Over-expression of extracellular superoxide dismutase in mouse synovial tissue attenuates the inflammatory arthritis

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Abstract

Oxidative stress such as reactive oxygen species (ROS) within the inflamed joint have been indicated as being involved as inflammatory mediators in the induction of arthritis. Correlations between extracellular-superoxide dismutase (EC-SOD) and inflammatory arthritis have been shown in several animal models of RA. However, there is a question whether the over-expression of EC-SOD on arthritic joint also could suppress the progression of disease or not. In the present study, the effect on the synovial tissue of experimental arthritis was investigated using EC-SOD over-expressing transgenic mice. The over-expression of EC- SOD in joint tissue was confirmed by RT-PCR and immunohistochemistry. The degree of the inflammation in EC-SOD transgenic mice was suppressed in the collagen-induced arthritis model. In a cytokine assay, the production of pro-inflammatory cytokines such as, IL-1β, TNFα, and matrix metalloproteinases (MMPs) was decreased in fibroblast-like synoviocyte (FLS) but not in peripheral blood. Histological examination also showed repressed cartilage destruction and bone in EC-SOD transgenic mice. In conclusion, these data suggest that the over-expression of EC-SOD in FLS contributes to the activation of FLS and protection from joint destruction by depressing the production of the pro-inflammatory cytokines and MMPs. These results provide EC-SOD transgenic mice with a useful animal model for inflammatory arthritis research.

Keywords: arthritis, experimental; reactive oxygen species; rheumatoid arthritis; superoxide dismutase; synovial membrane

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease involving hyper-proliferation of the synovial membrane and accumulation of activated T cells and macrophages, leading to progressive joint destruction. The initiating event of RA is followed by the induction of an immune response that results in inflammation in the synovial membrane and the lining of the joint, that is usually composed of macrophage and fibroblast-like cells known as synoviocytes (Sweeney and Firestein, 2004). These cells can produce pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8), which can enhance synovial inflam-

mation (Feldmann, 1994).

Oxidative stress such as reactive oxygen species (ROS) within the inflamed joint have been indicated as being involved as inflammatory mediators in the induction of arthritis (Goldring, 2004). These ROS include radicals such as superoxide (O_2^-), hydroxyl radicals (OH^-) and non-radical species, such as hydrogen peroxide (H_2O_2). The ROS derived from activated macrophage and granulocytes infiltrating arthritic joints are involved in the pathogenesis of RA. A high level of ROS can lead to direct damage to hyaluronic acid (McCord, 1974), lipids (Tiku *et al.*, 2000), cartilage matrix and DNA (Burkhardt *et al.*, 1986). The ROS contribute to disease through the induction of pro-inflammatory cytokines. After a period of hypoxia, re-oxygenated human monocytes produce large amounts of IL-1β stimulated by ROS (Koga *et al.*, 1992) and TNFα is produced by alveolar macrophages (Simeonova and Luster, 1995) and monocytes (Volk *et al.*, 1999) after stimulation with ROS. ROS are responsible for collagen hydrolysis and activation of metalloproteinase, leading to the degradation of the extracellular matrix.

The superoxide dismutase (SOD) are antioxidant enzymes that catalyze the dismutation of superoxide into hydrogen peroxide and oxygen (Fattman *et al.*, 2000). There are three different isoforms of SOD which exist in mammals, including cytoplasmic and nuclear CuZn-SOD (Tsan, 1997), mitochondrial Mn-SOD (Carlsson *et al.*, 1995) and extracellular SOD (EC-SOD). Unlike Cu/Zn- and Mn-SODs, EC-SOD is found in the extracellular matrix of tissue and extracellular fluids, such as serum, cerebrospinal, ascites, and synovial fluids (Ookawara *et al.*, 1998; Marklund, 2002). Correlations between EC-SOD and inflammatory arthritis have been shown in several animal models of RA. The decreased levels of IL-1β and TNFα in the sera were observed in the collagen-induced arthritis (CIA) by a gene transfer of EC-SOD (Iyama *et al.*, 2001), and EC-SOD deficient mice showed marked increases in mRNA levels of these cytokines in the joints (Ross *et al.*, 2004).

In the previous study, it has already been shown that the transfer of EC-SOD transgenic mouse embryonic fibroblast (MEF) represses the inflammatory arthritis (Yu *et al.*, 2008). However, there is a question whether the over expression of EC-SOD on arthritic joint could also suppress the progression of disease or not. In the present study, the effect on the synovial tissue of experimental arthritis was investigated using EC-SOD over-expression model.

Results

Over-expression of EC-SOD in the synovial tissue

First of all, the over-expression of EC-SOD was investigated in joint tissues from transgenic mice. RT-PCR of the joint tissues of each mouse were performed. As shown in Figure 1, EC-SOD was highly over-expressed in transgenic mice compared with wild type littermates. Furthermore, immunohistochemistry was also performed to confirm the distribution and localization of EC-SOD in joint tissues using an antibody to EC-SOD. The positive staining was mainly seen in the lining layer of the synovium and endotherial cells in the sublining.
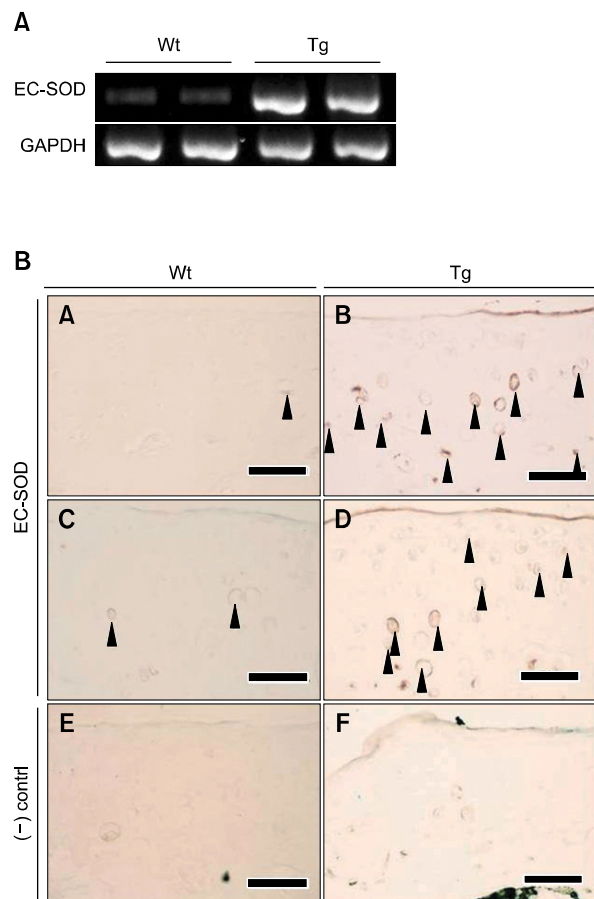

Figure 1. The EC-SOD over-expressed in joint tissue in transgenic mice. The over-expression of EC-SOD in transgenic mice was confirmed by RT-PCR (A). Total RNA was extracted from joint tissue of the transgenic and wild-type mice. GAPDH was used as control. Wt, wild type mice; Tg, EC-SOD transgenic mice. Further analysis of the location of over-expressed EC-SOD in transgenic mice was performed by immunohistochemistry (B). The synoviums were obtained from three different transgenic mice. Cells stained with anti-EC-SOD antibodies are shown in brown. Intense staining in the synovium observed in the endotherial surface of synovium (arrow). Left panel, magnification, ×20; right panel, magnification, ×40. Scale bars: in Left, 100 μm; in right, 200 μm.
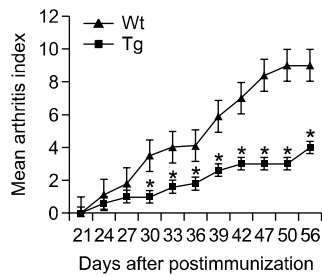
Figure 2. The overexprssion of EC-SOD in synovial tissue repressed inflammatory arthritis. After induction of arthritis in each mouse, disease development and severity were observed and determined as clinical score by blind manner of two trained inspectors. Disease severities were significantly reduced in transgenic mice on day 30 after primary immunization and this effect was maintained until all mice reach to terminal score (*$P < 0.05$ vs arthritic wild type mice).
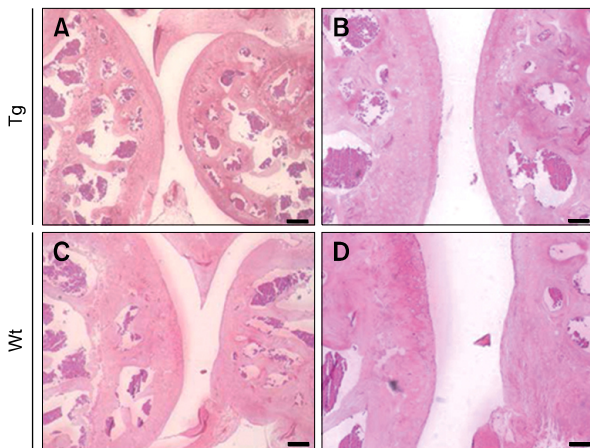

Figure 3. The over-expression of EC-SOD in the arthritic joint prevents destruction of cartilage and bone erosion. Histologic examination showed that the joints of wild type mice exhibit severe cartilage destruction and bone erosion. Mice were sacrificed on day 42 after the primary immunization. The left hind foot, which received collagen boosting, was excluded. The joint sections from transgenic mice (A, B) and wild type mice (C, D) are shown. The signs of tissue destruction were not seen in EC-SOD transgenic mice. Scale bars: in A, C, 100 μm; in B, D, 200 μm (A, B, magnification, ×20; C and D, magnification, respectively).

Pathological role of the EC-SOD over-expression in arthritic mice

To further analyze the pathologic role of the over-expression of EC-SOD in arthritic condition, the arthritis was induced by type II collagen immunization in transgenic mice. After arthritis induction, the clinical progressions were observed and scored until day 56. All mice showed detectable symptoms. However, transgenic mice showed delayed disease onset and the clinical scores were also significantly lower (Figure 2) as compared to the wild type mice ($P < 0.05$).
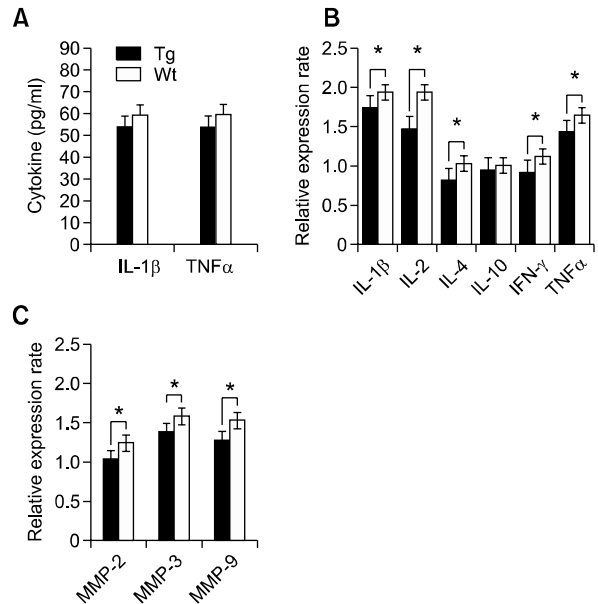

Figure 4. Cytokines and MMPs were decreased in FLS from EC-SOD transgenic mice during arthritis. The Levels of IL-1β and TNFα were determined in peripheral blood by ELISA (A). There is no significant difference between transgenic and wild type mice. For further examination, several cytokines (B) and MMPs (C) in FLS were tested by RT-PCR. Relative expression levels of each cytokines and MMPs were measured by the Image J software 1.38. Whole cytokines and MMPs were repressed in FLS from EC-SOD transgenic mice but not IL-10 (*$P < 0.05$ vs arthritic wild type mice).

Histological analysis

Histologic examination showed that the joints of wild type mice were heavily cartilage destruction and bone erosion (Figure 3, lower panel). In contrast, sections from transgenic mice showed no signs of tissue damage except for mild hyperplasia of the synovium (Figure 3, upper panel).

The change of cytokines and MMPs in peripheral blood and arthritic FLS

The levels of several cytokines and matrix metalloproteinases (MMPs) were also tested in transgenic mice. The concentrations of IL-1β and TNFα in peripheral blood were measured by ELISA. No significant difference between transgenic and wild type mice could be found (Figure 4A). Because EC-SOD was over-expressed in synovial tissue, various cytokines including IL-1β, TNFα and MMPs in fibroblast-like synoviocyte from transgenic mice were also tested. FLS from inflamed paws re-stimulated by type II collagen and RT-PCR was performed. The expressions of IL-1β and TNFα were suppressed in the FLS of transgenic mice (Figure 4B). In addition, the expression levels of IL-2, IL-4 and IFNγ were decreased in transgenic

mice. Moreover the expression patterns of MMPs in arthritic FLS were also depressed in transgenic mice FLS (Figure 4C).

Discussion

In the present study, the induction of arthritis in transgenic mice, which over-express EC-SOD in synovial tissue, showed suppressed incidence and development of disease. These protective effects were not limited to the inhibition of key pro-inflammatory cytokines, such as IL-1β and TNF-α, but included significant protection of the cartilage and bone.

The importance of maintaining a balance between oxidants and antioxidants in inflammatory diseases has been established by the amelioration of collagen-induced arthritis (CIA) by administration of EC-SOD through gene transfer or by treatment with an SOD mimetic (Salvemini *et al.*, 2001). The EC-SOD deficient mice had shown much more severity in CIA (Ross *et al.*, 2004). An imbalance in a disease state may be secondary to enhanced production of oxidants, to the decreased presence of antioxidants, or to a combination of these abnormal conditions. It is possible that decreased levels of EC-SOD in the joints of rheumatoid arthritis patients may contribute to more severe inflammation and tissue destruction (Slot *et al.*, 1986). Although these studies suggested the importance of EC-SOD in inflammatory arthritis, there were no transgenic researches of EC-SOD in synovial tissue.

In a previous study, EC-SOD transgenic mouse reduced the incidence of tumor formation in the DMBA/TPA two-stage carcinogenesis model (Kim *et al.*, 2005). Furthermore, engraftment of the EC-SOD transgenic mouse embryonic fibroblast repressed inflammatory cytokines and the CII-specific T cells (Yu *et al.*, 2008). In this study, the transgenic mice over-expressed EC-SOD in joint tissue and this protein was located in the lining layer of the synovium and endotherial cells in the sub lining. These results mean that EC-SOD transgenic mice could be a candidate for inflammatory arthritis. This hypothesis was tested in transgenic mice by CIA. During the disease progression, EC-SOD transgenic mice showed delayed disease incidence and the suppressed disease severity in CIA. Interestingly, no difference could be found between IL-1β and TNF-α in peripheral blood from both transgenic and wild type mice. But these cytokines were down-regulated in fibroblast-like synoviocyte (FLS) in transgenic mice. In addition, other pro-inflammatory cytokines such as IL-2,

IL-4, and IFN-γ were also decreased in FLS. This means the difference in disease severity was accompanied by changes in cytokine production, particularly in the joints. FLS play critical roles in normal embryogenesis and mature joint functioning. In RA, however, FLS take on both a different character and set of roles. The FLS in RA (RA-FLS) can be expended in cell culture over several passages and they escape from contact inhibition. These changes are often referred to as those of a tumor-like transformation since they result in aggressive and invasive behavior of RA-FLS in the adjacent cartilage and bone (Huber *et al.*, 2006). Activation of FLS by a broad array of soluble factors and cell surface interactions overrides homeostatic function, promoting synthesis of molecules that help mediate joint destruction and inflammation (Mor *et al.*, 2005). Inflammatory mediators that stimulate FLS include soluble, membrane-associated, and extracellular matrix-associated cytokines, chemokines, growth factors, and bioreactive lipids synthesized by both infiltlating leukocytes and FLS themselves. Several key cytokines that act on FLS such as TNF-α and IL-1β produced by macrophage (Bombara *et al.*, 1993; Nakahara *et al.*, 2003) , IL-17 and IFN-γ produced by T cells (Tasi *et al.*, 1996; Kehlen *et al.*, 2003; Hwang *et al.*, 2004). Furthermore, in a recent study, the capacity of synovial cells to produce the superoxide anion was probed. A low level of constitutive superoxide production in RA cells was also observed in immortalized chondrocytes and endothelial cells (Moulton *et al.*, 1997; Ago *et al.*, 2004) and the superoxide anion in synovial cells was produced via an NADPH oxidase pathway (Chenevier-Gobeaux *et al.*, 2006). These factors suggest the over-expression of EC-SOD in FLS would provide a better approach to treat RA.

The histological change and the expression of matrix metalloproteinases (MMPs) in FLS from arthritic mice were also analyzed. As shown in these results, tissue damages and bone erosion were not shown in EC-SOD transgenic mice and the levels of MMPs were also depressed in FLS. MMPs are Zn-containing endopeptidases that are involved most prominently in tissue remodeling. Pro-inflammatory cytokines, growth factors and matrix molecules induce the expression of MMPs via transcriptional activation. In one model, patient derived RA-FLS co-implanted with human cartilage plugs under the renal capsule of SCID mice showed histologic evidence of FLS-mediated cartilage erosion. Additionally, this cartilage erosion was inhibited by transfection of constructs that blocked matrix-degrading enzymes such as MMP-1 or cathepsin L (Rutkauskaite *et al.*, 2004;

Schedel *et al.*, 2004). Thus, histological protection in transgenic mice under *in vivo* arthritic condition would be due to the suppression of MMPs by EC-SOD over-expression in FLS.

In conclusion, these data suggest that the over-expression of EC-SOD in FLS contribute to the activation of FLS and protection from joint destruction by depressing the production of the pro-inflammatory cytokine and MMPs. These results provide EC-SOD transgenic mice would be a useful animal model for further inflammatory arthritis research.

Methods

Animals

DBA/1 mice, 6-8-week-old, were purchased from Charles River (Yokohama, Japan). The EC-SOD transgenic mice have been generated in a previous study (Kim *et al.*, 2005). These mice were backcrossed with wild type DBA/1 mice through at least 6 generations.

Over-expression of EC-SOD in the synovial tissue

The total RNA was extracted from the joint tissue of transgenic mice with TRIzol reagents (M.R.C, CA), according to the manufacturer's protocol. The cDNA synthesis was performed using a reverse transcription system (Promega, CA). Synthesized cDNA was amplified with the following primers; EC-SOD, forward: 5'-CGG TTG AGA TAG ACA GG-3', reverse: 5'-TTA AGT GGT CTT GCA CTC G-3'. Immunohistochemical staining of synovium was performed on 5 μm sections of formalin-fixed paraffin-embedded blocks. The sections were mounted on glass slides, deparaffinized in xylene, and rehydrated in a graded series of ethanol. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide. After blocking the nonspecific binding by treating the slides with blocking solution (Santa Cruz, CA) at room temperature for 30 min, the slides were incubated with goat anti-mouse SOD-3 anti-body (Santa Cruz, CA) at a 1/50 dilution overnight at 4°C. The sections were washed and incubated with the secondary anti-body, biotinylated goat anti-mouse IgG (Santa Cruz, CA). After washing and incubating, the sections were incubated with horseradish peroxidase conjugated streptavidin at room temperature for 30 min. Diaminobenzidine was added to reveal the Ag. The sections were dehydrated, cleared, and mounted.

Induction of arthritis and assessment

Age-and sex-matched male DBA/1 and EC-SOD transgenic mice were immunized with bovine type II collagen (CII, Chondrex, WA) at 8-12 weeks of age, as described previously. Briefly, CII was dissolved as 0.25% in 0.01 N-acetic acids. Mice were immunized on day 0 with an intradermal injection of 100 μg CII, emulsified in a complete Freund's adjuvant (CFA). On day 14, the mice were boosted into the left footpad with the same amount of CII in an incomplete Freund's adjuvant (IFA). Three weeks after primary immunization, the clinical scores of the mice were examined two to three times a week for the onset and severity of arthritis. The severity of arthritis was scored in a double-blind manner, with each paw exclude collagen injected paw, assigned a separate clinical score as follows: 0 = normal; 1 = erythema and mild swelling confined to the ankle joint and toes; 2 = erythema and mild swelling extending from the ankle to the midfoot; 3 = erythema and severe swelling extending from the ankle to the metatarsal joints; and 4 = ankylosing deformity with joint swelling (Myers *et al.*, 1993; Jung *et al.*, 2010).

Histological assessment of the arthritic mice

For a histological examination of the joints, the right hind limb was harvested from each mouse and fixed in 10% buffered formalin on day 42. The limbs were decalcified in a histological decalcifying agent (Calci-Clear Rapid, National Diagnostics, GA), embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H & E). The fixed and stained slides of the joint sections were read by a trained observer.

ELISA for IL-1β and TNFα in peripheral blood of arthritic mice

Blood samples from arthritic mice were prepared by the eye-bleeding method on day 42, post-immunization. The amount of IL-1β and TNFα in sera was measured by using commercially available ELISA kits (Quantikine Mouse IL-1β and TNFα immunoassay, R & D Systems, MN). Optical absorbance was measured using an ELISA reader at 450 and 540 nm.

Cytokine and matrix metalloproteinases (MMPs) activities in synovial tissue of arthritic mice

Fore and hind paws were removed at the ankle joint, the skin was removed from the inflamed paws, and the remaining tissue was carefully recovered with a scalpel in a volume of 1,000 μl PBS. The synovial tissue was digested with 2.5% collagenase (Sigma) for 4 h at 37°C to obtain a single cell suspension. The fibroblast-like synoviocyte (FLS), from passages 3 through 7 were seeded in 24-well plates at 2×10^4 cells/well in DMEM supplemented 10% FBS and cultivated at 37°C for 24 h. The cells were stimulated with type II collagen (Condrex) for 48 h. For the analysis of cytokine and MMPs patterns, total RNA was extracted from FLS. Synthesized cDNA was amplified with PCR. The primer sequences for RT-PCR are like Supplemental Data Table S1. PCR products were screened in 1% agarose gel and relative expression levels of each cytokines were measured by the Image J software 1.38.

Statistical analysis

Data are presented as mean $\pm$ SD. Comparisons of the numerical data between groups were performed by the paired or unpaired Mann-Whitney *U* test. *P*-values < 0.05 were considered statistically significant.

Supplemental data

Acknowledgements

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