarticular osteopenia[41]. We evaluated BV, BV/TV, BS/BV and Tb.Th. for bone destruction and periarticular osteopenia[32,33]. The significant correlation among these parameters supported the validity of three-dimensional micro-CT for evaluation of bone destruction due to the joint inflammation in CIA mice. We found that percutaneous application of both 0.5% and 1% Hph-1-MTX improved all parameters, suggesting that it might attenuate the severity of joint inflammation in CIA mice.

It has been reported serum concentration of cytokines involved in the pathophysiology of RA are correlated with their synovial concentration in RA patients[42,43]. We observed that the serum concentrations of inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IFN-γ were elevated in untreated CIA mice compared to controls, and significantly decreased in 0.1% and 1% percutaneous Hph-1-MTX-treated CIA mice, suggesting there might be a communicating network between those two different compartments and the local inflammation might affect the circulating inflammatory cytokines concentration. In addition, it has been reported that the increased concentrations of those cytokines can induce systemic manifestations including anemia and atherosclerosis in RA patients[44-46]. Surely, these secondary systemic complication of RA predominantly occur in patients who have relatively large joints affected, compared to those who have small inflamed joints, and the more

aggressive treatments such as the systemic dose escalation or intra-articular injection of MTX should be considered in these situations. However, it is difficult to exclude the possibility that those complications of RA might occur in RA patients with only several but refractory small joints affected, and increased dose of MTX might provoke other adverse effects. Therefore, we expect that percutaneous application of Hph-1-MTX can not only improve the arthritis, but also provide an additional advantage to decrease the frequency of secondary RA complications, in patients who have the refractory joint inflammation confined to several small joints where the intra-articular administration of MTX is unavailable or systemic diseases where the use of MTX is inadequate[47].

So far, there have been several efforts to develop a new skin-penetrating PTD[29,30], however, there was no reports on PTD that can permeate deeper into the joint. Here, we first elucidated the penetration of percutaneously applied PTD into the joint cavities using Hph-1-MTX conjugated with fluorescence. Confocal microscopy analysis confirmed that Hph-1-MTX efficiently penetrated the skin and its adjacent tissues in time-dependent manner, and reached the joint tissues within 24 hours after administration. Although the mechanism of the penetration of Hph-1-MTX through those tissues still remains controversial, it is obvious that Hph-1 actually can keep the stability of MTX and led it into the joint tissues of CIA mice. Moreover, 6-amino cupric acid (ACA), as a spacer, can overcome the structural component of MTX interfering its inhibitory action due to conjugation of tyrosine and alanine residues of Hph-1.

These data have three important clinical implications in the treatment of RA patients; first, percutaneous application of Hph-1-MTX can provide an opportunity to minimize the circulating concentration of MTX in RA patients who have diseases or conditions where use of MTX is not recommended[13,14]. Second, although toxicity experiment of this study cannot reflex almost all the complications of MTX, we could find that almost all the percutaneous Hph-1-MTX located at application site, and

any serious decrease in blood cell counts and deterioration of liver and kidney functions. Thus, it may lift a burden of anxiety from the rheumatologists that long-term use of MTX might develop serious and fatal adverse effects in RA patients who have only several but refractory small joints affected. Third, since it can permit a high dose of MTX to directly enter the joint cavity with less systemic complication, prior to an invasive procedure such as repetitive intra-articular injection which may induce joint damage, it can reduce a time lag to onset of action of MTX and quench the joint inflammation earlier, leading to less irreversible joint destruction and better functional status as well. In addition, the structural changes including atrophy or degeneration as adverse effect of MTX in the skin and its adjacent tissues were not observed.

V. CONCLUSION

In conclusion, these results indicated that percutaneous Hph-1 can directly deliver MTX to the inflamed joints of CIA mice with the similar efficiency to that of intraperitoneal MTX. Thus, percutaneous application of Hph-1-MTX may be a new therapeutic modality in patients with RA having several and refractory small joints affected in whom systemic administration of MTX may be expected to induce fatal complications. Furthermore, we expect that we can apply percutaneous Hph-1-MTX to other autoimmune inflammatory diseases such as psoriasis in which MTX is a mainstay for treatment, as a new therapeutic regimen.

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ABSTRACT (in Korean)

세포 투과성 메토트렉세이트 (methotrexate)를 피부 도포 시
콜라겐 유도성 관절염 쥐 모델에서의 관절염 완화 효과와
전신적 부작용 평가

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메토트렉세이트(methotrexate, MTX)는 류마티스 관절염
치료의 가장 중요한 약물이다. 하지만 좋은 효과에도 불구하고
다양하고 심각한 전신적인 부작용을 유발할 수 있다. 치료적
효과를 유지하면서 약제에 의한 부작용을 줄이기 위해서
메토트렉세이트에 인간 전사 인자의 일종인 Hph-1의 일부
단백을 이용한 새로운 단백 전달 영역 (protein transduction
domain, PTD)을 결합하여 세포 투과성을 지닌 메토트렉세이트
(Hph-1-MTX)를 개발하였다. Hph-1-MTX의 피부 투과
효능은 Franz cell 장치를 이용하여 입증하였고, Hph-1-MTX에
형광 물질을 결합한 뒤 피부에 도포하여 관절 주위로 침투하는
것을 확인하였다. 콜라겐 유도성 관절염 쥐 모델에서 Hph-1-
MTX을 피부에 도포하였을 때 약물의 농도에 비례하여
관절염의 증상을 호전시켰고, 삼차원 미세 컴퓨터 단층 촬영을
통해서 평가하였을 때 관절의 형태를 유의하게 보존하였다.
또한 Hph-1-MTX의 피부 도포는 염증성 사이토카인과 관절
파괴적인 효소들의 발현과 분비를 전신적인 부작용 없이, 관절
조직과 혈청에서 유의하게 감소시켰다. 따라서 세포 투과성
Hph-1-MTX는 류마티스 관절염의 새로운 치료 방법으로서의
의미를 지니며, 메토트렉세이트를 전신적으로 투여할 수 없는
환자의 경우나 약물에 불응적인 몇 개의 작은 관절에 국한된

염증을 가진 환자에서 전신적인 부작용 없이 안전하게 사용할
수 있을 것이다.

Abbreviations

ACA=6-amino cupric acid
ALE=alanine transminase
AST=aspartate transminase
BS=bone surface
BUN=blood urea nitrogen
BV=bone volume
CIA=collagen induced arthritis
COX-2=cyclooxygenase 2
Cr=creatinine
CT=computed tomography
CTLA=cytotoxic T lymphocyte antigen
DMARDs=disease modifying anti-rheumatic drugs
FITC=fluorescein isothiocyanate
Hb=hemoglobin
H&E=Hematoxylin and Eosin
IFN=interferon
IL=interleukin
iNOS=inducible nitric oxide synthase
MTX=methotrexate
PLT=platelet
PTD=protein transduction domain
RA=rheumatoid arthritis
Tb.Th=trabecular thickness
TNF=tumor necrosis factor
TV=tissue volume
WBC=white blood cell