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Involvement of tumor necrosis factor-α and interleukin-8 in antigen-induced arthritis of the rabbit temporomandibular joint

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BACKGROUND: In temporomandibular joint (TMJ) arthritis, knowledge is limited about the source of the inflammatory mediators. The aim of this study was to investigate the immunohistochemical role of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) in the development of the antigen-induced arthritis of the rabbit TMJ.
METHODS: Unilateral TMJ arthritis was induced in 28 adult rabbits. From 6 h to 6 weeks after induction of arthritis, the topology of TNF-α and IL-8 was observed.
RESULTS: Positive reaction for TNF-α of synovial cells was observed within 3 days after induction and at 3 weeks after induction. TNF-α positive vascular endothelial cells and chondrocytes were identified throughout the observation period. IL-8 was detected only during the acute stage.
CONCLUSIONS: The cytokines TNF-α and IL-8 were observed in specific cells depending on the stage. TNF-α was particularly related with angiogenesis and cartilage destruction and IL-8 was involved in the acute stage of inflammation.
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Keywords: antigen-induced arthritis; immunohistochemistry; interleukin-8; rabbit; temporomandibular joint; tumor necrosis factor-α

Introduction

Tumor necrosis factor-α (TNF-α) is one of the most important pro-inflammatory cytokines that is related to immune and inflammatory responses. TNF-α has various biologic functions, i.e. production of antibodies from B cells, induction of cytokines, and prostaglandin E2 with macrophage activation, production of collagenase in synovial cells and induction of osteoclastic bone resorption (1, 2).

IL-8 is a member of ECR CXC chemokines that activates leucocytes (3, 4). IL-8 has various biologic activities on neutrophils *in vitro*, including direct and transendothelial migration, release of storage enzymes, induction of oxygen metabolites, and expression of adhesion molecules (3). IL-8 also attracts T lymphocytes *in vitro* (5).

Rheumatoid arthritis (RA) is a systemic inflammatory condition of the joints, and about 30–50% of patients with RA will experience symptoms of the temporomandibular joint (TMJ; 6). Osteoarthritis (OA) is also accompanied by a local inflammatory condition and the TMJ is not seldom involved. Basically arthritic conditions might have similar mechanisms in the development of inflammation although the etiologic factors are different among them (7, 8). To investigate the mechanisms of arthritis, various animal models of arthritis have been developed. Concerning the TMJ, some models have been tried, but it has been difficult to develop chronic arthritis similar to human arthritis. With the injection of adjuvant it has been possible to induce acute arthritis but the effect disappeared within a couple of days (9). Thus, it has been difficult to induce chronic TMJ arthritis in conventional polyarthritis models (10, 11), and some years ago, a method for antigen-induced chronic monoarthritis of the TMJ was developed (12), resulting in, i.e. proliferation of synovium and destruction of cartilage. By the aid of this model, we studied pro-inflammatory cytokines, i.e. IL-1β and IL-1 receptor antagonist immunohistochemically (13). Findings from the prior investigation suggested that this model might have characteristics relating to both acute and chronic arthritis.

TNF-α and IL-8 were detected in synovial tissue and synovial fluid from patients with RA or OA (14–19). These cytokines were also detected in synovial fluid of patients with temporomandibular disorders (TMDs) (20, 21). Recently, the relation between these cytokines and clinical findings were discussed concerning the TMD (22–24).

The aim of this study was to immunohistochemically investigate the source and the role of the pro-inflammatory

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Table 1 Immunohistochemical summary

	Acute stage				*Chronic stage*		
	Early phase		*Late phase*		*Early phase*		
	6 h	*1 day*	*3 days*	*1 week*	*2 weeks*	*3 weeks*	*6 weeks (late phase)*
TNF-α							
Exuded inflammatory cells	+	+	+	+	−	−	−
Synovial cells	+	+	+	−	−	+	−
Subsynovial fibroblasts	+	+	+	−	−	±	−
Vascular endothelial cells	+	+	+	+	+	+	−
Chondrocytes	+	+	+	±	±	+	+
IL-8							
Exuded inflammatory cells	+	+	+	±	−	−	−
Synovial cells	+	+	+	−	−	−	−
Subsynovial fibroblasts	+	+	−	−	−	−	−
Vascular endothelial cells	+	+	±	−	−	−	−
Chondrocytes	−	+	+	−	−	−	−

Intensity of staining: +, positive; ±, slightly positive; −, negative.
Four animals were sacrificed at each time point.

cytokines TNF-α and IL-8 in an arthritic model, and to compare these cytokines with a prior study regarding IL-1β.

Materials and methods

Materials

Twenty-eight adult male New Zealand white rabbits (weighing 3.5–4.0 kg) were used in this study. The animals were bred in the Animal Care Center of Kyushu Dental College, and were kept at constant temperature and humidity during the experimental period. This animal experiment was performed under the guidelines and permission of the Animal Care Committee of Kyushu Dental College (2000-62).

Induction of arthritis

Induction of arthritis was performed as described previously (12). In brief, the animals were sensitized by two intracutaneous injections, 2 weeks apart, of ovalbumin (Sigma, St Louis, MO, USA) emulsified in Freund's complete adjuvant (Sigma, St Louis, MO, USA). After confirming the

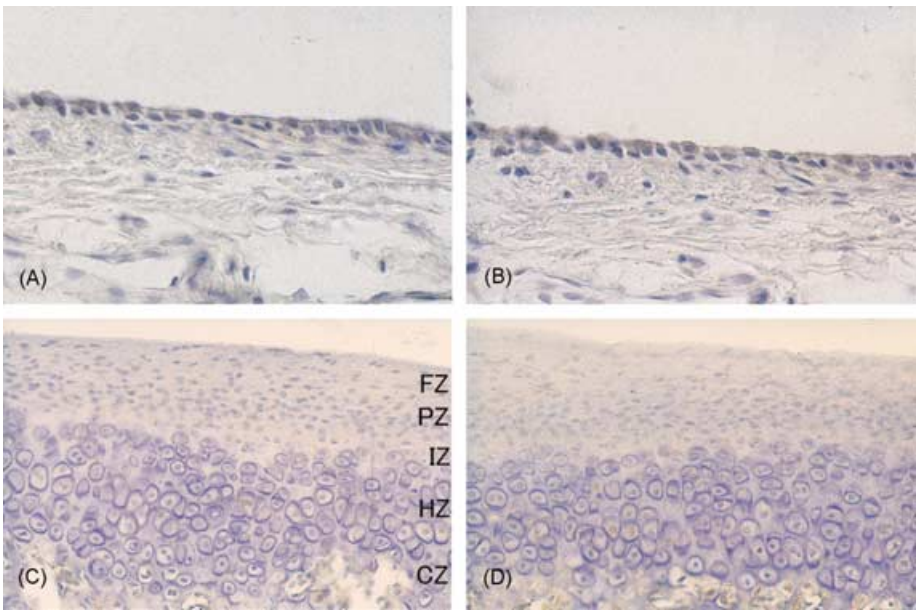

Figure 1 Immunohistochemical localization of TNF-α (left side) and IL-8 (right side) in control joint 3 weeks after sham induction. (A,B) The synovial and subsynovial layers are observed normally, synovial cells are in one layer, and no inflammatory cells infiltrate into these layers (×160). (C,D) The cartilage layer shows a normal morphology, which is divided into five layers from the surface: fibrous zone (FZ), proliferating zone (PZ), intermediate zone (IZ), hypertrophic zone (HZ), and calcifying zone (CZ) (×80). No cells are stained light brown, so they react negatively for TNF-α or IL-8 by immunohistochemistry.

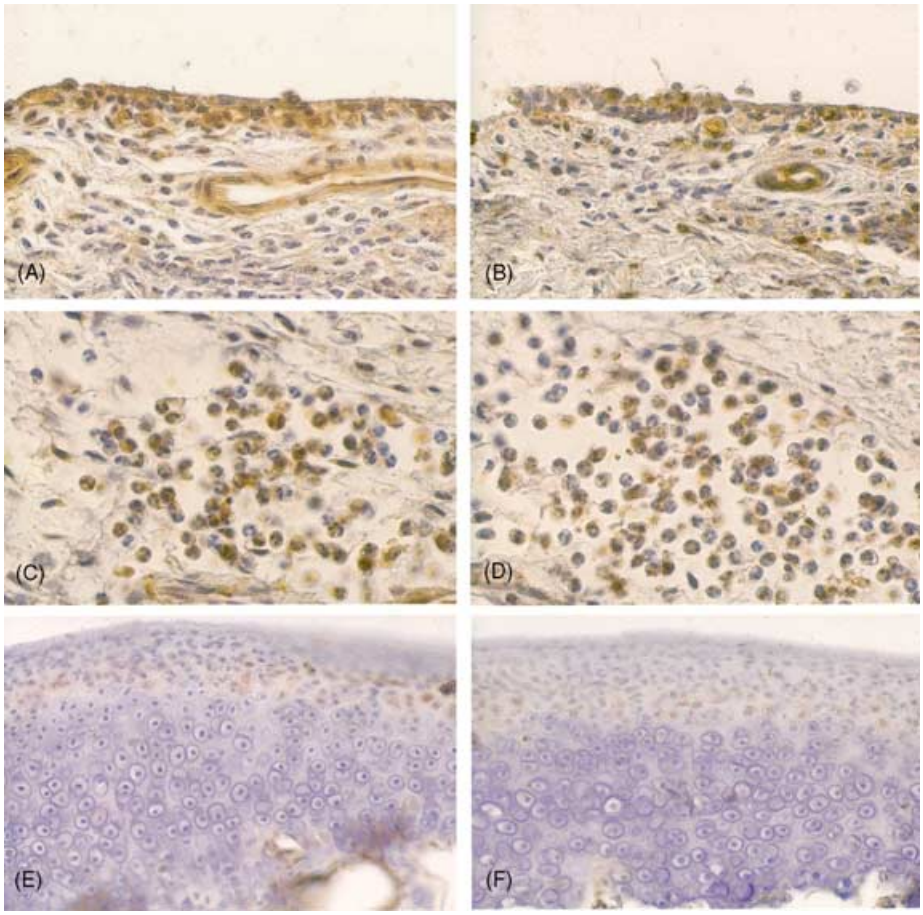

Figure 2 Immunohistochemical localization of TNF-α (left side) and IL-8 (right side) in arthritic joint 1 day after induction. All sections are stained with anti-rabbit TNF-α or anti-rabbit IL-8 antibody and the colors develop with DAB (positive cells are stained light-brown). Nuclei are counter-stained by hematoxylin. (A,B) Slightly thickened synovial layers accompanied with edema are positive for TNF-α and IL-8. Vascular endothelial cells in subsynovial layers are also positive for TNF-α and IL-8 (×160). (C,D) Exuded cells in subsynovial layers, which are predominantly polymorphonuclear leucocytes, react positively for TNF-α and IL-8 (×200). (E,F) The structure of the cartilage is normal, but chondrocytes in the proliferating zone are positive for TNF-α and IL-8 (×80).

establishment of a delayed hypersensitivity reaction, unilateral intra-articular injection of ovalbumin was administered into the TMJ. Saline was injected into the contralateral side as a sham induction.

Immunohistochemistry

Animals were sacrificed using an overdose of sodium pentobarbital (Nembutal®, Abbot Laboratory, IL, USA). Four animals were sacrificed at each of the following time points: 6 h, 1 day, 3 days, 1 week, 2 weeks, 3 weeks, and 6 weeks after induction of arthritis. Bilateral TMJ's samples were taken, fixed in 4% paraformaldehyde for 24 h, decalcified with a 10% EDTA solution and embedded in paraffin. Serial sagittal sections (4 μm) were cut. After blocking endogenous peroxidase with 0.3% H_2O_2 in methanol, the sections were treated with 5% normal rabbit serum and incubated overnight with 10 μg/ml of goat anti-rabbit TNF-α or 5 μg/ml of goat anti-rabbit IL-8 at 4°C (25). The sections were then rinsed and incubated for 30 min with 15 μg/ml biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA). The sections were rinsed again and incubated for 30 min with avidin–biotin–peroxidase complex (Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). As a chromogen, diaminobenzidine (DAB Reagent Set, KPL Laboratory, Gaithersburg, MD, USA) was used. Counter-staining was performed with hematoxylin. Negative control was obtained by replacing the first antibodies with diluted normal goat serum. All sections were reviewed under a light microscope. The immunostaining intensities of the sections were graded as negative (no staining in all sections), slightly positive (slightly visible staining in a few sections not but all sections), and positive (intense staining in almost all of the sections).

Results

The immunohistochemical results are shown in Table 1.

Sham-induced joints

At 6 h after saline injection, a few inflammatory cells were found in subsynovial layers. They were mainly composed of polymorphonuclear leucocytes and macrophages. Some of them reacted slightly positive against TNF-α and IL-8. No inflammatory changes and no TNF-α or IL-8 positive cells were observed in the joint (Fig. 1).

Six hours after induction of arthritis
Bleeding and edema were observed in the synovial and subsynovial layers. In a part of the synovium, two to three layers of proliferated synovial cells were also observed. The synovial cells, subsynovial fibroblasts, and vascular endothelial cells in the subsynovial layer reacted positively for TNF-α and IL-8. The infiltrated inflammatory cells were predominantly polymorphonuclear leucocytes, and some of them were macrophages. They reacted positively for TNF-α and IL-8. In the condylar cartilage, no change was found morphologically, while chondrocytes in the proliferating zone were positive for TNF-α.

One day after induction
Bleeding and exuded fibrin were still apparent in synovial and subsynovial layers. Synovial cells mostly proliferated to two to three layers with edema. Positive immunoreactivities for TNF-α in addition to IL-8 were found in synovial cells, subsynovial fibroblasts and vascular endothelial cells in the subsynovial layer. The exuded cells, which were positive for TNF-α and IL-8, were predominantly composed of polymorphonuclear leucocytes, but some macrophages and lymphocytes were also included. In the condylar cartilage, there was no observable morphologic change, but chondrocytes in the proliferating zone reacted positively against TNF-α and IL-8 (Fig. 2).

Three days after induction
Bleeding and edema in the subsynovial layer were less conspicuous and proliferation of fibroblasts and newly formed vascular endothelial cells were more obvious. Vascular endothelial cells were detected positive for TNF-α and slightly positive for IL-8. Infiltrated inflammatory cells were mainly lymphocytes and still positive for TNF-α in addition to IL-8. In the synovial layer, three to four layers proliferated and partly villi-formed synovium were observed. The synovium reacted positively against TNF-α in addition to IL-8. Subsynovial fibroblasts were only positive for TNF-α. In the condylar cartilage, there was no observable morphologic change, but chondrocytes in the proliferating and the intermediate zones reacted positively against TNF-α and IL-8 (Fig. 3).

One week after induction
Inflammatory cells, mainly lymphocytes, although decreased, were still observed in the subsynovial layer. They were positive for TNF-α and slightly positive for IL-8. The synovial layer thickened three to four layers, and villous

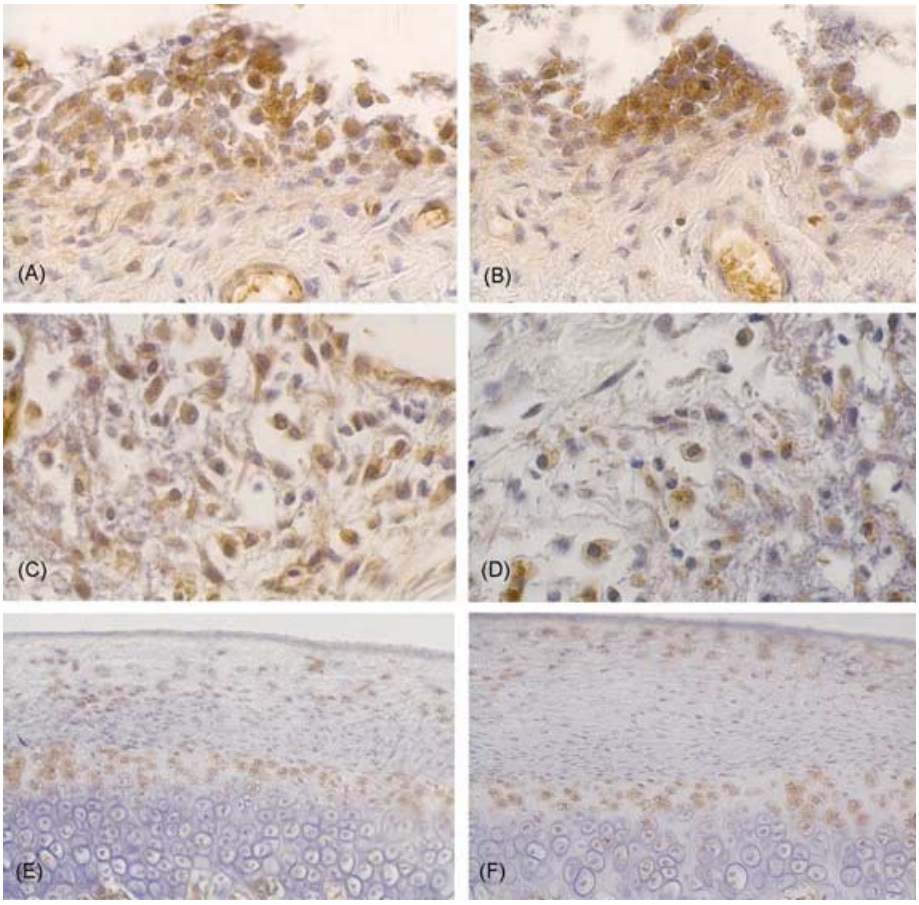

Figure 3 Arthritic joint 3 days after induction (left side; TNF-α, right side; IL-8). (A,B) Synovial cells proliferated three to four layers and peeled off, and vascular endothelial cells are positive for TNF-α and IL-8 (×160). (C) Most of subsynovial fibroblasts and infiltrated lymphocytes react positively for TNF-α (×200). (D) Infiltrated lymphocytes react positively for IL-8 but not subsynovial fibroblasts (×200). (E,F) TNF-α and IL-8-positive chondrocytes are mainly observed in the intermediate zone (×80). (Positive cells are stained light brown.)

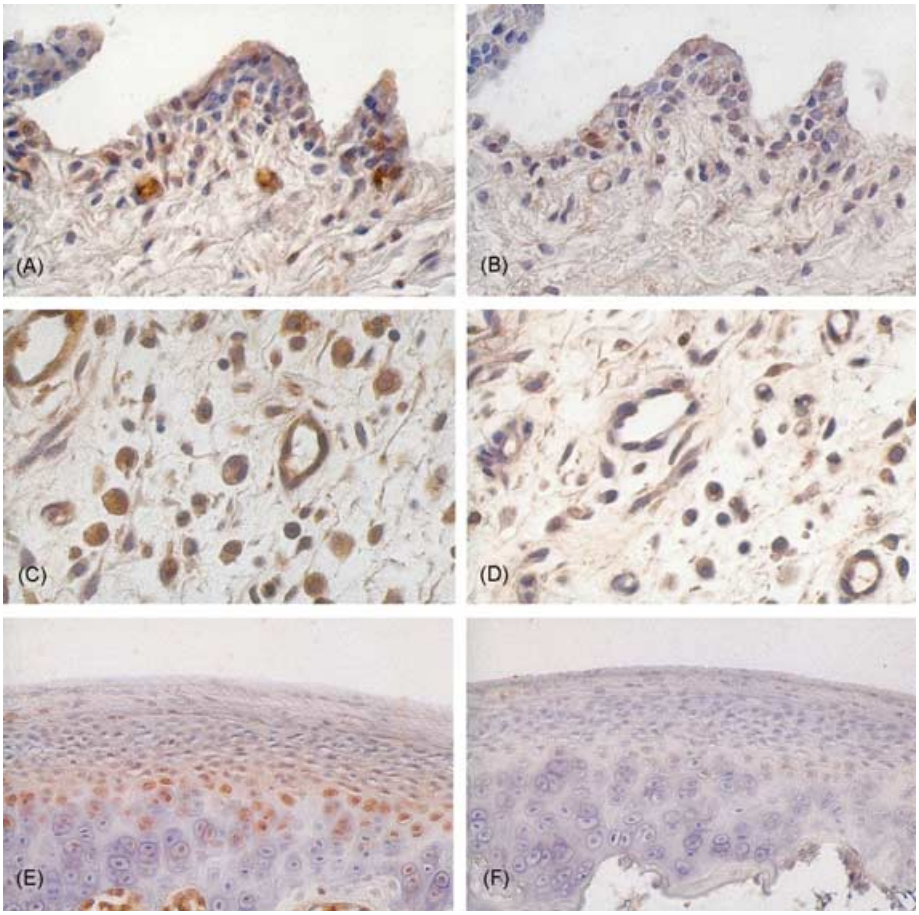

Figure 4 Arthritic joint 1 week after induction (left side; TNF-α, right side; IL-8). (A,B) Many newly formed vessels are observed in the areas of the villous formed synovium. Proliferated synovial cells are negative for TNF-α and IL-8. Vascular endothelial cells in subsynovial layers are positive for TNF-α, while negative for IL-8 (×160). (C,D) Most exuded inflammatory cells are positive against TNF-α but a few are positive against IL-8 (×200). (E,F) Chondrocytes from the intermediate zone to the hypertrophic zone react positively for TNF-α (×80). (Positive cells are stained light brown.)

formation was observed in some parts. These proliferated synovial cells and subsynovial fibroblasts were negative for TNF-α in addition to IL-8. It was clearly observed that newly formed blood vessels in subsynovial layers were positive for TNF-α. Chondrocytes that reacted slightly positively for TNF-β were identified from the intermediate zone to the hypertrophic zone (Fig. 4).

Two weeks after induction
A few lymphocytes, which reacted negatively against TNF-α and IL-8, were still observed in the subsynovial layer. In the synovium, three to four layers proliferated and appeared villous in part. Marked subsynovial fibrosis was observed. No positive immunoreactivities for TNF-α or IL-8 were found in the synovial and subsynovial layers except for the newly formed vascular endothelial cells in subsynovial tissue being positive for TNF-α. In the condylar cartilage, the fibrous zone tended to be thin. In some parts of the condylar cartilage, disorganization of chondrocytes was observed and they showed a slightly positive reaction against TNF-α.

Three weeks after induction
Inflammatory cells were scarcely observed and were negative for TNF-α and IL-8. The synovial layer thickened five to seven layers, and villous formation was observed in several areas. TNF-α positive cells could be observed in the synovial layers and in parts of the subsynovial fibroblasts. Positive reaction for TNF-α in the vascular endothelial cells was still observed. Chondrocytes of the cartilage were disorganized and were positive for TNF-α in various zones. No positive reaction for IL-8 in the components of the TMJ was found during this stage (Fig. 5).

Six weeks after induction
Four to five layers proliferated and villi-formed synovial layers were observed and were negative for TNF-α and IL-8. Severe disorganization of the collagen network between the chondrocytes resulting in cluster formation could be observed. TNF-α positive chondrocytes were mainly observed in the clustering areas. No positive reaction for IL-8 was found in this stage (Fig. 6).

Discussion

Biochemical analyses of various pro-inflammatory cytokines in synovial fluid from arthritic TMJ have revealed the mechanism of the arthritis (22–24, 26). However, there were some discrepancies among the results of these studies.

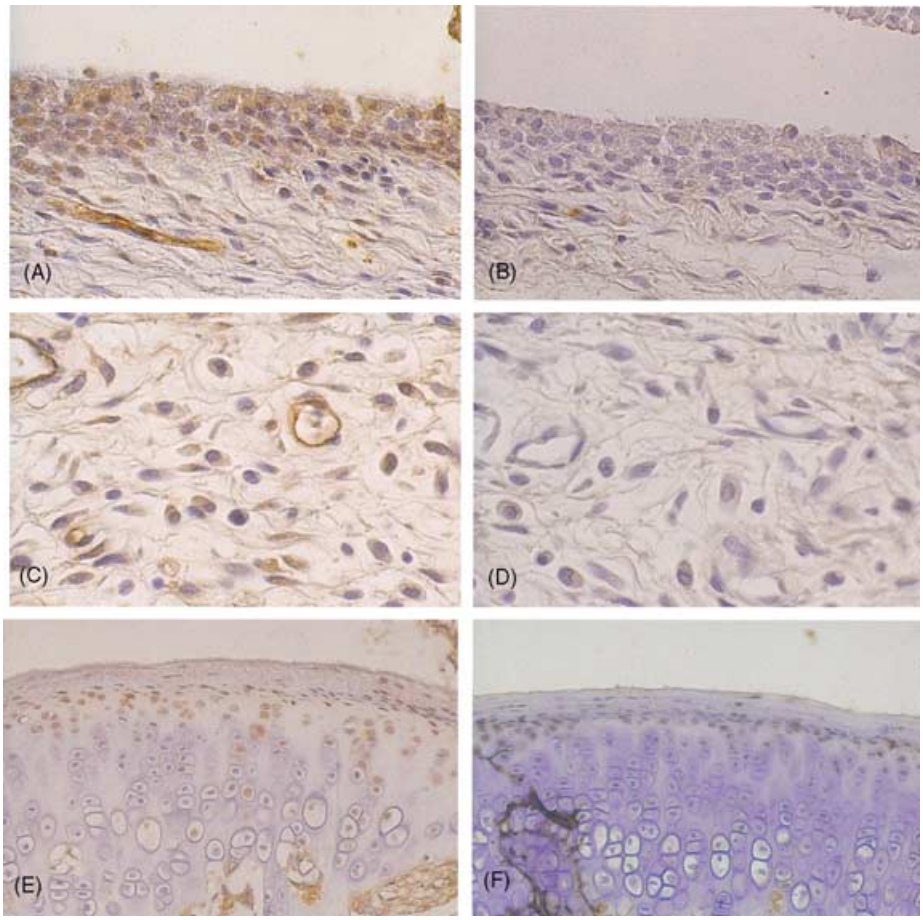

Figure 5 Arthritic joint 3 weeks after induction (left side; TNF-α, right side; IL-8). (A,B) Positive reaction against TNF-α in the thickened synovial layers is reidentified but negative reaction against IL-8 continues (×160). (C,D) Few scattered lymphocytes react positively for TNF-α and IL-8. Vascular endothelial cells are positive for only TNF-α (×200). (E,F) Chondrocytes in the condylar cartilage are disorganized, and TNF-α-positive chondrocytes are observed in various zones widely (×80). (Positive cells are stained light brown.)

The usefulness of human materials is limited because of the influence of any prior treatment or the uncertainty of the clinical stage of the arthritic condition at the time of sampling. Because of that, a proper animal arthritic model has been developed by the aid of an antigen that induces monoarthritis of the rabbit TMJ that mimics human TMJ arthritis (12).

An earlier study suggested that this model consisted of both acute and chronic stages (13). It also disclosed that the main sources of IL-1β were infiltrated inflammatory cells as well as synovial cells during the acute stage, and chondrocytes as well as hyperplastic synovial cells in the chronic stage. In the present study, TNF-α during the acute stage was produced by inflammatory cells, synovial cells, subsynovial fibroblasts, vascular endothelial cells, and chondrocytes. Although a positive reaction against TNF-α of vascular endothelial cells and chondrocytes continued to express until the chronic stage, the production of TNF-α derived from synovial cells was interrupted and reappeared during the chronic stage. This kind of biphasic production of pro-inflammatory cytokine was observed in our previous study regarding IL-1β (13). The acute reaction in this model appeared to be similar to that in single intra-articular injection of Freund's adjuvant or lipopolysaccharide (LPS), which disappeared rapidly (9, 27). The delayed reaction during the chronic stage might be caused by the accumulated antigen in pre-immunized animals (28). Therefore, the acute stage reaction corresponds to a direct inflammatory reaction against the injected antigen, and reproduction of pro-inflammatory cytokines during the chronic stage might be a delayed hypersensitivity reaction to the accumulated antigen.

The positive reaction against TNF-α in vascular endothelial cells was observed between the acute stage and the chronic stage. Almost all newly formed small blood vessels that were mainly observed in the subsynovial layer reacted positively against TNF-α. TNF-α would be related to vascular formation in the arthritic condition. Paleolog (29) reported that vascular endothelial growth factor (VEGF), which might play an important role in the mediation of the angiogenesis in RA, was induced by a stimulation of TNF-α. *In vitro* neutralization of TNF-α and IL-1 in cultured RA synovial membrane reduced VEGF release, and treatment of RA patients with anti-TNF-α significantly decreased serum VEGF (29, 30).

In the condylar cartilage, TNF-α has been discussed in relation to the degeneration of cartilage (31, 32). Cartilage

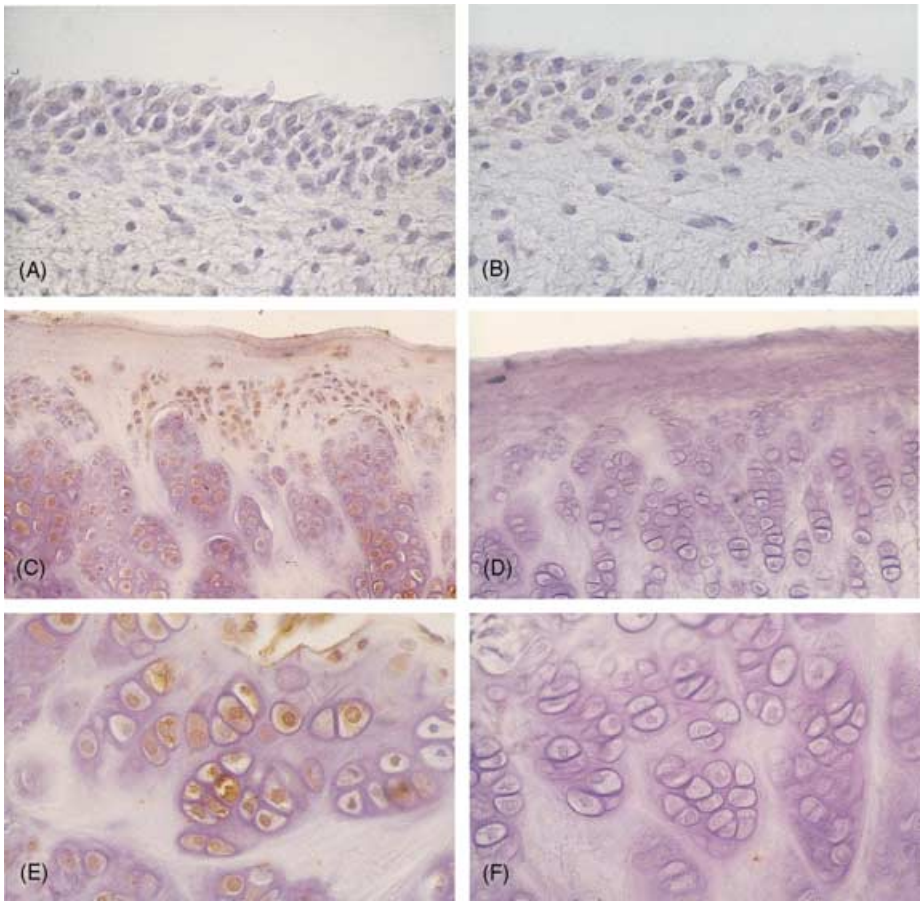

Figure 6 Arthritic joint 6 weeks after induction (left side; TNF-α, right side; IL-8). (A,B) No TNF-α- or IL-8-positive synovial cells are observed (×160). (C,D) Disorganized chondrocytes result in cluster formation. Clustering chondrocytes react positively against TNF-α and negatively against IL-8 (×80). (E,F) Photos in (E,F) are magnifications of those in (C,D) (×200). (Positive cells are stained light brown.)

destruction in arthritis is characterized by two major events: (i) reduced synthesis of matrix components; and (ii) increased breakdown of cartilage matrix. Saklatvala et al. (33) reported that TNF-α inhibited synthesis of proteoglycan in cartilage. Furthermore, enzymes such as matrix metalloproteinases, which were activated by pro-inflammatory cytokines, increased breakdown of the cartilage matrix (31). In the present study, TNF-α was characteristically observed in the cartilage where chondrocytes were disorganized and/or clustering. These findings suggested that the production of TNF-α was closely related to cartilage degeneration. In addition, during the acute stage of arthritis, TNF-α-positive chondrocytes were mainly observed in the surface layer of the condylar cartilage (the proliferating zone), and then the production site moved into the deeper layer (the intermediate zone to the hypertrophic zone). It was suggested that during the acute stage, the injected antigen directly stimulated the most active cells in proliferation and the chondrocytes that reacted positively for TNF-α differentiated to the deeper areas, and eventually, cartilage degeneration occurred. We previously reported that IL-1β-positive chondrocytes were detected later than 1 week after induction of arthritis in this model (13). In the present study, TNF-α-positive chondrocytes were detected at a stage earlier than that of IL-1β. Several studies reported that TNF-α was a cytokine related to early inflammation and TNF-α destroyed the articular cartilage with IL-1β (32, 34–36). Van den Berg et al. (31) mentioned that TNF-α induced inflammation either directly or through induction of IL-1β. These results suggested that TNF-α in addition to IL-1β played an important role in cartilage destruction.

In this model, IL-8 was produced by synovial cells and inflammatory cells composed of neutrophiles and macrophages during the acute stage, and the production of IL-8 disappeared at 1 week after the induction of arthritis. As IL-8 was identified only during the acute stage, it was supposed to be a cytokine related to the acute phase inflammation. Matsukawa et al. (25) showed that the number of neutrophiles and the concentration of IL-8 in synovial fluid increased in acute arthritis of the rabbit knee induced by LPS. They also reported that, immunohistochemically, the sources of IL-8 were infiltrated neutrophiles and synovial cells. Concerning the TMJ, there have been few studies regarding the origin of IL-8 in arthritic conditions. The results of the present study were consistent with those of the knee joint. Nishimura et al. (24) reported that IL-8 was detected in samples of synovial fluid from TMD patients who underwent arthroscopic or open TMJ surgery. However,

the samples involving detectable IL-8 were less correlated to cartilage degeneration than those involving the other pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α. The detectable IL-8 from the clinical sample might relate more to the acute aggravation of chronic arthritis because the materials in Nishimura's study had relatively high pain score.

In conclusion, TNF-α was closely related to cartilage degeneration and angiogenesis of inflammatory conditions, and IL-8 was mainly produced in infiltrating inflammatory cells and synovial cells during the acute stage. In clinical practice anti-cytokine therapies, particularly anti-TNF-α therapies, have recently focused in clinical practice on chronic arthritis, especially RA (37, 38). The results of the present study provide evidence for this kind of therapy of TMJ arthritis. However, IL-8 does not seem to be a candidate for this kind of therapy in chronic arthritis.

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