Alleviation of rheumatoid arthritis by cell-transducible methotrexate upon transcutaneous delivery

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1. Introduction

Rheumatoid arthritis (RA) is a prototypical systemic autoimmune disease that is characterized by inflammation in multiple joints [1]. In affected joints, diverse factors initiate the emergence of RA autoantigens including type II collagen, proteoglycans, human cartilage glycoprotein, citrullinated proteins, and heat-shock proteins which then provoke a series of immune reactions through the activation of CD4 T cells [1]. Activated CD4 T cells produce interferon (IFN)-γ and other inflammatory cytokines, which initially stimulate monocytes, macrophages and synovial fibroblasts. Subsequently, activated macrophages and synovial fibroblasts induce the overproduction of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-15, and IL-18 [1–3]. Activated CD4 T cells also stimulate B cells to differentiate into plasma cells producing autoantibodies including rheumatoid factor and anti-cyclic citrullinated peptide (anti-CCP) [1]. TNF-α, IL-1β and IL-6 stimulate osteoclasts, chondrocytes, neutrophils and synovial fibroblasts to produce various metalloproteinases and joint-destructive enzymes including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in inflamed joints. Finally these enzymes mediate cartilage degradation and bone erosion, resulting in pain and joint destruction; patients with inadequately treated RA, joint destruction is irreversible [1–3].

Among various disease-modifying anti-rheumatic drugs (DMARDs), the oral administration of methotrexate (MTX) is the most widely used for the treatment of RA [4–6]. MTX can slow the rate of joint destruction through its inhibitory effects on the cascade of events initiated by inflammatory cytokines and subsequent joint-destructive enzymes [7–9]. However, despite its therapeutic efficacy, the long-term administration of MTX may induce serious systemic complications, including infection, hepatitis, and bone marrow suppression [10–12]. Recently, new biological agents,
such as TNF-α blockade and anti-CD20 monoclonal antibody, have been developed and are being used clinically [13,14]. However, despite their potent and rapid therapeutic efficiency, these treatments may also provoke serious systemic complications, including infection, malignancies and autoimmune diseases [15,16]. Moreover, the formation of neutralizing antibodies to biological agents might decrease their therapeutic efficacy [17–19]. Therefore, it is suggested that MTX still remains a mainstay in the treatment of RA [18].

From a clinical point of view, increasing the dose of MTX in RA patients who have some refractory small joint diseases or continuing MTX in patients who have systemic diseases, such as pulmonary tuberculosis, interstitial lung diseases, viral hepatitis, liver cirrhosis or bone marrow dysfunction, may provoke more unwanted adverse effects. In these cases, the necessity of the local application of MTX has been raised, but to date, there has been no effective method reported to deliver MTX locally and percutaneously into joints.

In this study, we generated transcutaneous MTX (TC-MTX) to decrease the systemic toxicity of oral MTX, and to improve its therapeutic effect on affected joints. We used Franz static diffusion cells and skin from a hairless mouse, and in vivo in mice to confirm the effectiveness of transcutaneous delivery of MTX. TC-MTX was applied on inflamed joints of mice with collagen-induced arthritis (CIA), and both the changes in the severity of the arthritis and the levels of TNF-α, IL-1β, IL-6 and IFN-γ in inflamed joints as well as in the serum was investigated. Further, we compared the therapeutic potential of TC-MTX in vivo to that of the clinically used etanercept, an anti-TNF-α biological agent, for RA. Also, the toxicity of TC-MTX and its distribution in CIA mice after application was evaluated.

2. Materials and methods

2.1. Purification and generation of TC-MTX and dihydrofolate reductase (DHFR) assay

TC-MTX consists of Hph-1-PTD, ACA and MTX. First, Hph-1-PTD (YARVRRECPFR-PRH) was synthesized using solid phase techniques and commercially available fluorenyl-methoxycarbonyl (Fmoc) amino acids (Novabiochem, Darmstadt, Germany) on an Applied Biosystems 433 peptide synthesizer. ACA and MTX were coupled on the N-terminus of Hph-1-PTD sequentially. The TC-MTX conjugate was cleaved from the resin using 96% trifluoroacetic acid, 2% triisopropylsilane and 2% phenol for 12 h. Longer reaction times were necessary to completely remove the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pmbo-protecting groups) from the arginine. Subsequently, conjugates were filtered from the resin, precipitated using diethyl ether, purified using HPLC reverse-phase columns and characterized using MALDI-TOF (Bruker Daltomics, Bremen, Germany) (Fig. 1A). Because there are two carbonyl groups at α and γ positions of MTX, α and γ forms of TC-MTX were made. DHFR activity was compared to that of free MTX using a DHFR assay kit (Sigma-aldrich, Saint Louis, MO, USA) according to the manufacturer’s instruction.

2.2. Franz cell experiment

A 9-mm unjacketed Franz cell with a flat flange joint and clear glass was used for the experiment [20]. Dorsal skin of hairless mice was used as a membrane. The donor chamber was filled with 10 mg of TC-MTX in 0.4 ml of 0.1 M PBS (pH 7.4) or 10 mg of MTX in 0.4 ml of 0.5 M Tris buffer (pH 10.8). The receptor chamber was filled with 4.6 ml of PBS. They were incubated at 37 °C while stirring. MTX and TC-MTX residues (MTX-ACA-tydrosine-alanine) were collected from the receptor chamber with 5 h interval until 25 h and their permeated amount were measured by HPLC.

2.3. Fluorescence microscopic analysis of penetration of TC-MTX

For the Cy5.5 labeling of TC-MTX, N-hydroxy succinimide (NHS) Cy5.5 (GE healthcare, Buckinghamshire, UK) was coupled in N-methyl pyrolidone (NMP) solution for 5 h on the side chain of lysine that was added at the N-terminal end of Hph-1-PTD. The cleavage, purification, and characterization were performed using the same processes of TC-MTX synthesis. We percutaneously applied 1% Cy5.5-labeled TC-MTX on the joint of DBA/1 mice and obtained joint tissues at 3, 6, and 24 h after application. Cy5.5 fluorescence was detected using confocal microscopy.

2.4. Induction of CIA and assessment of arthritis severity

All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea. Arthritis was induced in DBA/1 mice at 8 weeks of age (SLC, Shizoka, Japan) and the severity of arthritis was assessed as described in our previous study [21].

2.5. Treatment protocol for CIA

Treatment began 4 weeks after the primary immunization and lasted 5 weeks. TC-MTX was mixed with sterile ointment at concentrations of 0.1%, 0.5% and 1% (mass/mass), and applied on the paws and knee joints of CIA mice in TC-MTX-treated groups in a total volume of 200 µl, twice per week. MTX was also mixed with ointment at a concentration of 1% (mass/mass) and administered to CIA mice in the percutaneous MTX-treated group with the same volume twice per week. Thirty-five mg/kg of MTX was intraperitoneally injected into CIA mice in the 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 TC-MTX on the paws and legs of CIA mice, and we intraperitoneally injected etanercept (5.5 mg/kg) and MTX (35 mg/kg) into CIA mice at week 4, twice per week, for 5 weeks as described above.

2.6. Histopathological and immunohistochemical examination

Paws and knee joint sections were prepared and stained with H&E and sequentially incubated with specific antibodies directed against murine TNF-α, IL-1β, IL-6, INOS or COX-2 (SantaCruz Biotechnology, Santa Cruz, CA, USA) followed by the appropriate secondary antibodies (ISU Axibs, Seoul, Korea); expression was evaluated as described in our previous study [21].

2.7. Micro-computed tomography (CT) imaging

A total of 35 mice were used for micro-CT imaging experiments. They were evenly divided into 5 groups (controls, untreated, and percutaneous 0.1%, 0.5% and 15% TC-MTX-treated CIA mice). The paws obtained from experimental mice were scanned, reconstructed into the three-dimensional structure with micro-CT (SkyScan 1076) (SKYSCAN, Kontick, Belgium) and evaluated as described in a previous study [21].

2.8. Measurement of serum level of TNF-α, IL-1β, IL-6 and IFN-γ

Male DBA/1 mice at 8 weeks of age (SLC, Shizoka, Japan) were evenly divided into 4 groups (controls, untreated, percutaneous 0.1% and 15% TC-MTX-treated CIA mice). The induction of arthritis, the treatment schedules, and animal sacrifice were all done according the same protocol mentioned above. The TNF-α, IL-1β, IL-6 and IFN-γ concentrations in the experimental CIA mice serum were measured by the sandwich ELISA (SantaCruz Biotechnology, Santa Cruz, CA, USA) performed according to the manufacturer’s instructions.

2.9. Distribution and toxicity of percutaneously administered TC-MTX in CIA mice

We applied 5 mg/kg of TC-MTX conjugated with radioactive 14C on the skin of mice and sacrificed mice at 1, 4, 24, 48 or 120 h after application. Skin from the application sites, kidney and liver of mice were harvested, and the concentration of radioactivity was determined with a radioactivity detector. Concentrations are presented as ng eq/g of TC-MTX. Blood samples taken at the time of sacrifice from controls, and 0.1% or 1% TC-MTX-treated CIA mice were examined for white blood cell (WBC) count, hemoglobin (Hb), platelet (PLT) count, and the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood nitrogen (BUN) and creatinine (Cr) to determine liver and kidney toxicity. To confirm the histopathological changes in the skin of CIA mice treated with TC-MTX, two pathologists and one dermatologist examined sections of joints; samples were blinded to prevent unconscious bias. Histological parameters included hyperkeratosis (thickening of the stratum corneum), parakeratosis, spongiosis and exocytosis and these parameters were scored as follows: 0 = normal; 1 = equivocal; 2 = mild; 3 = moderate; and 4 = severe [22].

2.10. Statistics

All statistical analyses were conducted using SPSS package for Windows, version 15 (SPSS Inc., Chicago, IL, USA). All results and measurements are expressed as the mean ± standard deviation. Statistical analysis of group differences was examined using the Mann-Whitney U test and a t-test. Correlations were calculated using Spearman’s correlation coefficient. *P < 0.05 was considered to be significant.
3. Results

3.1. Inhibition of DHFR activity by TC-MTX and its percutaneous delivery

To generate a TC-MTX that could facilitate the percutaneous delivery of MTX, we sequentially conjugated 6-amino cupric acid (ACA) and Hph-1-Protein Transduction Domain (PTD) to a carboxyl group of MTX. In TC-MTX, ACA, a small and flexible spacer, was inserted to allow MTX to interact with the binding pocket of dihydrofolate reductase (DHFR) without steric hindrance (Fig. 1A). To eliminate the concern that the chemical conjugation of MTX could influence the interaction between MTX and DHFR, the inhibitory potential of TC-MTX on DHFR activity was compared...
with that of free MTX using a DHFR assay kit. There are two carboxyl groups (α and γ positions) available for chemical conjugation in MTX. The γ form of TC-MTX inhibited DHFR activity more efficiently than the α form of TC-MTX and its inhibitory activity was comparable to that of free MTX. The half maximal inhibitory concentration (IC50) of TC-MTX was 813 nM, 51.7 nM and 23.6 nM, respectively (Fig. 1B). Therefore, the γ form of TC-MTX was chosen for the evaluation of the therapeutic potential of TC-MTX upon percutaneous delivery. To examine the skin-penetrating efficiency of TC-MTX, we conducted experiments using the dorsal skin of hairless mice and Franz cells [20]. The donor chamber was filled with 10 mg of TC-MTX dissolved in phosphate-buffered saline (PBS, pH 7.4) or 10 mg of MTX dissolved in Tris buffer (pH 10.8). The permeated amount in the receptor chamber was measured by high performance liquid chromatography (HPLC) at 5 h interval until 25 h. The skin-penetrating efficiency of TC-MTX was significantly higher than that of MTX (Fig. 1C). To test whether TC-MTX can be delivered into joints in vivo, we applied TC-MTX labeled with Cy5.5 on the skin of the paws and legs of mice and examined fluorescence in joint-sections at 3, 6 or 24 h after the application. While Cy5.5 was confined to the skin and muscle layers at 3 and 6 h, strong Cy5.5 fluorescence was detected in the periosteal and periarticular tissues at 24 h, thus demonstrating the time-dependent skin-penetrating efficiency of TC-MTX (Fig. 1D). Taken together, these data demonstrate that TC-MTX efficiently and specifically penetrate into the joints through the skin in vivo.

3.2. The severity of arthritis by TC-MTX in vivo

To assess the therapeutic activity of TC-MTX on arthritis, we applied TC-MTX (0.1%, 0.5% or 1%) or MTX percutaneously on the paws and legs of CIA mice, or intraperitoneally injected MTX at week 4, twice a week for 5 weeks. Controls were normal mice which did not received type II collagen. Macroscopic evidence of arthritis such as erythema or swelling was clearly observed in untreated and percutaneous MTX-treated CIA mice. In contrast, percutaneous TC-MTX significantly decreased both mean arthritis score and paw thickness in a dose-dependent manner (Fig. 2A). The histopathological evaluation of the joint-sections of untreated and percutaneous MTX-treated CIA mice revealed inflammatory cell infiltration, synovial hyperplasia, and partial bone destruction. However, in CIA mice percutaneously treated with TC-MTX, the severity of the observed pathological changes was significantly reduced (Fig. 2B and C). With these results, we conclude that TC-MTX can be delivered through the skin into the joints, where it can effectively inhibit inflammatory cell infiltration and markedly alleviate arthritis in CIA mice.

3.3. The therapeutic efficiency of TC-MTX analyzed by Micro-CT

We performed three-dimensional micro-CT imaging analysis to quantitatively investigate the level of bone alterations. Severe bone destruction was observed in untreated CIA mice, but TC-MTX-treated CIA mice exhibited the dose-dependent preservation of bone integrity; the examination of bones in 1% TC-MTX-treated CIA mice found them to be comparable to naïve mice without arthritis (Fig. 3A). Four parameters were analyzed: bone volume (BV), bone volume/tissue volume (BV/TV), bone surface areas adjusted to BV (BS/BV) and trabecular thickness (Tb.Th). The BV and BV/TV measurements allowed us to compare the bone samples of different sizes, when determining the extent of bone preservation. The BS/BV parameter reflects the loss of bone surface due to erosion. Tb.Th is inversely correlated with periarticular osteopenia induced by joint inflammation [23]. TC-MTX-treated CIA mice showed significantly higher BV, BV/TV, Tb.Th and lower BS/BV values in a dose-dependent manner compared to those in untreated mice (Fig. 3B). To clarify the validity of these parameters, we evaluated the correlation coefficients among four parameters, and found that Tb.Th was correlated with BV/TV and BS/BV (r2 = 0.757 and r2 = -0.698, p < 0.001, respectively), and that BV/TV was also well correlated with BS/BV (r2 = -0.925, p < 0.001) (Fig. 3C). With these results, we conclude that MTX can effectively alleviate arthritis and preserve bone volume and integrity in CIA mice. To clarify the validity of these parameters, we evaluated the correlation coefficients among four parameters, and found that BV/TV was almost equivalent to that of intraperitoneally injected MTX or controls (Fig. 4A and B). No positive immunohistochemistry was detected in the periosteal and periarticular tissues at 24 h, thus demonstrating the time-dependent skin-penetrating efficiency of TC-MTX (Fig. 1D). Taken together, these data demonstrate that TC-MTX efficiently and specifically penetrate into the joints through the skin in vivo.

3.4. Reduction of inflammatory cytokines by TC-MTX

In inflamed joints, the inflammatory cytokines TNF-α, IL-1β, and IL-6, and the joint-destructive enzymes iNOS and COX-2 are the pivotal messengers in the pathophysiology of RA [1,2,8]. To examine whether TC-MTX could modulate the expression of inflammatory cytokines and enzymes in inflamed joints, we performed immunohistochemistry on the joint-sections of CIA mice. The immunohistochemical analysis of the joint-sections of untreated and percutaneous MTX-treated CIA mice showed positive staining for TNF-α, IL-1β, IL-6, iNOS, and COX-2. In contrast, TC-MTX significantly reduced their expression in a dose-dependent manner. The extent of the expression of TNF-α, IL-1β, IL-6, iNOS, and COX-2 in CIA mice percutaneously treated with 0.5% or 1% TC-MTX was equivalent to that in CIA mice intraperitoneally injected with MTX or controls (Fig. 4A and B). No positive immunohistochemical staining was detected in joint-sections, when isotype-matched irrelevant antibodies were used as controls (Supplementary material, S1). The serum concentrations of inflammatory cytokines have a tendency to be proportional to the extent of joint inflammation in CIA mice [24,25]. To investigate whether TC-MTX could decrease serum levels of inflammatory cytokines, we measured the concentrations of TNF-α, IL-1β, IL-6 and IFN-γ in the serum of CIA mice percutaneously treated with 0.1% or 1% TC-MTX. TC-MTX significantly reduced serum concentrations of these cytokines in a dose-dependent manner. In comparison with untreated CIA mice, 1% MTX reduced concentrations of these inflammatory cytokines in the serum by over 70% (Fig. 4C). Taken together, TC-MTX can significantly decrease levels of inflammatory cytokines in the serum and in inflamed joints of CIA mice. Further, TC-MTX significantly reduced levels of joint-destructive enzymes within inflamed joints of CIA mice.

3.5. Comparison of therapeutic efficacy between TC-MTX and etanercept

To compare the in vivo therapeutic efficacy of TC-MTX to that of a TNF-α blockade, etanercept, we percutaneously applied 1% MTX or 1% TC-MTX on the paws and legs of CIA mice, and intraperitoneally injected etanercept (3.5 mg/kg) or MTX (35 mg/kg) into CIA mice, twice per week for 4 weeks for 5 weeks. We assessed arthritis score, paw thickness, and histopathological alterations in these mice. Significant joint inflammation was observed in untreated CIA mice or CIA mice percutaneously treated with MTX. Mean arthritis score and paw thickness results revealed that the therapeutic efficacy of 1% TC-MTX was almost equivalent to that of intraperitoneally injected-etanercept or -MTX (Fig. 5A). Also TC-MTX and etanercept significantly decreased histopathological alterations including inflammatory cell infiltration and bone erosion to similar
degrees (Fig. 5B and C). We conclude that the therapeutic efficacy of TC-MTX on arthritis in CIA mice is comparable to that of etanercept.

3.6. In vivo distribution and cytotoxicity of TC-MTX

To examine the distribution of percutaneously applied TC-MTX, we labeled the ACA linker with radioactive $^{14}$C, and then applied 5 mg/kg of radio-labeled TC-MTX on the skin of the shaved right thighs of mice. The concentration of radioactivity in the application site (skin), kidney, and liver was examined at different time points after administration. A high concentration of radioactivity was detected at the application site during the entire experimental period with peak levels at 48 h. In contrast, very low levels of radioactivity were detected in kidney and liver at 24 and 48 h, with no radioactivity being detected at 120 h (Table 1). We conclude that the majority of TC-MTX stays and exerts its effects at the application site, and its contribution to the systemic circulation is minimal. To evaluate the toxicity of TC-MTX in vivo, we percutaneously applied 0.1% or 1% TC-MTX on the paws and legs of CIA mice and examined parameters reflecting the functions of liver, kidney, and bone marrow. There were no differences in blood cell counts and hemoglobin (Hb) levels or in liver and kidney functions among controls, 0.1% and 1% TC-MTX-treated CIA mice (Fig. 6A).

To investigate the toxicity and the signs of skin irritation, various doses of TC-MTX was subcutaneously injected, or percutaneously applied was percutaneously applied or subcutaneously injected on the shaved skin of experimental animals, respectively. No toxicity including animal death or no significant irritation signs were observed (Supplementary material, S2). In addition, immunotoxicity was not detected in terms of skin sensitization, active systemic anaphylactic shock or passive cutaneous anaphylaxis in Guinea pigs (Supplementary material, S2). The genotoxicity of TC-MTX was not
observed in the bacterial reverse mutation assay (Supplementary material, S3), the chromosomal aberration test in cultured Chinese Hamster lung cells (Supplementary material, S4) or in the bone marrow micronucleus test in ICR male mice (Supplementary material, S5). Reproductive toxicity was not observed in studies of fertility, embryo-fetal development and pre- and postnatal development (data not shown). Histological changes were examined semi-quantitatively on the skin of CIA mice following the percutaneous administration of TC-MTX. No significant differences were observed in the histological scores among CIA mice percutaneously treated with 0.1% or 1% TC-MTX and controls (Fig. 6b). With these combined results, we conclude that TC-MTX does not induce serious systemic cytotoxic complications.

4. Discussion

MTX is a mainstay for the treatment of RA, but the oral administration of MTX involves risk of systemic and cytotoxic complications. There have been several efforts to develop skin-permeable MTX in order to diminish systemic complications [26,27]. However, no report has been published on the transcutaneous delivery of MTX into joints, or its therapeutic efficacy in an animal model of RA. In this study, we generated a skin-penetrating form of MTX (TC-MTX). The chemical conjugation of Hph-1-PTD and the spacer ACA to a carboxyl group of MTX significantly enhances the solubility of MTX, and the ACA spacer allows MTX to interact comfortably with the binding sites of the substrate without any steric hindrance. There are two carboxyl groups (α and γ positions) available for chemical conjugation in MTX; the α carboxyl group is important for the interaction of MTX to the binding site of DHFR. The poly-glutamation of MTX, which makes MTX toxic to the cells, frequently occurs at the γ position. In agreement with this notion, the inhibitory activity of γ form of TC-MTX on DHFR is comparable to free MTX and higher than the α form of TC-MTX.

The efficiency of the percutaneous delivery of TC-MTX was confirmed in experiments using Franz cells with the hairless mouse skin and human cadaver skin. A low level of MTX delivery was
Fig. 4. The level of inflammation-related factors was markedly reduced in inflamed joints and in the serum of CIA mice treated with TC-MTX. Reduced expression of inflammatory cytokines and joint-destructive enzymes in the affected joints and their serum concentrations in CIA mice treated with transcutaneous MTX (TC-MTX). TC-MTX (0.1%, 0.5% or 1%) was percutaneously applied twice per week for five weeks. Controls were normal mice which did not received type II collagen. Immunohistochemical staining for TNF-α, IL-1β, IL-6, iNOS or COX-2 in the joint-sections of CIA mice (original magnification ×100). (PC, percutaneous; TC, transcutaneous; IP, intraperitoneal) (A). The semi-quantitative analysis of the degree of stain-positivity of immunohistochemical staining (B). TC-MTX (0.1% or 1%) was percutaneously applied on CIA mice and serum concentrations of TNF-α, IL-1β, IL-6 or IFN-γ were measured (C). Values are given as mean ± SD, n = 5, asterisks represent significance compared to untreated mice with *p < 0.05.

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detected in the Franz cell experiment, but this was most likely due to the high pH Tris buffer used to dissolve MTX that damaged the skin layer. When fluorescence-labeled TC-MTX was applied on the joints of mice, it efficiently penetrated the skin in a time-dependent manner, reaching the joints within 24 h after administration. These results clearly demonstrate that TC-MTX can be percutaneously delivered ex vivo and in vivo, significantly improving the physi-chemical properties of MTX.

TC-MTX ameliorated the severity of arthritis in CIA mice, including marked improvement in the histopathological condition of the joints. Further, there was a significant reduction in inflammatory cell infiltration, synovial hyperplasia, and bone destruction; the decreased levels of TNF-α, IL-1β, IL-6, iNOS, and COX-2 in inflamed joint were also observed. The therapeutic efficacies of TC-MTX were similar to those observed in intraperitoneal MTX-treated CIA mice, in which a substantially higher than the suggested in vivo dose of MTX was injected. Moreover, the dose-dependent reduction in the severity of arthritis by TC-MTX provides additional evidence that the improvement was mediated by the pharmacologic action of MTX.

To confirm our results, we measured BV, BV/TV, BS/BV, and Tb.Th, four parameters reflecting the degree of bone destruction and periarticular osteopenia, using highly quantitative micro-CT and three-dimensional reconstruction [28–30]. The typical radiological findings in RA are bone erosions and periarticular osteopenia [31]. The level of bone destruction and periarticular osteopenia due to joint inflammation in CIA mice was quantitatively well correlated with these parameters, supporting the validity of three-dimensional micro-CT. Consistent with our in vivo data shown in

**Fig. 5.** Comparison of therapeutic efficacy between transcutaneous MTX (TC-MTX) and etanercept. 1% MTX or 1% TC-MTX were applied on the paws and legs of CIA mice, and etanercept (5.5 mg/kg) or MTX (35 mg/kg) were intraperitoneally injected into CIA mice on day 28 after primary immunization (red ▼), twice per week for five weeks. Controls were normal mice which did not received type II collagen. Macroscopic evidence of arthritis such as erythema or swelling was assessed by arthritis score and paw thickness. (PC, percutaneous; IP, intraperitoneal) (A). Histological findings of the joint-sections were evaluated (original magnification ×100) (B). The semi-quantitative analysis of histological findings of the inflamed joints such as infiltrate score and erosion score (C). Values are given as mean ± SD, n = 5, asterisks represent significance compared to untreated mice with p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**

<table>
<thead>
<tr>
<th>Radioactivity concentration in tissues after a single application of 14C-transcutaneous MTX (TC-MTX) on the skin of non-fasting male rats (dose: 5 mg/kg).</th>
<th>Radioactivity concentration (ng eq. of TC-MTX/g tissue)</th>
</tr>
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<tbody>
<tr>
<td>Tissue</td>
<td>1 h</td>
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<tr>
<td>Application site</td>
<td>13,213</td>
</tr>
<tr>
<td>Kidney</td>
<td>BLD</td>
</tr>
<tr>
<td>Liver</td>
<td>BLD</td>
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Upper limit of quantification was 79,045 ng eq. of TC-MTX/g tissue and lower limit of quantification was 40 ng eq. of TC-MTX/g tissue. BLD, below the limit of detection.
Figs. 2 and 4, TC-MTX improved all measured parameters in a dose-dependent manner, and the extent of bone preservation by 1% TC-MTX was comparable to that observed in controls. Biological agents, such as TNF-α blockades or anti-CD20 monoclonal antibody, have been demonstrated to have therapeutic efficacy in RA and are widely used [13,14]. However, they also can induce serious systemic complications. The use of these biological agents must be limited in areas endemic for tuberculosis and viral hepatitis, or in patients suffering from these diseases. In this study, we compared the therapeutic efficacy of TC-MTX to that of etanercept, one of the most widely used biological agents for RA treatment. We found that TC-MTX was comparable to etanercept in the alleviation of arthritis in CIA mice. With these results, we concluded that TC-MTX has similar therapeutic potential to etanercept and it can be safely used in RA patients who have systemic diseases or conditions where the use of biological agents is limited.

In this study, most TC-MTX remained at the application site with significantly low levels of TC-MTX being detected in kidney and liver. In line with these results, systemic complications including genotoxicity, immunototoxicity and skin irritation were not observed. The high toxicity of MTX is caused by the formation of polyglutamate complexes through the carboxyl group of MTX and the prolonged retention of poly-glutamated MTX in the cytoplasm [32,33]. Hph-1-PTD and ACA occupy the γ-carboxyl group of MTX, where poly-glutamation occurs, leading to the decreased formation of poly-glutamated MTX. The significant reduction of toxicity and the rapid excretion of TC-MTX that was observed was most likely due to enhanced solubility and decreased poly-glutamation of TC-MTX as compared to MTX.

The results of our studies have three important clinical implications in the treatment of RA patients. First, TC-MTX can provide an equivalent level of therapeutic potential to that of oral MTX and etanercept, as well as an opportunity to minimize the circulating concentration of MTX in RA patients who have diseases or conditions that MTX treatment is not feasible such as liver diseases or interstitial lung diseases [11,12]. Second, we observed that most TC-MTX remained at the application site without significant skin irritation, histological changes, or dysfunction in major organs. Thus, it may relieve the concern of rheumatologists that the long-term systemic use of MTX might result in serious complications in RA patients who have refractory small joint inflammation. Third, because TC-MTX allows a high dose of MTX to directly and rapidly delivery, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.10.079.
enter the joint cavity, it can reduce the lag-time to the onset of its therapeutic action. Therefore, TC-MTX will reduce irreversible joint destruction and allow better functional status without the concerns of the systemic complications of MTX.

5. Conclusion

Our study demonstrates that TC-MTX can be percutaneously delivered to inflamed joints of CIA mice with similar therapeutic efficiency to that of intraperitoneally injected MTX and -etanercept without the complications associated with the systemic administration of MTX. Thus, TC-MTX may be a new therapeutic modality for RA patients with refractory small joint inflammation or systemic diseases where the use of MTX is unavailable. Furthermore, we expect that TC-MTX can be effectively used to treat other autoimmune inflammatory diseases such as psoriasis, where MTX is one of the first-line drugs.

Acknowledgments

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.10.079.

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