The role of TNF-α in the pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera

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Abstract

Objective. In order to elucidate which cytokine preferentially stimulates the synovium in patients with rheumatoid arthritis (RA), we investigated the roles of tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6) using SCID mice engrafted with human RA tissue (SCID-HuRAg).

Methods. The SCID-HuRAg mice were prepared according to our previously described method. First, SCID-HuRAg mice were treated with chimeric anti-TNF-α monoclonal antibody (mAb, 100 µg/mouse) and histological changes were examined 4 weeks after the initial treatment. Secondly, a total of 100 µg of recombinant TNF-α or IL-6 (0.6 µg/h) was administered daily to mice using an osmium pump. The histological changes and serum cytokine levels were examined 4 weeks after the initial administration. Human immunoglobulin G (IgG) was administered to mice as a control.

Results. Synovial inflammatory cells were significantly decreased after the anti-TNF-α mAb treatment; conversely, the degree of synovial inflammation was significantly exacerbated by TNF-α administration. The levels of both IL-6 and TNF-α in sera were significantly increased by recombinant TNF-α administration, while TNF-α levels were unchanged by IL-6 administration. This suggests that TNF-α controls IL-6 production. Despite the profound changes in inflammation, we found no effects on bone and no articular cartilage damage was produced by TNF-α.

Conclusion. This study provides strong evidence that TNF-α is a key molecule in the control of the inflammatory changes that occur in the RA synovium. In addition, TNF-α regulates IL-6 production. However, other inflammatory pathways independent of TNF-α may contribute to the bone and cartilage damage seen in RA.

Key words: Rheumatoid arthritis, SCID mouse, Tumour necrosis factor, Interleukin-6, Pathology.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of the synovium and an excess of inflammatory cells, leading to progressive destruction of the joints. In RA, several cytokines, e.g. interleukin (IL)-1, IL-6, IL-8, IL-12, IL-17, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and granulocyte–macrophage colony-stimulating factor (GM-CSF), are involved in almost all aspects of articular inflammation and destruction [1]. TNF-α has been considered a pivotal cytokine in the pathogenesis of RA, as significant clinical and laboratory evidence has been obtained by TNF-α blockade [1–3]. Moreover, it has been verified that neutralized monoclonal antibodies (mAb) against TNF-α can reduce the production of other proinflammatory cytokines, such as IL-1 and GM-CSF, in cultured RA synovia [4]. In the clinical trials performed to date, anti-TNF-α mAb has been effective in the majority of treated RA patients. Moreover, anti-TNF-α mAb may reduce tissue perfusion in the inflamed synovium, according to the results.

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of a concomitant study using nuclear magnetic resonance monitoring [5]. However, there is only one paper that has shown that the characteristic histopathological findings of RA change after treatment with anti-TNF-\( \alpha \) mAb [6]. Recently, many researchers and clinicians have considered whether anti-TNF-\( \alpha \) mAb treatment should focus on patients with elevated concentrations of C-reactive protein (CRP) or IL-6 [7].

IL-6 has also been considered to correlate with disease activity in RA, as it has been shown to stimulate the secretion of immunoglobulin by plasmacytes, activate and promote the proliferation of T lymphocytes, increase neutrophils and platelets, induce acute-phase proteins such as CRP, fibrinogen, haptoglobin and serum amyloid-A, regulate the proliferation and differentiation of osteoclasts, and induce bone resorption [8]. However, there is no direct comparative study in humans that has addressed the importance of TNF-\( \alpha \) vs IL-6 in the pathogenesis of cartilage and bone destruction. Therefore, in order to examine the roles of these cytokines in RA patients, we conducted a treatment study using the severe combined immunodeficiency (SCID) mouse, into which human RA tissues have been grafted (SCID-HuRAg) [8–10]. In addition, we examined the relationship between TNF-\( \alpha \) and IL-6 levels in the sera of SCID-HuRAg mice.

Materials and methods

Cytokine concentration in cultured synovial supernatant

The effects of TNF-\( \alpha \) and IL-6 on cytokine (TNF-\( \alpha \), IL-6 and IL-1) production in synoviocytes were examined as described in our previous reports [11, 12]. Samples of human synovia were collected from RA patients who fulfilled the revised criteria of the American Rheumatism Association at the time of total knee replacement surgery or synovectomy. We obtained informed consent from each patient before entry into the study. The synoviocytes were isolated and were cultured as described in our previous report [11, 12]. Briefly, synovial tissues were minced into small pieces and digested with 1 mg/ml of collagenase (type I; Sigma, St Louis, MO, USA) in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Rockville, MD, USA) for 2 h at 37°C. Synoviocytes were extensively washed, and then cultured in Ham’s F-12 (Gibco BRL) supplemented with 10% foetal bovine serum (FBS) (Cell Culture Technologies, Zurich, Switzerland), penicillin, streptomycin and 2.5 \( \mu g/ml \) of fungizone (F10F) in a humidified 5% CO\(_2\) atmosphere at 37°C. The synoviocytes were used from passages 2–5 in this experiment.

The large mononuclear cells were adjusted to a concentration of 1 \( \times 10^6/ml \) and 1 ml of this suspension per well was dispensed in 24-well plates. After overnight incubation, the plates were washed with phosphate-buffered saline (PBS) and the cells were resuspended in 1 ml of culture medium. The resulting adherent cells were cultured for 12 h at 37°C in the presence or absence of the indicated dose of recombinant human TNF-\( \alpha \) (100 ng/ml) or recombinant human IL-6 (100 ng/ml). Purified recombinant human TNF-\( \alpha \) was a gift from Asahi Chemical Industry (Tokyo, Japan). It was cloned from HL-60 cells and expressed in Escherichia coli. Recombinant human IL-6 was obtained from Ajinomoto (Tokyo, Japan). After incubation, the culture supernatants were collected and the cytokines were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (TNF-\( \alpha \) and IL-6, Gibco BRL, Island, NY, USA; IL-1, Sigma, St Louis, MO, USA). The results are expressed in ng/ml relative to standards included in the test kits.

Preparation of SCID-HuRAg mouse

SCID-HuRAg mice, which we had developed previously, were evaluated as a model for this study [8–10]. Briefly, 6–7-week-old male SCID mice (CB.17.Icr; Charles River Japan, Tokyo, Japan), which had been bred under specific pathogen-free conditions at our University Animal Center, were used for the experiments. Pannus tissue from the synovial membrane, articular cartilage and bone, collected as a mass from RA patients at the time of surgery, was used for implantation. A piece of transplant tissue was trimmed to a block of 4–8 mm in diameter prior to implantation. The tissue implants were grafted subcutaneously into the back of the mouse. After the subcutaneous tissue had been exposed, the oblique paraspinal muscle was scraped with a scalpel until the muscle bled. The graft was then placed on the muscles at the level of the 4th–6th lumbar vertebra. All surgical procedures were performed under sterile conditions. Optimal transplantation was achieved when all operative procedures were completed within 1 h. Successful implantation of human RA tissue was observed by visual assessment 4 weeks after implantation, after which experimentation became possible [8–10].

Protocol of anti-TNF-\( \alpha \) mAb treatment

The chimeric anti-TNF-\( \alpha \) mAb (cA2; Centocor, Great Valley Parkway Molvet, PA, USA) treatment protocol with SCID-HuRAg mice was also performed as described in our previous reports [8–10]. Sixteen SCID-HuRAg mice were used for this treatment study. The initial treatments were started 4 weeks after implantation, when it was demonstrated that the implanted tissue was accepted by the SCID-HuRAg mouse [8–10]. The anti-TNF-\( \alpha \) mAbs were administered by single subcutaneous injections into the implanted tissue at a dose of 100 \( \mu g \) in eight SCID-HuRAg mice. The other eight SCID-HuRAg mice were injected with the same dose of anti-human IgG1 mAbs (BioPur; Bubendorf, Switzerland) as a control. The mice were anaesthetized with methoxyflurane, according to the guidelines established by our university’s animal ethics committee, and were euthanized 4 weeks after injection in order to allow removal of the implanted tissue. After the implanted tissue had been removed, it was fixed in 4%
paraformaldehyde. After decalcification with EDTA, each tissue sample was cut into sections 6 µm thick, and stained with haematoxylin and eosin (HE) at room temperature.

The degree of synovial inflammation was assessed as described in our own and others’ previous reports [13–16]. The histopathological findings were divided into individual findings (destruction of articular cartilage, synovial giant cell, lymphocytes follicle, fibrosis, diffuse lymphocytes infiltration, neovascularization, synovial hyperplasia), and each relative histological finding was then assessed using an arbitrary score on a scale from 0 to 3, where 3 indicated the histological findings of control mice, 2 indicated lesser features compared with the control mice, 1 indicated minimal features, and 0 indicated that such features were not detectable.

Histological assessments were studied under double-blind conditions. Animal researchers and pathologists recorded the data on separate case record forms without exchanging any information until the conclusion of the study. Finally, the data reported by each doctor were compiled for analysis of results. Furthermore, in accordance with our previous reports [8], the number of inflammatory cells per unit was counted using Mac-scope (Mitani, Fukui, Japan), computer graphic analysis software used for histopathological specimens. With this system, a target cell is first selected on a computer display, and cells with similar characteristics (colour, size and shape) are counted. When the cell selection is done precisely, the specificity and sensitivity are close to 1. Three measurements were made for each unit area, and the mean values were then calculated.

Administration protocol of TNF-α and IL-6

Twenty-four SCID-HuRAg mice were used for the cytokine administration study. Four weeks after transplantation of rheumatoid synovium, recombinant human TNF-α (Asahikasei Chemical Industry, Tokyo, Japan) and IL-6 (Ajinomoto, Tokyo, Japan) were administered to SCID-HuRAg mice using osmium pumps (Alza, CA, USA). The mice were anaesthetized with methoxyflurane and each animal received a surgically inserted osmium pump on its back. All surgical procedures were performed under sterile conditions. Each cytokine was diluted in 100 µl of endotoxin-free RPMI-1640 containing 10% foetal calf serum (FCS) (Gibco, New York, NY, USA) and a total of 100 µg of recombinant TNF-α or IL-6 was administered daily to each mouse using the osmium pump (0.6 µg/h, total 7 days), as similar doses of cytokines have been shown to have proinflammatory effects [2, 8, 12, 17, 18]. The control mice received RPMI containing 10% FCS instead, but in the same manner. Eight SCID-HuRAg mice were used for each treatment group. The mice were anaesthetized with methoxyflurane and were euthanized 4 weeks after cytokine administration in order to allow removal of the implanted tissues. After the implanted tissues had been removed, the tissues were fixed in 4% paraformaldehyde. After decalcification with EDTA, each tissue block was cut into sections 6 µm thick and stained with HE. The histopathological findings were divided into the individual findings described above. Each animal’s histological findings were assessed using an arbitrary score from 0 to 3. However, in this analysis, the histological findings of the control mice were scored as grade 1, non-detectable was represented by a score of 0, 2 indicated a more severe condition compared with control mice, and 3 indicated the most severe condition.

Measurement of human TNF-α and IL-6 in mouse serum

The serum levels of TNF-α or IL-6 were measured according to the method described in our previous report [8]. Briefly, mouse blood was obtained by heart puncture on week 4 after cytokine administration. IL-6 was measured by the chemiluminescence enzyme technique (Fuji Rebio, Osaka, Japan), with a two-step sandwich method using anti-human IL-6 mAbs with a solid phase on a ferrite particle, in which only human-type IL-6 was measured. TNF-α was also measured by ELISA using a specific human TNF-α mAb [19].

Statistical analysis

Each in vitro experiment was performed five times. The results are expressed as the mean ± s.d. (standard deviation). The Mann–Whitney test was used to examine the differences between two independent groups. A multiple comparative study was conducted with Dunnett’s test. P < 0.05 was considered to be statistically significant.

Results

Measurement of cytokines in synovial culture medium

The production of cytokines (TNF-α, IL-6 and IL-1) in cultured synoviocytes was examined after human recombinant TNF-α and IL-6 administration. TNF-α stimulated the production of cytokines (TNF-α, IL-6 and IL-1) in cultured synoviocytes, though IL-6 did not have this effect on cytokine production in cultured synoviocytes (Fig. 1).

Features of implanted tissues after anti-TNF-α mAb treatment

The implanted tissue grew 1.0- to 2.0-fold in size by 8 weeks after implantation in the control group, just as we noted in our previous study [8]. In the anti-TNF-α-treated group, the volume of the implanted tissue was significantly reduced to 0.6 of the value in the control group (Fig. 2A). The histological features of RA (such as the proliferation of synovial villi, stratification of synovial cells, infiltration of inflammatory cells and formation of lymphoid follicles) were preserved in the control group. In contrast, synovial tissues were transformed to a great extent into adipose or fibrous tissues in the anti-TNF-α mAb-treated group (Fig. 3). The number of inflammatory cells (monocytes, lymphocytes and granulocytes) was significantly decreased by
the anti-TNF-α mAb treatment (Fig. 2B). Moreover, the degrees of neovascularization and synovial hyperplasia were markedly decreased and the number of synovial giant cells and the degree of fibrosis were moderately decreased by treatment with anti-TNF-α mAb. However, the degree of articular cartilage destruction and lymphoid follicle were unchanged compared with control mice (Fig. 4). Simultaneously, tartrate-resistant alkaline phosphate staining was performed in order to investigate the number of osteoclast cells, as described in our previous report [8]. However, a statistically significant difference was not observed between the control mice and anti-TNF-α mAb-treated mice (data not shown).

Features of implanted tissues after TNF-α and IL-6 administration
Two of the TNF-α-treated mice died during the administration part of the study. Therefore, six mice were used for the analysis of TNF-α administration and eight were used in the study involving IL-6 and control vehicle administration. The implanted tissue grew 1.0- to 2.0-fold in size after implantation in the control group; however, no significant differences were found among the treated groups (recombinant human TNF-α, recombinant human IL-6 and control) in the volume of implanted tissues 8 weeks after engraftment (Fig. 5A). In contrast, the degree of synovial inflammation was clearly different between the cytokine-treated groups and the control group. In the group that received both recombinant human TNF-α and recombinant human IL-6, a significant increase in the number of inflammatory cells was noted compared with the control group (Figs 5B and 6).

Histological features of RA were exacerbated by treatment with TNF-α and IL-6
Among the characteristic histological features of RA, synovial hyperplasia, neovascularization and diffuse lymphocyte infiltration were markedly changed by TNF-α administration. Moreover, synovial giant cells and fibrosis were moderately changed by TNF-α
administration. However, the destruction of articular cartilage and lymphoid follicle were unchanged by TNF-α. Marked diffuse lymphocytic infiltration was also observed after IL-6 administration. In addition, moderate changes in fibrosis, neovascularization and synovial hyperplasia were induced by IL-6 administration. In contrast, changes in the destruction of articular cartilage, lymphoid follicles and synovial giant cells induced by IL-6 were inconspicuous. The synovial histological changes were more remarkable after TNF-α administration than after IL-6 administration (Fig. 7).

Levels of TNF-α and IL-6 in sera

As expected, human TNF-α and IL-6 were not detected in the ungrafted SCID mouse sera (Fig. 8). However, the levels of these cytokines in the sera were increased significantly in the transplanted SCID mice, suggesting new production by the grafts, as we have reported previously [8]. Moreover, in the SCID-HuRAg mice treated with recombinant human TNF-α, the levels of both TNF-α and IL-6 were significantly increased compared with the untreated control SCID-HuRAg mice. On the contrary, in the SCID-HuRAg mice treated with recombinant human IL-6, the levels of IL-6 were significantly increased but the levels of TNF-α were unchanged compared with control animals.

Discussion

Both TNF-α and IL-6 have been considered important cytokines in the pathogenesis of RA [1, 8]. Therefore, there have been numerous studies on these cytokines themselves and the use of anti-cytokine therapies in animal models of arthritis and in patients with RA [1–8, 19–21]. In particular, TNF-α has been considered the pivotal cytokine in the pathogenesis of RA, as TNF-α is present at biologically significant levels in RA synovial tissue and fluid, but not in osteoarthritis synovia or systemic lupus erythematosus kidney tissue. Furthermore, the levels of TNF-α in RA synovia seem to parallel the extent of both inflammation and bone erosion [22, 23]. Recent studies have suggested that the biological effects of TNF-α are caused by direct actions on multiple target tissues and the induction of other cytokines, such as IL-1, IL-6 and IL-8 [1, 24].

IL-6 is another proinflammatory cytokine with multiple functions and is believed to play a pivotal pathogenic role in RA, given that it is closely related to disease activity in such patients [8, 25–27]. However, it has been pointed out that TNF-α and IL-6 have overlapping and synergistic actions, even though some of the effects of these two cytokines are regulated by distinct mechanisms [1, 24].
Therefore, we would like to try to determine the individual role of each cytokine in vitro and in vivo. In cultured synoviocytes, the production of cytokines (TNF-α, IL-6 and IL-1) was significantly induced by TNF-α administration. However, IL-6 did not have this effect. In SCID-HuRAg mice, synovial inflammation was grossly exacerbated by the administration of recombinant TNF-α and IL-6. However, the degree of synovial histological changes was more severe after TNF-α administration compared with IL-6. Moreover, this study indicates that TNF-α can increase the production of IL-6 while IL-6, in contrast, does not increase the production of TNF-α in vivo. These results confirm in vivo a hierarchy between TNF-α and IL-6, as previously reported in vitro [1, 4, 24]. Therefore, the SCID-HuRAg mouse is considered to be a useful model for the histological examination of biological agents. However, there is considerable heterogeneity among RA synovial tissues concerning the distribution of cells and the production of cytokines. To solve the problem of heterogeneity in individual samples, for each examination RA tissue samples were obtained from the same RA patients and were compared, i.e. individual RA samples were divided into groups of the same size and were implanted into control groups and treatment groups.

On this basis, a series of therapeutic trials using TNF-α blocking agents has been carried out. Recently, TNF-α blockade has been reported to prevent cartilage erosion and bone destruction in RA patients [1, 28].
Therefore, the second aim of our study was to investigate the relationship between TNF-α and bone and cartilage destruction. As mentioned, the synovial inflammatory condition was markedly improved by TNF-α blockade and, conversely, the degree of inflammation was significantly exacerbated by TNF-α administration in our model. However, no evidence of a preventive effect on bone and articular cartilage damage by TNF-α administration was observed.
by anti-TNF-α mAbs was obtained. In addition, direct TNF-α administration to the grafts again showed no evidence of bone and articular cartilage damage. It is possible that these results were related to the fact that the observation period was too short. However, it is also possible that pathological pathways other than TNF-α may be involved in the process of cartilage and bone destruction. This possibility is supported by the clinical observation that approximately 25% of patients treated with TNF-α-blocking agents do not respond to therapy [28]. In addition, several studies have indicated that perhaps IL-1 is more directly responsible for the development of joint erosions [29, 30]. Therefore, further studies using our SCID-HuRAg mouse model may be useful to confirm or refute this possibility. We considered a treatment study of the SCID-HuRAg mouse involving human chimeric IL-1 and/or IL-6 mAb, but unfortunately we cannot obtain these human chimeric mAbs. Further investigation using these mAbs is warranted.

References


