Thioredoxin protects against joint destruction in a murine arthritis model

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Abstract: Thioredoxin (TRX) is an oxidative stress-inducible biological antioxidant that is highly expressed in the synoviocytes of rheumatoid arthritis (RA) patients. There is much evidence that oxidative stress plays a key role in the inflammation and destruction of RA joints; the functional relationship between TRX and RA remains unknown, however. We therefore investigated the role played by TRX in the inflammatory and joint-damaging processes of RA using a murine model in which arthritis was induced by administering a mixture of anti-type II collagen monoclonal antibodies (mAb) and lipopolysaccharide (LPS). In Wt mice mAb/LPS injection induced neutrophil infiltration, cartilage destruction and chondrocyte apoptosis within the joints, all of which were dramatically suppressed in TRX transgenic (TRX-Tg) mice. Moreover, the 8-hydoxy-2'-deoxyguanosine (8-OHdG) expression seen in Wt mice after mAb/LPS injection was almost completely

inhibited in TRX-Tg mice. The administration of recombinant TRX also suppressed mAb/LPS-induced joint swelling in Wt mice. Taken together, these results suggest that TRX protects against arthritis and is a plausible candidate with which to develop novel therapies for the treatment of RA.

Dr. Matthew Grisham

Associate Editor

Free Radical Biology & Medicine

December 13, 2005

Dear Dr. Grisham:

We are grateful to Reviewers and You for favorable consideration of our manuscript "Thioredoxin protects against joint destruction in a murine arthritis model" (FRBM-D-05-00675) by Goh Tsuji, Masahiro Koshiba, Hajime Nakamura, Hidekazu Kosaka, Saori Hatachi, Chiyo Kurimoto, Masahiro Kurosaka, Yoshitake Hayashi, Junji Yodoi, and Shunichi Kumagai.

As suggested in your decision letter of August 15, 2005, we have modified our manuscript and have incorporated additional data to fully address the reviewer (#2)'s comments. We take this opportunity to point out changes made in the revised manuscript.

We thank the editor and the referees for their thoughtful comments and prompt review. The points raised have strengthened the manuscript. We hope that you will find our revised manuscript acceptable for publication in the Free Radical Biology and Medicine and we look forward to hearing from you at your earliest convenience.

Expecting to hear from you soon.

Sincerely yours,

Dr. Shunichi Kumagai

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Reviewer #2:

1. From the manuscript it is not clear how many separated experiment were performed or that all data is based on a single in vivo experiment. The authors need to clarify this and include the number of separated experiments (anticollagen Ab induced arthritis or Tg mice experiment) that were performed.

Answer to Comment #1:

As suggested by the reviewer, the number of separated experiments (anti-collagen Ab induced arthritis or Tg mice experiment) performed was clearly mentioned in Results section (page 13, line 12 and page 13, line 11 of the revised manuscript) as well as the figure legends for Figs. 3 and 5.

2. In addition to the observed local effects, such as decreased 8 OHdG expression demonstrated by immunohistochemistry, the authors need to show whether or not systemic TRX therapy influences systemic immunity and disease activity. Does TRX therapy influence systemic ROS or cytokine levels?

Answer to Comment #2:

We have investigated the effect of TRX on TNF-alpha levels both in the joints (local effect) and serum (systemic effect). As shown in the Results section (page 16, line 1 of the revised manuscript) and Figs. 9C and 9D which were newly added, TRX did not influence the systemic TNF-alpha level but completely suppressed the increase of the cytokine in joints. As discussed in the Discussion section (page 20, line18 of the revised manuscript), we believe that this could further enhance the beneficial effect for the possible future TRX-mediated treatment of RA.

3. The authors may also consider another anti-oxidant (i.e. vitamin E) as a control to address the concern as to what is so special about TRX.

Answer to Comment #3:

As suggested we addressed the possibility for another anti-oxidant (i. e. vitamin E) as a control, and added the sentences to the Introduction (page 5, lines 11-page 21, line 315 of the revised manuscript) that the anti-oxidants other than TRX reportedly have little therapeutic effects for RA, which we explain the standing point of the specialty about TRX.

4. The histology results have some problems in this paper. None of the histology panels has a scale bar. The other problem is that the sections shown in Figs. 1 A-D are from very different parts of the paw, and therefore, are not comparable.

Answer to Comment #4:

As the reviewer suggested, each of the histology panels now contains a scale bar.

As described in the legend for Fig. 1, the sections shown in Figs. 1 A and 1B were from the human patient with RA and OA, respectively (namely, not the parts of mouse paws). The corresponding parts of the joint from these patients are shown, but the sections have a different appearance due to the difference of the diseases. The similar situation is true for the sections shown in Figs. 1 C and 1D; these are from the similar parts of the joints but taken from the untreated (Fig. 1C) or treated (1D) mice, that is the reason for the different appearance of the figures.

5. When do authors start rTRX treatment in arthritic mouse? The clarification is required.

Answer to Comment #5:

We started the rTRX treatment from day 1, which is now mentioned in the Materials and Methods section (page 8, line 10 of the revised manuscript) as was suggested.

6. The authors focused on neutrophil function in the pathogenesis of RA. To our best knowledge, infiltrating lymphocytes plays more dominant role compared to neutrophil in RA.

Answer to Comment #6:

We completely agree with the reviewer's notion that the lymphocytes play a dominant role in RA.   It has been reported, however, that the neutrophils may have a dominant role in our RA model, which made us to discuss the TRX effects on neutrohils. According to the reviewer's suggestion, we now included the discussion for the TRX and lymphocytes to the Discussion section (page 19, lines 12-19 of the revised manuscript).

Thioredoxin protects against joint destruction in a murine arthritis model [1]

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Acknowledgement

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Running title: TRX ameliorates arthritis

Thioredoxin protects against joint destruction in a murine arthritis model

Abstract

Thioredoxin (TRX) is an oxidative stress-inducible biological antioxidant that is highly expressed in the synoviocytes of rheumatoid arthritis (RA) patients.   There is much evidence that oxidative stress plays a key role in the inflammation and destruction of RA joints; the functional relationship between TRX and RA remains unknown, however.   We therefore investigated the role played by TRX in the inflammatory and joint-damaging processes of RA using a murine model in which arthritis was induced by administering a mixture of anti-type II collagen monoclonal antibodies (mAb) and lipopolysaccharide (LPS).   In Wt mice mAb/LPS injection induced neutrophil infiltration, cartilage destruction and chondrocyte apoptosis within the joints, all of which were dramatically suppressed in TRX transgenic (TRX-Tg) mice.   Moreover, the 8-hydoxy-2'-deoxyguanosine (8-OHdG) expression seen in Wt mice after mAb/LPS injection was almost completely inhibited in TRX-Tg mice.   The administration of recombinant TRX also suppressed mAb/LPS-induced joint swelling in Wt mice.   Taken together, these results suggest that TRX protects against arthritis and is a plausible candidate with which to develop novel therapies for the treatment of RA.

Keywords: thioredoxin, rheumatoid arthritis, animal model, oxidative stress, antioxidants

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by the proliferation of synovial cells and infiltration of the joints by a variety of inflammatory cells [1,2]. During the course of inflammation, cell-derived inflammatory mediators, including various cytokines [e.g., interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF-α)], autoantibodies and lipid metabolites, induce the pathological outgrowth of synovial fibroblasts and the production of proteolytic enzymes that catalyze the local destruction of cartilage and bone [3]. Neutrophils, which play a key role in the pathogenesis of the synovial inflammation seen in RA, constitute more than 90% of the cellular exudate in synovial fluid (SF) and are present in the inflamed synovial tissue at the interface of the cartilage and the pannus [4,5]. They are recruited to and activated within RA joints by proinflammatory cytokines and by complement 5a generated when the complement pathways are activated by the immune complexes formed by autoantibodies in the joint. Once activated, neutrophils cause damage to the joints by releasing granules containing collagenases and elastases and by generating reactive oxygen species (ROS) [6].

ROS are produced in RA joints by polymorphonuclear cells, including neutrophils, as well as by macrophages and mechanical reperfusion, and contribute to the destruction of the cartilage in various ways [7-9]. For example, hydrogen peroxide (H_2O_2) inhibits cartilage proteoglycan synthesis by interfering with ATP, so that intra-articular injection of H_2O_2-generating systems causes severe joint damage in animals [10-12]. Hypochlorous acid (HOCl) can activate latent forms of neutrophil collagenases and gelatinases [13], while

H_2O_2 and superoxide ($O_2^{\cdot-}$) both accelerate bone resorption by osteoclasts [14,15]. Nitric oxide (NO) has long been considered the primary inducer of chondrocyte apoptosis mediated by caspase-3 and tyrosine kinase activation, though it has recently become clear that, by itself, NO cannot initiate apoptosis and that concomitant production of $O_2^{\cdot-}$ is required, suggesting a role for peroxynitrite (ONOO) in this process [16,17].

Other evidence suggesting the involvement of oxidative stress in RA includes the findings that degradation of hyaluronic acid, levels of uric acid oxidation products and protein carbonyls are all increased in the SF of RA patients [18-20]. Also increased are blood levels of the lipid peroxide malondialdehyde and urinary levels of 8-hydoxy-2'-deoxyguanosine (8-OHdG), a marker of oxidatively damaged DNA [21-23]. Quite a few reports indicate the decreased anti-oxidants such as ascorbic acid, vitamin E, thiols and glutathione in RA patients [21,24], but the effectiveness of anti-oxidant therapy to RA is yet to be clarified. For example, ascorbic acid and vitamin E have been shown to have only low evidence of effectiveness [25]. On the other hand, previous reports have also shown that levels of the antioxidant thioredoxin (TRX) are elevated in the plasma and SF of RA patients, and that the elevation of TRX correlates with the elevation of urinary 8-OHdG [23,26,27].

TRX is a small (12 kDa), ubiquitous, multifunctional protein that contains a redox (reduction/oxidation)-active disulfide/dithiol within its conserved active site [28,29], and is representative of an expanding family of proteins that share a similar active sequence (-Cys-Xxx-Yyy-Cys-). In the present study, we refer to the conventional cytosolic TRX-1 as TRX, since TRX-2 is now known to exist only in the mitochondria. Notably, the TRX

gene contains a *cis*-regulatory element in its promoter region that is responsive to oxidative stress [30]; indeed, TRX is constitutively expressed in most cells of the body and is induced by a variety of cellular stresses, including viral infection, ultraviolet irradiation, oxidative stress and postischemia reperfusion [31].   Once expressed, TRX acts as a scavenger of ROS [28,29], and when secreted it has cytokine-like properties that are of importance in immunogenic responses.   TRX also exerts growth-promoting and anti-apoptotic effects [32], and its overexpression in mice leads to resistance against oxidative stress and life span extension that is accompanied by no apparent abnormalities [33].   Our earlier observation that TRX is expressed on synovial cells and cells infiltrating RA joints prompted us to hypothesize that TRX has a protective function in RA joints and may be able exert a therapeutic effect there [23].   To test that idea, we evaluated the antiarthritic effects of TRX in a mouse arthritis model.

Materials and Methods

Animals

TRX transgenic (TRX-Tg) mice were originally provided by the Oriental Yeast Co. Ltd. (Tokyo, Japan); wild-type (Wt) C57BL/6 mice were obtained from Charles River Japan (Tsukuba, Japan).   Both Wt and TRX-Tg mice were housed in the Kobe University animal facility at a constant temperature and were provided with laboratory chow and water.   All procedures were carried out in accordance with the recommendations of the Institutional Animal Care Committee of Kobe University.

Anti-collagen antibody-induced arthritis

Arthritis antibody kits were obtained from Immuno-Biological Laboratories (IBL; Gunma, Japan), after which arthritis was induced by following the manufacturer's instructions [34]. Briefly, 6 to 7-week-old Wt and TRX-Tg mice were injected i.p. with a mixture of four anti-type II collagen monoclonal antibodies (mAb) at 4 mg/ml/body on days 1 and 2, followed by i.p. injection of 50 µg/100 µl/body lipopolysaccharide (LPS) (0111:B4; IBL) on day 4.   As a control, saline was injected in place of the mAb and LPS (mAb/LPS). Following mAb injection, the severity of the macroscopic arthritis in each of the four limbs of the mice was graded on a 1-4 scale as described previously [35].   The grading criteria were: 0, normal; 1, swelling and/or redness in one joint; 2, swelling and/or redness in more

than one joint; 3, swelling and/or redness in an entire paw; and 4, maximal swelling. Thus,

the maximum score obtainable from the four limbs of each mouse was 16 (4 x 4). These

evaluations were made by two trained laboratory personnel blinded to the treatment

protocol. In addition, the volume of the left hind paw of each mouse was measured using

a plethysmometer (Neuroscience, Osaka, Japan) on days 4 and 9.

In vivo treatment of mice with rTRX

Human recombinant (r)TRX protein was provided by Ajinomoto Inc. (Kawasaki, Japan).

Normal C57BL/6 mice and those with mAb/LPS-induced arthritis were treated daily from

day 1 with an i.p. injection of 40 μg rTRX suspended in 100 μl of sterile PBS; 40 μg of

ovalbumin (OVA) (Nacalai Tesque, Inc., Kyoto, Japan) was used as a control.

Histology

After sacrificing groups of mice, their left hind paws were removed, fixed in 4%

paraformaldehyde in PBS, decalcified in EDTA, embedded in paraffin and sectioned.

Some were then stained with hematoxylin and eosin (H & E) or toluidine blue. Others

were immunohistochemically analyzed. The right hind paws of the mice were used for

frozen sections. In addition, with the approval of the Committee for Human Research at

Kobe University, human synovial tissue samples were obtained from patients who had

undergone synovial resection for the treatment of RA or osteoarthritis (OA). Excised

specimens were immediately placed in neutral buffered formalin for 24 h.   After fixation,

the human samples were embedded in paraffin.

Immunohistochemistry

Paraffinized tissue sections were deparaffinized in xylene and then dehydrated through a

series of graded alcohols, after which the antigen was unmasking by autoclaving the

sections for 5 min.   Frozen sections were fixed in 4% formalin in PBS.   All samples

except those used for 8-OHdG staining were incubated in 0.3% H_2O_2 for 30 min at room

temperature to inactivate endogenous peroxidase.   The sections were then incubated for 1

h at room temperature first with anti-8-OHdG antibody (Japan Institute for the control of

Aging, Shizuoka, Japan), anti-human (h)TRX mAb (Redox Bioscience, Inc., Kyoto, Japan),

anti-mouse (m)TRX mAb (Redox Bioscience, Inc., Kyoto, Japan), or control antibody

(mouse IgG) and then with peroxidase-conjugated goat anti-mouse IgG (Simple Stain

MAX-PO, Nichirei, Tokyo, Japan).   The resultant signals were visualized using

3,3'-diaminobenzidine tetrahydrochloride (DAB) for paraffinized sections or

aminoethylcarbazole (AEC) for frozen sections.

Serum TNF-α Levels

Serum samples were obtained on day 4, 1 h after i.p. injection with LPS, and TNF-α levels

were measured with an ELISA system kit according to the manufacturer's protocols (Amersham Pharmacia).

Quantification of TNF-α expression in the joints by real-time RT-PCR

Expression levels of TNF-α mRNA were estimated by the real-time quantitative RT-PCR method.   Briefly, frozen joint tissues from the hind paws of TRX-Tg (n = 3) and Wt (n = 3) mice killed on day 10 were homogenized using Tissue Lyser (Qiagen, Valencia, CA), total RNAs were isolated using RNeasy Mini Kit (Qiagen), and cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).   Each PCR reaction was run in triplicate using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The primer pairs used in these reactions were Mm00443258-m1 (Applied Biosystems) . The amplification reactions, data acquisition, and analyses were performed with the ABI Prism 7900 HT instrument (Applied Biosystems).   With Mm99999915-g1 primer pairs (Applied Biosystems), glyceraldahyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene against which all the samples were normalized for differences in the amount of total RNA added to each cDNA reaction and for variation in the reverse transcriptase efficiency among the different cDNA reactions.

Detection of apoptosis

The incidence of apoptosis was evaluated by *in situ* terminal deoxynucleotidyl transferase

(TdT)-mediated dUTP-biotin nick end labeling (TUNEL) using an Apoptag Kit S7101

(Cosmo Bio, Tokyo, Japan) according to the manufacturer's instructions.   Briefly,

deparaffinized sections were incubated with proteinase K (20 µg/ml) for 15 min at room

temperature and then washed with distilled water, after which endogenous peroxidase was

inactivated by incubation in 3% H_2O_2 for 5 min at room temperature.   The sections were

then washed with PBS, immersed in working strength TdT enzyme solution also containing

digoxigenin-nucleotides, and incubated under a humidified atmosphere for 1 h at 37°C.

After washing again with PBS, the sections were incubated for 30 min at room temperature

with peroxidase-conjugated anti-digoxigenin Ab, and the resultant signals were visualized

with DAB.

Statistical analysis

Data were expressed as means ± SEM.   Statistical comparisons were made using

Mann-Whitney's U test.   Values of $P < 0.05$ were considered significant.

Results

8-OHdG is expressed on both the synovia of RA patients and the joints of arthritic model mice

We initially examined the synovial tissues from two RA and two OA patients for expression of 8-OHdG using an anti-8-OHdG antibody.   We found that 8-OHdG was expressed on the surface of the synovial lining and sublining layers in RA patients, whereas little expression was detected in tissues from OA patients (Fig. 1A and 1B).   The expression of 8-OHdG was confirmed in synovial cells and chondrocytes from mAb/LPS-treated arthritic mice, but was not observed in Wt mice (Fig. 1C and 1D), which suggests the involvement of oxidative stress in these arthritic model mice as well as in RA patients.

TRX is expressed in the joints of mice after induction of arthritis

Joint tissues from Wt mice and mAb/LPS-treated arthritic mice were examined for expression of endogenous mTRX using an anti-mTRX mAb.   mTRX was expressed on the surface in synovial cells and chondrocytes isolated from mAb/LPS-treated arthritic mice (Fig. 2B and 2D), but was not observed in untreated Wt mice (Fig. 2A and 2C), which suggests expression of mTRX within the joints is an arthritis-related event.

Treatment with rTRX reduces joint swelling in arthritic mice

A recent *in vitro* study showed that, when added extracellularly, rTRX is able to enter cells and suppress the effects of oxidative stress [36].   That finding prompted us to test whether administration of rTRX could effectively suppress the development and/or progression of mAb/LPS-induced arthritis.   Because i.p. injection of 40 μg rTRX every other day was previously shown to suppress cytokine- and bleomycin-induced lung injury in C57BL/6 mice [37], we used the same dose to treat the arthritic mice.   On the other hand, although the mean serum level of TRX was 5090 ng/ml 1 h after injection, levels remained over 100 ng/ml for only 12 h (half-life, 2.29 h; data not shown).   We therefore administered rTRX daily.   We found that in both rTRX-treated and control (OVA-injected) mice, joint swelling began on day 6 after the first mAb injection, and that all of the mice eventually developed arthritis.   However, administration of rTRX significantly reduced the severity of the arthritis, with the arthritis score peaking at 12.2 on day 11 in the control group and at 10.3 on day 9 in the rTRX group (Fig. 3; representative data from two independent experiments are shown).

Arthritis is suppressed in TRX-Tg mice

To further confirm the hypothesis that TRX is protective against the joint inflammation and destruction observed in RA, we next investigated the development and progression of mAb/LPS-induced arthritis in TRX-Tg mice.   The TRX-Tg mice used in this study carried a transgene encoding hTRX under the control of the β-actin promoter and systemically expressed hTRX at levels up to ten times higher than the endogenous mTRX [38].   The

expression of hTRX was confirmed in synovial cells and chondrocytes from TRX-Tg mice (Fig. 4C and 4D), but was not observed in Wt mice (Fig. 4A and 4B).   The mean serum hTRX concentration in TRX-Tg mice was 2612.5 ng/ml, while the level in Wt mice was negligible (data not shown).

Following administration of mAb/LPS, joint swelling was observed in all of the Wt mice, but only 4 of 6 TRX-Tg mice developed any swelling.   In Wt mice, the arthritis score began to increase on day 5 and peaked at a value of 14.8 on day 8.   In TRX-Tg mice, by contrast, the arthritis score did not start to rise until day 6, and the arthritis was less severe (Fig. 5B-5E), so that the arthritis score never exceeded a value of 7 (Fig. 5A). These observations were true on the 3 independent experiments and thus the representative data are shown.   Also diminished in TRX-Tg mice was the inflammation-related increase in paw volume (Fig. 6).

TRX suppresses cell infiltration beginning at an early stage of arthritis development
To examine the effects of TRX on the development of arthritis in more detail, histological examination was carried out on joints from Wt and TRX-Tg mice sacrificed on day 5 after administration of mAb, when there was no apparent joint swelling, or on day 9, at the peak of swelling.   On day 5, TRX-Tg mice showed no cell infiltration or angiogenesis, whereas articular synovial proliferation, cell infiltration and angiogenesis were all readily observable in Wt mice (Fig. 7A and 7C).   On day 9, severe damage to cartilage and bone tissue was apparent in Wt mice, as were infiltration of mainly neutrophils and fibrin

coagulation within the joint space and synovial tissues.   TRX-Tg mice showed only slight synovial proliferation, and the neutrophil infiltration and joint destruction were markedly suppressed (Fig. 7B and 7D).

Destruction of articular cartilage is suppressed in TRX-Tg mice

Toluidine blue staining of the articular cartilage was very much reduced in mAb/LPS-injected Wt mice, as compared to saline injected Wt mice, which indicates a loss of proteoglycan from the cartilage matrix (Fig. 8A and 8B).   By contrast, toluidine blue staining in mAb/LPS-injected TRX-Tg mice was similar to that seen in saline-injected Wt and TRX-Tg mice, suggesting TRX exerts a protective effect that mitigates cartilage destruction (Fig. 8C and 8D).

8-OHdG expression on chondrocytes is suppressed in TRX-Tg mice even after induction of arthritis

The expression of 8-OHdG on articular cartilage tissue from Wt and TRX-Tg mice after induction of arthritis was assessed using an anti-8-OHdG Ab.   We found that 8-OHdG was expressed on most chondrocytes from mAb/LPS-treated arthritic Wt mice, but was rarely observed in mAb/LPS-treated TRX-Tg mice (Fig. 9A and 9B).   Given that chondrocytes from saline-treated Wt (Fig. 1C) and TRX-Tg (data not shown) mice express little 8-OHdG, these results suggest that the contribution made by oxidative stress to the development of arthritis in this model was diminished in TRX-Tg mice.

TNF-α production induced by mAb/LPS in TRX-Tg mice was diminished only in the joints

Examined next was whether or not TRX influences the level of TNF-α, one of the major inflammatory cytokines contributed in RA.   Previous reports showed that serum TNF-α level in this mouse model was elevated only 1 h after LPS injection (39).   Indeed our preliminary experiments indicated that the serum level of TNF-α was increased 1 hour after LPS injection and decreased to below the detection limit within 3 hours after LPS injections in Wt mice (data not shown).   Thus we compared the serum levels of TNF-α between in TRX-Tg and Wt mice 1 hour after LPS injection.   The mean serum level of TNF-α was 3615 ± 968 pg/ml in TRX-Tg mice and not significantly different in Wt mice, the levels on which was 4382 ± 1380 pg/ml, indicating that systemic TNF-α response to mAbs/LPS was not impaired in TRX-Tg mice (Fig. 9C).   On the other hand, the increase of TNF-α mRNA expression in the joints of mAb/LPS treated Wt mice was completely suppressed in the joints of mAb/LPS treated TRX-Tg mice (Fig. 9D), suggesting that TRX suppressed the up-regulation of TNF-α in the joints but not systemically.

Chondrocyte apoptosis in articular cartilage is suppressed in TRX-Tg mice

To clarify the mechanism(s) by which TRX suppresses cartilage destruction, the incidence of apoptosis among chondrocytes was examined using TUNEL assays.   We found that mAb/LPS administration significantly increased the numbers of TUNEL-positive

chondrocytes in Wt mice (Fig. 10A, 10B and 10E) but not in TRX-Tg mice (Fig. 10C, 10D and 10E). This suggests that the abundant expression of TRX attenuated cartilage destruction by preventing the apoptotic cell death of chondrocytes.

Discussion

Although it has frequently been reported that oxidative stress is increased and levels of antioxidants are decreased in RA joints [18-25, 40] , we previously observed that levels of the antioxidant TRX are actually increased in the joints of RA patients.   Apparently, the increased expression of TRX, which is in response to the oxidative stress within RA joints, is not sufficient to fully counteract the progression of the disease.   We hypothesized, however, that a sufficient amount of TRX might suppress joint inflammation and destruction in RA; after all, it does suppress tissue damage caused by interstitial pneumonia and ischemic brain damage, the pathogeneses of which are thought to be related to oxidative stress [37,38].

We used an anti-collagen mAb/LPS-induced arthritis model in the present study because C57BL/6 mice are largely not susceptible to collagen-induced arthritis, which is the more commonly used RA model [39].   That 8-OHdG was expressed in the joints of arthritic mice (Fig. 1D), just as it is in the joints of RA patients (Fig. 1B), suggests that oxidative stress is a contributing factor in both, and that TRX could play an important role mitigating its effects.   Consistent with that idea, rTRX significantly suppressed the severity of the arthritis, though the difference between the joint destruction seen in rTRX-treated and OVA-treated mice was not clear on H & E-stained histological sections (data not shown).   For that reason, we next examined TRX-Tg mice, which overexpress hTRX in serum, articular synoviocytes and chondrocytes (Fig. 4).   We found that TRX-Tg

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mice were more resistant to mAb/LPS-induced arthritis than were Wt mice treated with

rTRX (Fig. 5, 6). This suggests that the stable elevation of serum TRX levels (> 2000

ng/ml) seen in TRX-Tg mice may provide more resistance to mAb/LPS-induced arthritis

than the more variable levels seen with rTRX treatment. Alternatively, it may be that

intracellular TRX within articular tissues prevents joint destruction in TRX-Tg mice, and

rTRX is not translocated into chondrocytes or synoviocytes in Wt mice, regardless of the

presence of arthritis (data not shown). In addition, the arthritis model we used was

characterized by, among other things, infiltration of neutrophils into the joints [39]. Such

neutrophil infiltration was strongly suppressed in TRX-Tg mice, even before joint swelling

became apparent (Fig. 7), which is consistent with an earlier finding that elevated levels of

circulating TRX prevented chemotaxis induced by LPS and/or chemokines in a standard

chemotaxis (air pouch) model [41]. RA is considered to be a lymphocyte-mediated

autoimmune disease characterized by T cell activation. Recent reports on the efficacy for

RA of new drugs targeting lymphocytes such as FK506 and rituximab (anti-CD20) support

this hypothesis [42, 43]. TRX was also reported to regulate the function of many kinds of

cells including lymphocytes. The model used here, however, is thought to be mediated

mainly by the neutrophils, and not by the lymphocytes since this model of arthritis could be

successfully induced on the SCID mice that do not carry the lymphocytes [39]. Thus the

possible role of TRX on lymphocytes in RA patients should be clarified.

It is the cartilage loss and subsequent joint failure that eventually causes disability among

RA patients. The mechanisms responsible for the cartilage erosion in RA are thought to

involve the chronic secretion of inflammatory mediators and proteolytic enzymes by

infiltrating inflammatory cells (e.g., neutrophils) and synovial cells [16,44,45]. Moreover,

in a recent *in vitro* study chondrocytes were shown to undergo apoptosis upon addition of

NO or the coupling of Fas/Fas ligand, suggesting they possess an inherently programmed

cell death pathway that can be activated by various stimuli [46,47]. In that regard,

oxidative stress has been shown to be capable of initiating apoptosis by inducing

cytochrome c release from mitochondria and by activating various caspases, p53, apoptosis

signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase and p38 mitogen-activated

protein kinase (MAPK). TRX, on the other hand, inhibits apoptotic signaling by

scavenging intracellular ROS in cooperation with the glutathione system and by inhibiting

the activities of ASK1 and p38 MAPK [48]. Our present findings thus suggest that TRX

suppresses apoptosis among chondrocytes by directly suppressing oxidative stress, which is

evidenced by the reduced expression of 8-OHdG on these cells in arthritic TRX-Tg mice,

and by negatively regulating intracellular apoptotic signaling (Fig. 8-10), which in turn

mitigates cartilage loss and joint damage.

Also contributing to the inflammatory response within RA joints are the

proinflammatory cytokines TNF-α, IL-1β and IL-6, the levels of which are significantly

elevated in the hind paws of arthritic mice in this model [39]. We demonstrated in

TRX-Tg mice the up-regulation of local TNF-α expression after LPS injection was

complete suppressed without affecting the systemic TNF-α levels (Fig. 9C, 9D). This

observation suggests that the treatment of RA patients with TRX may only suppress the

overproduction of the inflammatory cytokines in the joints. If this is true TRX treatment should have a great benefit for the treatment of RA since these cytokines are also known to be important for the host-defence mechanisms against the pathogenic organisms. It is noteworthy that the synthesis of these cytokines is regulated by NF-κB, which is activated via ROS-mediated dissociation of I-κB [49]. Thus, by scavenging ROS, cytosolic TRX likely inhibits the dissociation of I-κB from NF-κB, thereby suppressing activation of NF-κB and, in turn, synthesis of the aforementioned proinflammatory cytokines.

Finally, the activities of TRX make it a plausible candidate with which to develop novel therapies for the treatment of RA. Since a recent report has shown that anti-TNF-α therapy does not suppress neutrophil chemotaxis or ROS production in patients with RA [50], we would expect TRX, alone or together with anti-TNF-α, to have more beneficial effect than anti-TNF-α alone. This is because TRX has already been shown to suppress neutrophil chemotaxis, chondrocyte apoptosis and ROS levels. In addition, TRX reportedly enhances resistance to pneumonia caused by microorganisms such as influenza [41] and suppresses cytokine-induced interstitial pneumonia [38], which are major complications of currently-used immunosuppressant RA treatments.

Acknowledgement

Abbreviations

TRX- thioredoxin, RA- rheumatoid arthritis, mAb- monoclonal antibody, mAb/LPS- a mixture of anti-type II collagen monoclonal antibodies and lipopolysaccharide, Tg- transgenic, Wt- wild type, IL- interleukin, TNF- tumor necrosis factor, SF- synovial fluid, ROS- reactive oxygen species, H_2O_2- hydrogen peroxide, $O_2^{\cdot-}$- superoxide, NO- nitric oxide, 8-OHdG- 8-hydroxy-2'-deoxyguanosine, OVA- ovalbumin, H & E- hematoxylin and eosin, rTRX- recombinant TRX, hTRX- human TRX, mTRX- mouse TRX, DAB- 3,3'-diaminobenzidine tetrahydrochloride, AEC- aminoethylcarbazole, OA- osteoarthritis, GAPDH- glyceraldahyde-3-phosphate dehydrogenase, TUNEL- terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling, ASK1- apoptosis signal-regulating kinase 1, MAPK- mitogen-activated protein kinase, NF-κB- nuclear factor kappa B, I-κB- inhibitor-NF-κB

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Figure Legends

FIGURE 1.   8-OHdG expression within human synovia and the joints of arthritic mice.

Immunohistochemical analyses confirmed that 8-OHdG was expressed on synovial cells

from RA patients (**B**), but not on those from OA patients (**A**).   8-OHdG expression was

also observed on synovial cells and chondrocytes from mAb/LPS-treated arthritic mice (**D**),

but not on those from untreated-mice (**C**).   Representative data from 6 mice in each group

are shown.

FIGURE 2.   Endogenous mTRX expression within the joints of arthritic mice.

mTRX was expressed on synovial cells (**B**) and chondrocytes (**D**) from mAb/LPS-treated

arthritic mice, but not on those from untreated-mice (**A** and **C**).   Representative data from

6 mice in each group are shown.

FIGURE 3.   Effect of rTRX administration on joint swelling.

Time-dependent changes in mean arthritis scores were determined as described in the

Material and Methods for mice receiving the indicated treatment protocol.   Note that the

severity of the arthritis was significantly suppressed by rTRX.   Representative data from

two independent experiments are shown.   Data are means ± SEM (n =6 in each group); *P

< 0.05, rTRX vs. OVA in mice injected with mAb/LPS.

FIGURE 4.   hTRX expression in the joints of TRX-Tg mice.

Immunohistochemical analysis confirmed that hTRX was expressed in synoviocytes (**C**)

and chondrocytes (**D**) in TRX-Tg mice, but not in either cell type in Wt mice (**A** and **B**).

Representative data from 6 mice in each group are shown.

FIGURE 5.   Joint swelling in Wt and TRX-Tg mice following injection with mAb/LPS.

A, Arthritis was induced and scored as described in the Materials and Methods.   Data are

means ± SEM; *$P < 0.05$, TRX-Tg vs. Wt mice administered mAb/LPS.   Note that the

severity of the arthritis was significantly suppressed in TRX-Tg mice.   Representative data

from three independent experiments are shown.   **B-E**, Photographs of joint swelling in

fore and hind paws taken on day 15: **B**, Wt mice injected with mAb/LPS; **C**, TRX-Tg mice

injected with mAb/LPS; **D**, Wt mice injected with saline; and **E**, TRX-Tg mice injected

with saline.   Images shown are representative of 6 in each group.

FIGURE 6.   Left hind-paw volumes measured on days 4 and 9 using a plethysmometer.

Data are means ± SEM (n=6 in each group); *$P < 0.05$, TRX-Tg vs. Wt mice on day 9.

FIGURE 7.   Sections of hind-paw joints obtained on days 5 and 9 (H & E staining).

The sections were prepared from Wt (**A** and **B**) and TRX-Tg (**C** and **D**) mice injected with

mAb/LPS.   On day 5, there were no signs of arthritis in samples from TRX-Tg mice (**C**),

whereas articular synovial proliferation, cell infiltration and angiogenesis were noted in

samples from Wt mice (**A**).   On day 9, modest synovial proliferation was detectable in

TRX-Tg mice (**D**), whereas severe destruction of cartilage and bone tissue, infiltration of

mainly neutrophils, and fibrin coagulation within the joint space and synovial tissues were

readily detectable in Wt mice (**B**).

FIGURE 8.   Proteoglycan-staining in articular cartilage.

Shown are representative sections of articular cartilage from the hind-paw joints of Wt (**A**

and **B**) and TRX-Tg (**C** and **D**) mice stained with toluidine blue to visualize proteoglycan

levels following injection with saline (**A** and **C**) or mAb/LPS (**B** and **D**).   Significant loss

of proteoglycan was observed in Wt mice injected with mAb/LPS (**B**); no such loss was

detected in TRX-Tg mice injected with mAb/LPS (**D**).   Representative data from 6 mice

from each group are shown.

FIGURE 9.   8-OHdG and TNF-α expression within the joints of arthritic mice.

Immunohistochemical analyses confirmed that 8-OHdG was expressed on most

chondrocytes from Wt mice (**A**), but not on those from mAb/LPS-treated TRX-Tg mice (**B**).

Representative data from 6 mice in each group are shown.   Serum TNF-α level induced by

LPS injection were compatible in Wt (n = 4) and TRX-Tg (n = 4) mice (**C**).   TNF-α

mRNA was elevated in the joints of mAb/LPS treated Wt mice (n=3), but not in those of

mAb/LPS treated TRX-Tg mice (n=3) (**D**).   Data are means ± SEM; *P < 0.05, TRX-Tg

vs. Wt mice administered mAb/LPS.

FIGURE 10.   Incidence of apoptosis among chondrocytes evaluated using TUNEL

assays.

A-D, Representative sections showing that TUNEL-positive cells were detected in samples

from Wt mice injected with mAb/LPS (**B**) but not in those from TRX-Tg mice injected with

mAb/LPS (**D**); Wt and TRX-Tg mice injected with saline did not carry TUNEL-positive

cells (**A** and **C**, respectively).   **E**, Quantification of TUNEL-positive cells.   Data are

expressed as means ± SEM (n=6); *$P < 0.05$, saline vs. mAb/ LPS in Wt mice.
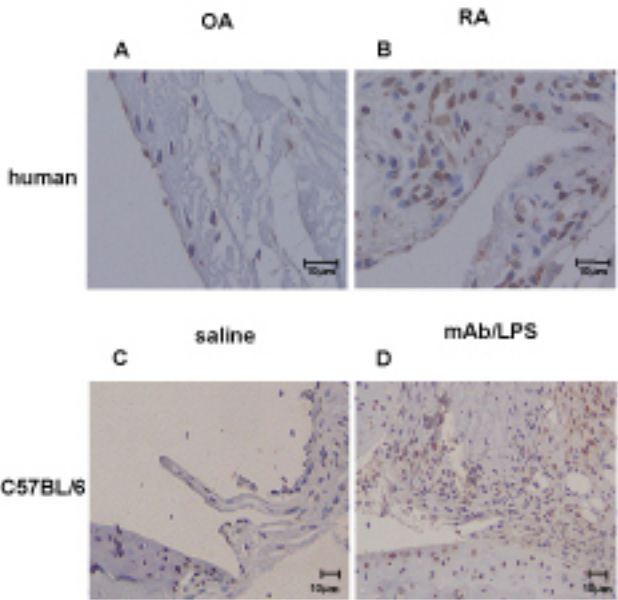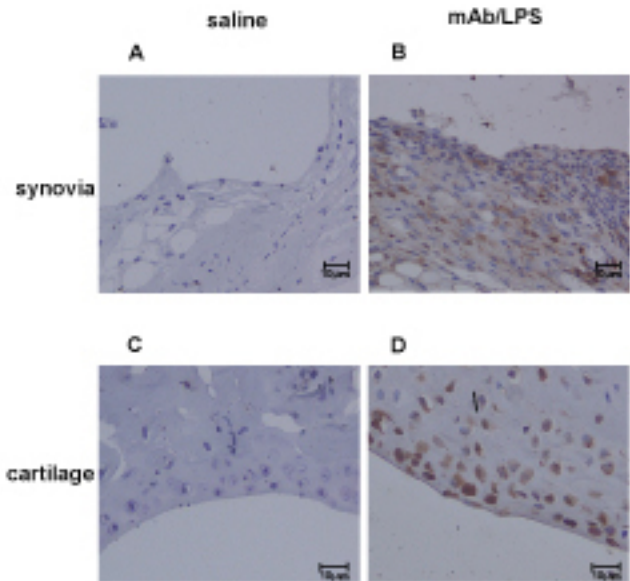
Figure(s)

Fig.1, Goh Tsuji, top

A — OA   B — RA

human

C — saline   D — mAb/LPS

C57BL/6

Fig.2, Goh Tsuji, top

A — saline   B — mAb/LPS

synovia

C — saline   D — mAb/LPS

cartilage

Fig.3, Goh Tsuji, top

Legend:
- saline and OVA
- saline and rTRX
- mAb/LPS and OVA
- mAb/LPS and rTRX

y-axis: Mean arthritis score (0, 4, 8, 12, 16)
x-axis: Days (1–15)

Fig.4, Goh Tsuji, top

A — Synovium   B — Cartilage

Wt

C — Synovium   D — Cartilage

TRX-Tg

Fig.5, Goh Tsuji, top

A

Legend:
- Wt, saline
- TRX-Tg, saline
- Wt, mAb/LPS
- TRX-Tg, mAb/LPS

y-axis: Mean arthritis score (0, 4, 8, 12, 16)
x-axis: Days (1–15)

B, C, D, E

Fig.6, Goh Tsuji, top

Legend:
- Wt, mAb/LPS
- TRX-Tg, mAb/LPS

y-axis: Volume of lt. Paw (m l) (0.15–0.35)
x-axis: Day4, Day9

Fig.7, Goh Tsuji, top

A — Day 5   B — Day 9

Wt

C   D

TRX-Tg

Fig.8, Goh Tsuji, top

A — saline   B — mAb/LPS

Wt

C   D

TRX-Tg

Fig.9, Goh Tsuji, top

A — Wt   B — TRX-Tg

C — serum TNF-alpha (pg/ml)

y-axis: 0, 2000, 4000, 6000
x-axis: Wt, TRX-Tg

D — mRNA of TNF-alpha (%)

y-axis: 100, 300, 500
x-axis: Wt, TRX-Tg (saline); Wt, TRX-Tg (mAb/LPS)

Fig.10, Goh Tsuji, top

A — saline   B — mAb/LPS

Wt

C   D

TRX-Tg

E

y-axis: TUNEL positive cells / chondocytes (%) (0, 10, 20, 30)
x-axis: Wt, TRX-Tg (saline); Wt, TRX-Tg (mAb/LPS)