Chapter V

Involvement of Deoxyadenosine and Adenosine Deaminase in the Methotrexate-Induced Suppression of Inflammatory Bone Destruction[*]

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Abstract

Methotrexate (MTX) is an anti-tumor medicine classified into the anti-folate drugs. MTX suppresses DNA synthesis through inhibiting

dihydrofolate reductase (DHF) of *de novo* nucleic acid pathway, which suppresses proliferation of malignant tumors. MTX is also utilized as for anti-rheumatic medicine as the lower doses of MTX are able to suppress effectively the inflammation of the synovial tissues in RA patients. In our previous study, plasma adenosine level was markedly increased during induction of inflammation in rats with adjuvant-induced arthritis (arthritic rats). MTX-induced suppression of osteoclastogenesis was canceled by the addition of adenosine *in vitro* as well as *in vivo*. Deoxyadenosine (dAdo) is known to regulate proliferation of immune cells and partly share metabolic pathways with adenosine. Adenosine deaminase (ADA) is a key enzyme which metabolize both of adenosine and dAdo to produce inosine and deoxyinosine perpetually. Here we examined the regulatory role of dAdo in MTX-induced suppression of inflammatory bone destruction and investigated a possible involvement of ADA as a target molecule of MTX. In rats with adjuvant-induced arthritis, in whole bone marrow cultures for evaluating osteoclastogenesis, MTX markedly suppressed osteoclastogenesis and dAdo completely canceled its suppression. This cancellation was partially blocked by caffeine, antagonist for the adenosine receptors, A_1AR, $A_{2a}AR$ and A_3AR. Semi-quantitative RT-PCR showed that MTX suppressed expression of RANKL without affecting osteoprotegerin (OPG) expression. Addition of dAdo clearly recovered the expression of RANKL and slightly suppressed OPG expression, which result in augment in osteoclastogenesis. In arthritic rats, MTX-induced suppression of inflammatory bone destruction was completely cancelled by the injection of dAdo. A marked induction of ADA was apparent in bone marrow cells observed in the bone destruction sites around ankle joints in arthritic rats. MTX strikingly suppressed expression of ADA in the corresponding area. Immunoblot analysis also showed a clear induction of ADA protein in arthritic rats and a marked suppression of ADA protein when arthritic rats were treated with MTX. In bone marrow cultures for evaluating osteoclastogenesis, expression of ADA mRNA was markedly suppressed by MTX and dAdo partially cancelled its inhibition. These observations suggest that ADA is involved in the MTX-mediated inhibition of inflammatory bone destruction regulated by adenosine and dAdo. Suppression of ADA by MTX may contribute to the stability of dAdo.

A list of abbreviation: AA, adjuvant-induced arthritis; ADA, adenosine deaminase; AR, adenosine receptor; CFA, complete Freund adjuvant; dAdo, deoxyadenosine; MNC, multinucleated cells; MTX, methotrexate; RA, rheumatoid arthritis; RANK, receptor activator NF-kB ligand, TRAP, tartrate acid-resistant phosphatase.

Keywords: ADA, Adjuvant-induced arthritis, adenosine receptor, Deoxyadenosine, MTX, Osteoclastogenesis

Rheumatoid arthritis (RA) is an inflammatory disease with chronic inflammation in joints which often associates with severe bone destruction [1, 2]. Inflammatory bone destruction is generally accompanied with abnormal increase in osteoclastogenesis in rheumatoid arthritis as well as in periodontal diseases [3].

Osteoclastogenesis is basically controlled by the differentiation factor, the receptor activator NF-κB ligand (RANKL) and the inhibitory factor, osteoprotegerin (OPG) [4]. Inflammatory cytokines are also involved in the pathological augments in osteoclastogenesis [1, 2].

Methotrexate (MTX), a monomethylated form of Aminopterin, is widely utilized as an anti-inflammatory medicine. MTX and Aminopterin have been utilized as the effective anti-tumor drugs as these drugs suppress DNA synthesis through inhibiting the dihydrofolate reductase, a key enzyme required for *de novo* synthesis of nucleic acids [5]. In the area of bone-related diseases, low dose of MTX is utilized to suppress articular inflammation in RA patients [6-9]. However, it has also been recognized that MTX is ineffective to some RA patients [10, 11]. It is likely that MTX suppresses abnormal proliferation of cells present in the synovial membrane observed in RA patients, however, precise target molecule of MTX is ambiguous. Therefore, so far, no one knows the detailed molecular entity of the resistance against MTX.

Intracellular adenosine is the precursor molecule for ATP. Extracellular adenosine is recognized as an important regulator of inflammation [12]. Generally, adenosine is formed from the extracelluar ATP, which is mainly released from apoptotic cells, by a sequential action of cell surface phosphatases, CD39 and CD73 [13].

Extracellular adenosine acts as an inflammatory mediator through cell surface receptors for adenosine. So far four types of adenosine receptors (ARs), A_1AR, $A_{2a}AR$, $A_{2b}AR$, and A_3AR have been identified. We have detected a rapid increase in the plasma adenosine level during the incidence of adjuvant-induced arthritis in rats [14]. Plasma adenosine level correlated with the severity of arthritis in rats. We have also shown that MTX-induced suppression of osteoclastogenesis is canceled by the administration of adenosine through $A_{2b}AR$, the low affinity AR and that MTX suppressed osteoclastogenesis by suppressing the level of RANKL without affecting OPG production [14].

The level of OPG expression was suppressed without affecting the level of RANKL expression when MTX-suppressed cultures were treated with

adenosine, which resulted in the increase in the ratio of RANKL/OPG and augment in osteoclastogenesis.

MTX-treatment seems to have some pivotal relationship with the metabolism of nucleic acids, however, target molecules concerning the MTX-induced suppression of inflammatory bone destruction remains unclear. In our previous study, we have found that deoxyadenosine (dAdo) is the only deoxynucleoside which clearly canceled MTX-induced suppression of osteoclastogenesis [14].

As dAdo is known to regulate proliferation of immune cells and partly share metabolic pathways with adenosine. Adenosine deaminase (ADA) is a key enzyme which metabolize both of adenosine and deoxyadenosine to produce inosine and deoxyinosine perpetually. Deficiency in ADA activity results in a marked elevation of intracellular level of these nucleosides to cause death of immune cells, which leads to the incidence of immune deficiency.

Here we investigated on a possible involvement of ADA in the regulation of MTX- induced suppression of osteoclastogenesis in rats with adjuvant-induced arthritis (arthritic rats). We also examined a possible regulatory role of dAdo, another substrate for ADA, in the MTX-induced suppression of inflammatory bone destruction.

We have obtained lines of evidence suggesting that ADA is one of the target molecules for MTX. MTX-induced suppression of inflammatory bone destruction observed in arthritic rats was clearly canceled by the administration of dAdo.

Materials and Methods

Materials

α-Minimum Essential Medium (αMEM; cat#12000-022) and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY). RANKL was purchased from Peprotech EC (London, UK). 1α, 25-dihydroxy vitamin D_3 (1α, 25(OH)$_2$ D_3) was from Biomol Research Laboratories (Plymouth Meeting, UK). MTX, deoxyadenosine, caffeine and MRS1754 were obtained from Sigma (St.Louis, MO). Heat-killed *Mycobacterium butyricum* and mineral oil were obtained from Difco Laboratories (Detroit, MI). Anti-adenosine deaminase antibody (H-300) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Second antibody: Alexa Fluor® 488 Goat

Anti-Rabbit IgG (H+L) for immunofluorescence was obtained from Life Technologies Corporation (Tokyo, Japan). Peroxidase-conjugated anti-rabbit IgG antibody and enhanced chemiluminescence (ECL) kits were purchased from Amersham Biosciences (Buckinghamshire,UK)

Animal Experiments and Ethics Committee Approval

Sprague Dawley (SD) rats and Lewis rats were obtained from Kyudo Co. Ltd. (Tosu, Japan). All animal experiments were performed according to the guide-lines for "Care and Use of Animals of Kyushu University.

Bone Marrow Culture System

Formation of osteoclast-like multinucleated cells (MNCs) from rat bone-marrow cells was performed as described previously [15, 16]. Briefly, bone-marrow cells were obtained from the tibia and femur of 4-6 weeks old male SD rats. Cells were seeded into 24-well culture plates (10^6 cells/well) and cultured in αMEM containing 15% FBS in the presence of 10^{-8}M 1α, 25(OH)$_2$ D $_3$ and 10% (V/V) heat-treated ROS17/2.8 cell conditioned medium (ht-ROS CM). After 4 or 5 days of culture in the presence of various concentrations of MTX, cells were fixed with acetone-citrate-formaldehyde, and stained for TRAP using a Leukocyte acid phosphatase kit. TRAP-positive cells containing more than three nuclei were counted as osteoclast-like multinucleated cells (MNCs). Osteoclast were also detected by staining with osteoclast-specific anti-Kat1-antigen monoclonal antibody [17, 18]. For stromal cell-free cultures, bone marrow stromal cells were depleted by Sephadex G-10 (GE Healthcare, Uppsala, Sweden) column as described previously [15]. Non-adherent bone marrow cells were cultured in the presence of 10^{-8} M 1 α, 25(OH)$_2$ D $_3$, 20 ng/ml sRANKL and 10% (V/V) ht ROSCM. Cells were cultured in 96-well culture plates (3 $\times 10^5$cells/well) for 4 days. Cells were fixed and stained for TRAP.

Induction of Adjuvant Arthritis in Lewis Rats and μCT Analysis

Rats with adjuvant-induced arthritis was prepared as described previously [14, 18]. Briefly, 5 weeks-old female Lewis rats were intradermally injected at

the base of the tail with complete Freund adjuvant (CFA) consisting of 25mg/kg heat-killed *M. butyricum* (Difco Laboratories, Detroit, MI) suspended in mineral oil. In control experiments, rats were injected with mineral oil alone. All animals were anesthetized with diethyl ether or isoflurane prior to adjuvant injections. MTX (1mg/kg/week) or vehicle (phosphate buffered saline, PBS) was injected intraperitoneally. These injections were performed once a week beginning at 3 days after the adjuvant injection. Deoxyadenosine or vehicle (PBS) was injected into the ankle-joint cavities of CFA-injected rats. Thirty-gauge needles were used to inject 80 µg dAdo (in 30 µL PBS) around the articular cavity of the left ankle joint. As an internal control, 30 µL PBS was injected around the right ankle joint. Injections were performed every 3 days beginning on the same day as MTX-injections (3 days after the adjuvant injection). The rats were sacrificed on day 21 after the first injection of adjuvant of the same rat. The level of bone destruction of hind paws was imaged by using a µCT analysis system (SKYSCAN-1076KHS Inc., SkyScans, Kontich, Belgium) and SKYSCAN software.

Histological Analysis

After fixation by perfusion with 4% ($^{W}/_{V}$) paraformaldehyde/PBS, tissue blocks were taken from the hind paws (tarsal bones and tibia) of Lewis rats and immersed in the same fixative for overnight at 4 ℃, followed by washing in PBS overnight at 4 ℃. After decalcification in 10% (W/V) ethylene diamine tetraacetate (EDTA) for 3 weeks at 4 ℃ and dehydration in ethanol series, the tissue blocks were embedded in paraffin. Sections (6µm) of ankle joints including tibia (*Ti*) and talus (*Ta*) were also stained for TRAP or hematoxylin-eosin (HE).

Semi-quantitative RT-PCR

Total cellular RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and subjected to semi-quantitative RT-PCR using RT-PCR kit (Takara, Kyoto, Japan) according to the manufacturer's protocol. The primers used for PCR were as follows: rat adenosine deaminase forward, 5'-TCC TGG CCA AGT TCG ATT AC-3'; rat adenosine deaminase reverse, 5' - GCC CTG GTT TAC AAG ATC CA-3';

rat adenosine A_1 receptor forward, 5'- CAT TGG GCC ACA GAC CTA CT-3'; rat adenosine A_1 receptor reverse, 5'- CAA GGG AGA GAA TCC AGC AG-3'; rat adenosine A_{2a} receptor forward, 5'-TAC CTG GCC ATC ATT CTC TC-3'; rat adenosine A_{2a} receptor reverse, 5'-TGG GTA GGA AGC TCC ACA TC-3'; rat adenosine A_{2b} receptor forward, 5'-CTT CTG CAC GGA CTT TCA CA-3'; rat adenosine A_{2b} receptor reverse, 5'- GGT GGC ACG GTC TTT ACT GT-3'; rat adenosine A_3 receptor forward, 5'- CGG CTT GGA TTA CAT GGT CT-3'; rat adenosine A_3 receptor reverse, 5'-CAG CAC AAG GCA AAC AAG AA-3'; rat RANKL forward, 5'-CGT TTG CTC ACC TCA CAA TC-3'; rat RANKL reverse, 5'-ACG CTA ATT TCC TCA CCA GC-3'; rat OPG forward, 5'-ATT GGC TGA GTG TTC TGG TG-3'; rat OPG reverse, 5'-GCC CAG TGA CCA TTC CTA AC-3'; rat NFATc1 forward, 5'- CAA CGC CCT GAC CAC CGA TAG-3'; rat NFATc1 reverse rat, 5'-GGC TGC CTT CCG TCT CAT AGT-3' ; rat ADA forward, 5'-TCCTGGCCAAGTTCGATTAC-3', rat reverse, 5'-GCCCTGGTTTACAA-GATC CA-3'; GAPDH forward, 5'-GGT GAT GCT GGT GCT GAG TA-3'; rat GAPDH reverse, 5'-ACT GTG GTC ATG AGC CCT TC-3'. The PCR products were subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining with UV light illumination.

Western Blotting

Areas of ankle joint containing distal tibia and talus were dissected from Lewis rats and were frozen immediately in liquid nitrogen. Frozen tissues were ground into powder under liquid nitrogen using small mortar and pestle. Then the total proteins were extracted in lysis buffer containing 20 mM Tris-HCl (pH7.2), 10 mM EDTA, 0.3 M NaCl, 0.1% Triton X-100, 0.05%Tween 20 and protease inhibitor cocktail (Sigma) overnight at 4 ℃. The lysates, containing equal amounts of proteins, were applied on 13% SDS–PAGE followed by transfer to nitrocellulose membranes. After blocking with 5% nonfat dry milk, the membranes were probed with anti-ADA rabbit polyclonal antibody and then reacted with peroxidase-conjugated anti-rabbit IgG antibody followed by detection with ECL kit. The Same membranes were stripped and reprobed with anti-GAPDH.

Immunofluorescence Staining

Deparaffinized sections (6μm) were blocked with 10% normal goat serum for 1 hr at room temperature. These sections were incubated with anti-ADA rabbit polyclonal antibody (H-300) diluted with 1% normal goat serum (1:200 dilution) in a humidified chamber for overnight at 4℃. After washing with PBS, sections were incubated with goat anti-rabbit IgG (H+L) (A11034) conjugated with Alexa Fluor® 488 diluted with 1% normal goat serum (1:500) for 1 h at room temperature. After rinsing, observation was performed by a fluorescence microscope (Carl Zeiss; Axiovert 100, Germany).

Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was performed using Student's *t*-test. Data shown are representative results from three independent experiments.

Results

Deoxyadenosine Canceled MTX-induced Suppression of Osteoclastogenesis

In rat whole bone marrow cultures, formation of TRAP-positive MNCs was markedly suppressed by MTX. This suppression was completely abolished by the addition of dAdo in a dose-dependent manner, as shown in (Figure 1a and b). MTX markedly inhibited the formation of MNCs expressing Kat1-antigen, a specific marker for rat osteoclasts, and addition of dAd completely abolished its suppression in the formation of Kat1-antigen-positive osteoclasts (Figure 1c).

When bone marrow cells were cultured in the absence of MTX, addition of dAdo did not affect osteoclastogenesis (Figure 1d). When stromal cell-free non-adherent bone marrow cells (NABMCs) were utilized, neither MTX-induced suppression nor dAdo-mediated recovery of osteoclastogenesis was observed (Figure 1e). These data suggest that MTX-induced suppression and cancellation by deoxyadenosine in osteoclastogenesis was mediated through marrow stromal cells.
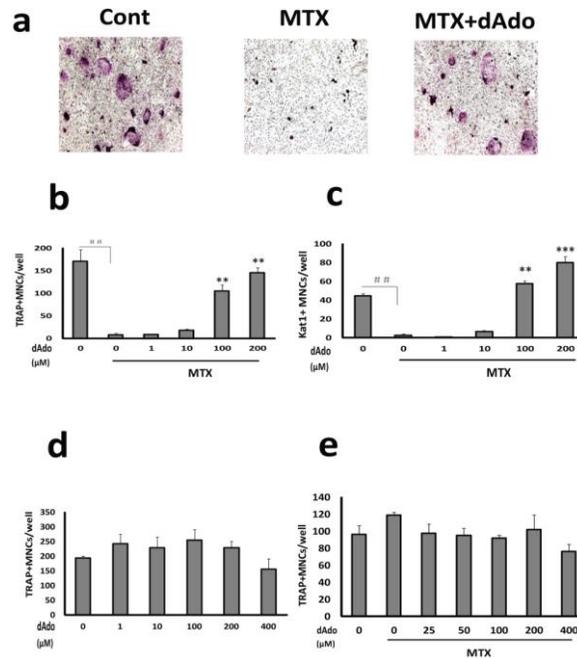
Figure 1. Deoxyadenosine canceled MTX-induced suppression of osteoclastogenesis. (a-c) Abolishment of MTX-induced suppression of osteoclastogenesis by dAdo. Bone-marrow cells were cultured for forming osteoclasts in 24-well culture plates (10^6 cells/well) as described in MATERIALS AND METHODS with or without 1μM of MTX in the presence of various concentrations of dAdo. After 4 days of culture, the cells were stained for TRAP (a,b) or for Kat1-antigen (c) as described in MATERIALS AND METHODS. (a) Microscopic observation of the TRAP-positive MNCs. Cont: control in the absence of MTX, MTX: in the presence of 1μM MTX, MTX+dAdo: in the presence of 1 μM MTX and 200 μM dAdo. (b,c) Abolishment of MTX-induced suppression of osteoclastogenesis by dAdo in a dose-dependent manner. Number of TRAP-positive MNCs were counted (b) and that of Kat1-positive MNCs were counted (c). Data present means±SEM from four cultures (n=4). Data were analyzed by Student's *t*-test * $p<0.05$, ** $p<0.01$, *** $p<0.001$ if compared with the control culture in the absence of MTX. # $p<0.05$, ## $p<0.01$ if compared among indicated pairs. (d) Effect of dAdo on osteoclastogenesis in the absence of MTX. (e) MTX–induced suppression of osteoclastogenesis was dependent on bone marrow stromal cells. For stromal cell-free cultures, bone marrow stromal cells were depleted by Sephadex G-10 (GE Healthcare, Uppsala, Sweden) column as described in MATERIALS AND METHODS. The stromal cell-free bone marrow cells were cultured in 96-well culture plates (3×10^5 cells/well) in the presence of various concentrations of dAdo with or without 1μM MTX. After 4 days of culture, the cells were fixed and stained for TRAP. Data present means±SEM from four cultures. Data were analyzed by Student's *t*-test * $p<0.05$, ** $p<0.01$, *** $p<0.001$ if compared with the control culture in the absence of MTX.

Recovery Effect of dAdo was Partially Prevented by Caffeine, Antagonist for High Affinity Adenosine Receptors

To investigate a possibility that ARs are involved in the abolishment of MTX-induced suppression of osteoclastogenesis by dAdo, we assessed the effects of adenosine receptor antagonists (Figure 2). MTX-induced suppression of osteoclastogenesis was partially blocked by the addition of caffeine, an antagonist for the high affinity adenosine receptors, A_1AR, $A_{2a}AR$ and A_3AR (Figure 2a).
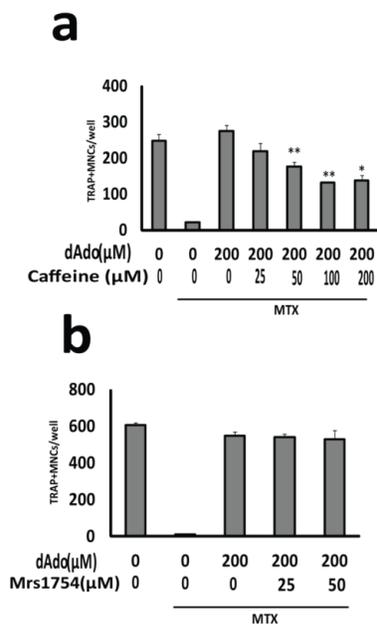

Figure 2. The recovery effect of dAdo was partially blocked by caffeine but not by MRS1754. Bone-marrow cells were cultured for forming osteoclasts in 24-well culture plates (10^6cells/well) with or without MTX in the present or absence of 200μM dAdo. These cultures were treated with various concentration of caffeine (a) or MRS1754 (b). After 4 days of culture, cells were fixed and stained for TRAP and TRAP-positive MNCs were counted. Data present means±SEM from four cultures. Experiments were performed 3 times and the representative data were shown. Data were analyzed by Student's *t*-test. [###]p<0.001 if compare with the control culture in the absence of MTX. *P<0.5, **P<0.01, ***P<0.001 if compared with culture treated with dAdo in the absence of caffeine (for (a)) or MRS1754 (for (b)).

However, as shown in (Figure 2b), MRS1754, the selective antagonist for the low affinity receptor $A_{2b}AR$ did not affect this canceling effect of dAdo

from MTX-induced suppression of osteoclastogenesis (Figure 2b). These data suggested that dAdo-mediated abolishment of MTX suppression is partly mediated through high affinity adenosine receptors, A_1AR, $A_{2a}AR$ or A_3AR.

Deoxyadenosine Significantly Abolished the Therapeutic Effects of MTX on Inflammatory Bone Destruction in Arthritic Rats

Arthritic rats were sacrificed on day 21 after the first injection of adjuvant with or without MTX- treatment. The level of bone destruction in the hind paws injected with or without dAdo was imaged by μCT analysis. As shown in (Figure 3a), MTX markedly suppressed inflammatory bone destruction in arthritic rats. Injection of dAdo around the ankle-joint cavities of arthritic rats completely diminished the therapeutic action of MTX on inflammatory bone destruction (Figure 3a). TRAP-staining data clearly demonstrated that MTX markedly suppressed osteoclastogenesis in the distal tibia and talus of arthritic rats and that dAdo-treatment completely abolished the suppressive effect of MTX in osteoclastogenesis *in vivo*. Quantitative μCT analysis of the talus was shown concerning four parameters: percent bone volume (BV/TV) (%), trabecular thickness (Tb.Th) (mm), trabecular number (Tb.N/mm) and trabecular separation (Tb.Sp) (mm). As shown in Figure 3b, μCT analysis clearly showed that the marked bone loses observed in arthritic rat was significantly suppressed by MTX-treatment and that injection of dAdo around the articular joints completely cancelled MTX-induced suppression.
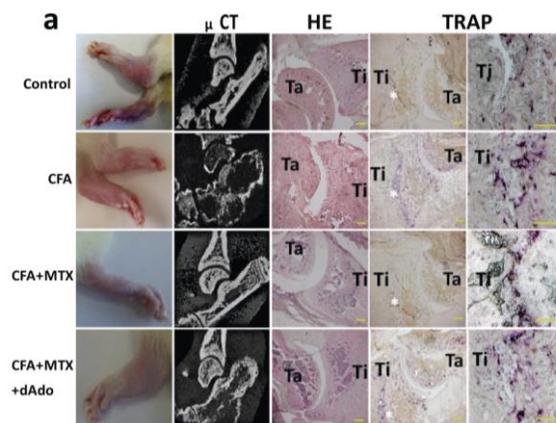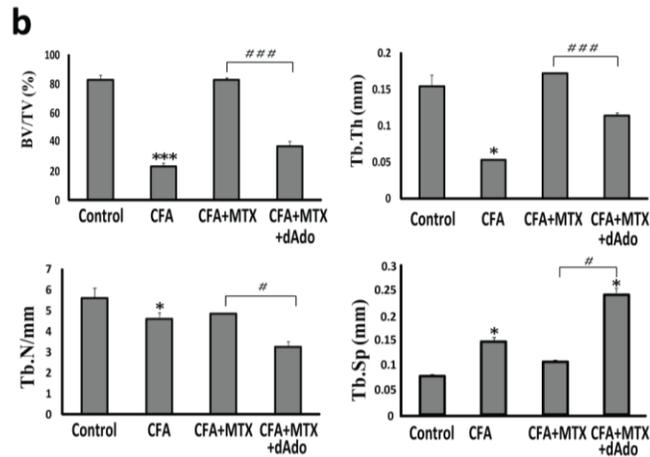

Figure 3. (Continued).

Figure 3. Deoxyadenosine significantly abolished the therapeutic effects of MTX on inflammatory bone destruction in rats with adjuvant-induced arthritis.
(a) MicroCT analysis and histochemical analysis: Arthritic rats were induced by the injection of complete Freund's adjuvant (CFA) as described in MATERIALS AND METHODS. Arthritic rats were treated with MTX (1mg/kg/week) (CFA+MTX) or MTX plus dAdo (CFA+MTX+ dAdo). As the control, rats were injected mineral oil alone (Control). After analyzing the ankle joints by use of μCT (μCT), bone tissues were processed for histological analysis. Paraffin sections (6μm) were prepared and stained for hematoxylin eosin (HE) or for TRAP activity (TRAP). (In TRAP staining data, the right panels are the high magnification view of the left panel). Ti: tibia, Ta: talus. Bars 50 μm. (b) Quantitative demonstration of μCT analysis: The talus was analyzed by μCT concerning the four parameters: Percent bone volume (BV/TV) (%), trabecular thickness (Tb.Th)(mm), trabecular number (Tb.N)/mm and trabecular separation (Tb.Sp)(mm). Data present mean$\pm$SEM for four rats. *p<0.05, ***p<0.001 if compared with control (Control). #P<0.05, ###P<0.001 if compared among indicated pairs.

Suppression of RANKL Expression Mediated by MTX Was Cancelled by dAdo

In bone marrow cultures for evaluating osteoclastogenesis, MTX-induced suppression of osteoclastogenesis was canceled by the addition of dAdo (Figure 1a). In the same condition, the total RNA was extracted and analyzed by semi-quantitative RT-PCR (Figure 4). MTX did not affect expression of OPG mRNA expression but markedly suppressed expression of RANKL

and NFATc1. Addition of dAdo almost completely recovered expression of RANKL and NFATc1. Furthermore, dAdo treatment inhibited expression of OPG mRNA. Therefore dAdo-treatment markedly increased the expression-ratio of RANKL/OPG (Figure 4a). We have examined the expression of ARs. Although MTX did not affect the expression of mRNA for A_1AR and $A_{2b}AR$, MTX augmented expression of mRNA for $A_{2a}AR$ and slightly increased expression of A_3AR (Figure 4b). The level of mRNA expression of ARs were not affected by the administration of dAdo.
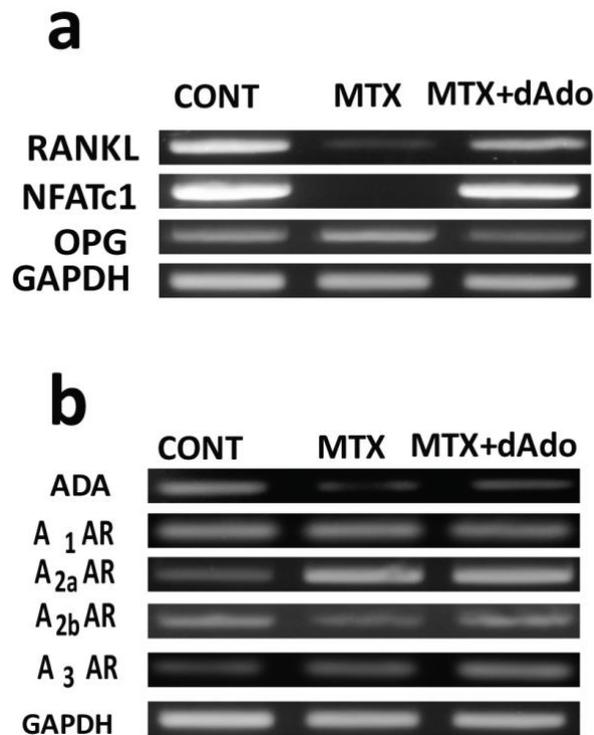

Figure 4. Semi-quantitative RT-PCR analysis of gene expression concerning osteoclastogenesis and adenosine metabolism. Bone marrow cells were cultured for forming osteoclasts as described in MATERIALS AND METHODS with or without 1μM MTX (+MTX) in the presence or absence of 200μM dAdo. Total RNAs were extracted and semi-quantitative RT-PCR was performed using primer sets for RANKL, NFATc1, OPG, GAPDH (a) or those for ADA, A_1AR, $A_{2a}AR$, $A_{2b}AR$, A_3AR and GAPDH (b). PCR products were analyzed by 2% agarose gels.
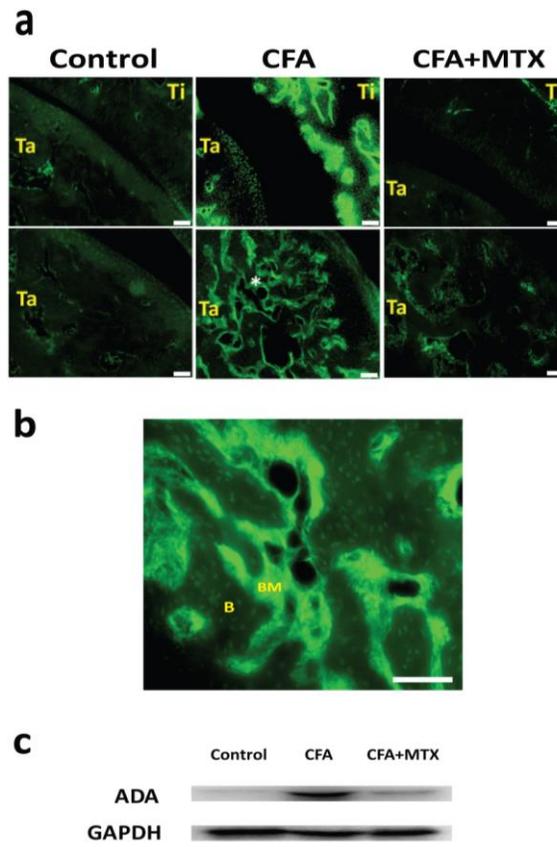
Figure 5. MTX inhibits ADA expression in bone destruction sites of arthritic rats.
(a) Immunohistochemical analysis. Hind paw-joint sections of control rats (Control),
arthritic rats (CFA), arthritic rats injected with MTX (CFA+MTX) were incubated with
anti-ADA polyclonal antibody (rabbit) followed by detection with anti-rabbit IgG
conjugated with AlexaFluor 488 as described in MATERIALS AND METHODS.
Upper panels are the immunofluorescence observation of the ankle joint. Lower panels
are the immunofluorescence view of talus. Ti: tibia, Ta: talus. Bars: 50μm. (b)
Detection of intense ADA immunoreativity in bone marrow area in arthritic rats. The
high magnification view of the asterisk area of the lower central panel of (a). B:bone,
BM: bone marrow. Bars 50 μm. (c) Western blot analysis. Protein samples of the area
of the ankle joints containing distal tibia and talus were prepared from control rats
(Control), arthritic rats (CFA), arthritic rats injected with MTX (CFA+MTX) as
described in MATERIALS AND METHODS. The cell lysates containing equal
amounts of proteins were subjected to 13% SDS-PAGE followed by immunoblotting
as described in MATERIALS AND METHODS. Membrane sheets were reacted with
anti-ADA antibody (ADA) or anti-GAPDH antibody (GAPDH).

MTX Suppressed Expression of ADA in Adjuvant-Induced Arthritis

Our previous report showed that MTX markedly suppressed osteoclastogenesis and adenosine cancels its suppression [14]. As ADA is a key enzyme regulating the intracellular level of adenosine and dAdo, we have examined the expression of ADA in MTX-induced suppression of bone destruction. To know whether ADA is involved in the mechanisms of MTX-mediated inhibition of inflammatory bone destruction, we have investigated the expression of ADA in the joint tissue of the arthritic rats by use of immunofluorescence methods.
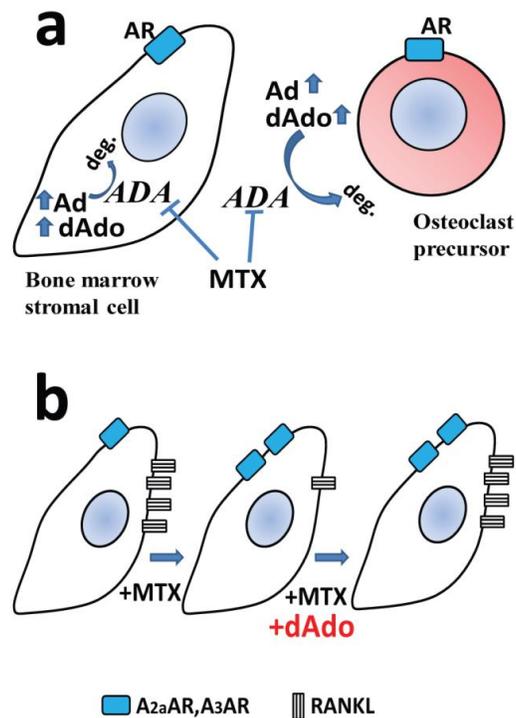

Figure 6. A possible Involvement of ADA and dAdo in the regulation of MTX-induced suppression of osteoclastogenesis: Illustration diagram. (a) Inhibition of ADA-expression by MTX. (b) MTX-induced suppression of RANKL- expression was canceled by dAdo.

As shown in Figure 5, immunoreactivity of ADA was quite intense in bone destruction area of arthritic rats, while control rats showed minimum level of ADA expression. High magnification view of ADA-positive area of arthritic rats showed an intense immunoreactivity in bone marrow area. A diffuse staining pattern was observed in the bone marrow area, in which not only bone marrow cells but also extra-cellular spaces were stained. MTX-treatment markedly reduced the staining level with anti-ADA antibody in the corresponding area. Western blotting analysis confirmed a clear induction of ADA protein in arthritic rats and a marked suppression of ADA by MTX (Figure 5c). Semi-quantitative RT-PCR using bone marrow cultures for evaluating osteoclastogenesis showed that the expression of ADA mRNA was detected in cultures for evaluating osteoclastogenesis and it was markedly suppressed by MTX treatment. MTX-induced suppression of ADA gene expression was slightly canceled by the addition of dAdo, although its expression level was still significantly suppressed.

Discussion

In the current study, we have demonstrated a marked induction of ADA in the bone destruction sites of arthritic rats. As a diffuse immunoreactivity of ADA was observed in the bone marrow area, ADA seems to be localized not only in the producer cells but also in the extracellular spaces of the bone marrow cavity in arthritic rats. Nakamachi et al. [19] reported the specific increase in the enzyme activity of ADA in rheumatoid synovial fibroblasts. Our finding concerning a high level of protein in bone marrow cavity is supposed to be related to a direct function of ADA in bone metabolism [20]. MTX-treatment markedly suppressed expression of ADA in the current study, which was also confirmed by immunobloting. Our findings clearly demonstrated an involvement of ADA in the regulation of inflammatory bone destruction. MTX is reported to increase extracellular adenosine concentration [6, 21]. Suppression of ADA expression by MTX is supposed to result in a marked accumulation of adenosine and dAdo. In this condition, exogenously administered dAdo is supposed to be markedly accumulated without degradation as the level of ADA is maintained in a very low level by MTX. In patients with ADA-deficiency combined with severe immune-deficiency disease, it has been reported that adenine deoxynucleosides and deoxynucleotides are overproduced in these patients [22]. Erer et al. [23]

reported that serum ADA levels in RA patients are found to be higher than healthy controls. Their clinical observations are well consistent with findings in the current study in which we have detected abundant ADA in the bone destruction sites of arthritic rats.

By use of marrow culture system for forming osteoclasts, we have shown that MTX-induced suppression of RANKL expression was clearly canceled by the addition of dAdo. In this condition, expression of OPG was slightly suppressed by the addition of dAdo. These observations strongly suggest that dAdo markedly augment in the ratio of RANKL/OPG, which allows abundant osteoclastogenesis in the presence of MTX (Figure 6). In MTX-suppressed conditions, dAdo-induced induction of osteoclastogenesis was partly blocked by the addition of caffein, an antagonist for the high affinity adenosine receptors, A_1AR, $A_{2a}AR$ and A_3AR, but not blocked by the addition of antagonists for the low affinity receptor, $A_{2b}AR$. Among these receptors, MTX-treatment stimulated $A_{2a}AR$ and A_3AR and further treatment with dAdo did not change their expression level. It has been so far unclear that dAdo binds to ARs, our current research could predict a possibility that dAdo utilizes high affinity ARs in some conditions. Kara et al. [24] demonstrate that A_1AR is required for normal and pathological osteoclastogenesis. Ochaion et al. [25] reported a highly selective agonist for A_3AR effectively suppressed inflammation and bone destruction accompanying arthritis. Rath-Wolfson et al. [26] reported on an involvement of A_3AR in bone resorption in rats with adjuvant-induced arthritis. These data combined with our findings suggest that high affinity receptors for adenosine are involved in the dAdo-mediated osteoclastogenesis. In contrast, Mediero et al. [27] recently reported that $A_{2a}AR$ agonist, CGS21680, inhibited osteoclast differentiation and function. Femurs of $A_{2a}AR$ gene deficient mice show a significantly decreased bone formation with an increased TRAP-positive osteoclasts, strongly suggesting that $A_{2a}AR$ acts as a negative regulator for osteoclastogenesis. Thus ARs could act as the positive or negative regulators in osteoclastogenesis [28].

Among nucleotides, adenine-bound nucleotides show unique regulatory activities for osteoclastogenesis as well as for bone resorption. Pellegatti et al. [29] has shown that P2X7, the receptor for ATP, is involved in osteoclastogenesis. Receptors for ADP, P2Y1 and P2RY12, are also demonstrated to be involved in the regulation of osteoclastogenesis [30, 31]. The regulatory system using adenine-bound nucleosides and nucleotides in osteoclastogenesis might have been evolutionally developed in vertebrate. In general, extracellular ATP is utilized as a neurotransmitter in neuro-synapse and it has also been shown that ATP secreted from apoptotic cells acts as a

"find-me signal" for phagocytes through $P2Y_2$ ATP receptors expressed on macrophages [32].

Our previous report showed MTX-induced suppression of osteoclastogenesis was canceled by the addition of adenosine through the low affinity adenosine receptor, $A_{2b}AR$ [14]. In this case, MTX-induced suppression of RANKL expression was not canceled by adenosine but adenosine-treatment markedly suppressed expression of OPG, which finally resulted in an increase in the ratio of RANKL/OPG and subsequent augment in osteoclastogenesis. Major cause of the difference in the influence on RANKL expression between adenosine and dAdo could be attributed to a difference in the receptors utilized, although cell surface specific receptors for dAdo has not so far been identified. Extracellular dAdo could also been incorporated into cells through nucleoside transporters [33]. Details remains to be clarified concerning the sensing mechanism of osteoclast precursors to extracellular dAdo. In general, intracellular pool of deoxynucleosides is supplied by *de novo* synthesis of deoxynucleosides or uptake of extracellular deoxy-nucleosides through nucleoside transporter. Major source of extracellular deoxynucleosides is DNA from apoptotic cells [34]. For normal cell growth, well balanced deoxynucleoside pool is essential. When pool of some deoxynucleoside lapse into beeing insufficient (hypo-conditions) or extra-sufficient (hyper-conditions), "replication stress" occurs and DNA synthesis is inhibited. Replication stress has recently shown to be a potent driver of functional decline in aging hematopoietic stem cells [35]. Austin et al. [36] have demonstrated that the deoxycytidine kinase plays a critical role to avoid "replication stress" in hematopoietic cells. This enzyme phosphorylates deoxycytidine to form dCMP. It is utilized as a precursor of dCTP in the process of DNA synthesis. Deoxycytidine kinase is able to catalyze dAdo and deoxyguanosine to convert into dAMP and dGMP, respectively. Deficiency in this enzyme severely affects differentiation of hematopoietic cells. In our current study, dAdo-mediated stimulation of osteoclastogenesis in the presence of MTX could explain by the action of deoxycytidine kinase to metabolize dAdo. In general, a fraction of the cellular pool of ribonucleosides is converted into deoxyribonucleosides by the ribonucleoside reductase [37]. Prompt phosphorylation of dAdo formed from adenosine by ribonucleoside reductase would also contribute to avoid "replication stress". Involvement of dAdo in the regulation of osteoclastogenesis could be related to the regulation of "replication stress". Further investigations are required to elucidate the mechanism of induction of osteoclastogenesis by dAdo-treatment.

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