J Oral Pathol Med (2006) 35: 91–8

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Relationship between TNF-α and TUNEL-positive chondrocytes in antigen-induced arthritis of the rabbit temporomandibular joint

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BACKGROUND: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL) staining is a widely accepted method for the detection of DNA fragmentation in nuclei of apoptotic cells. Tumor necrosis factor (TNF)-α is closely associated with changes in condylar cartilage and modulates apoptosis in various tissues including cartilage. The aim of this study was to investigate the relationship between apoptotic chondrocytes and TNF-α in a rabbit model of arthritis.

METHOD: Unilateral temporomandibular joint (TMJ) arthritis was induced in 20 adult New Zealand White rabbits. From 1 day to 6 weeks after the induction of arthritis, immunohistochemical analysis for TNF-α and TUNEL was performed.

RESULTS: In condylar cartilage, TNF-α-positive cells and TUNEL-positive cells were localized together. TNF-α-positive chondrocytes seemed to precede TUNEL-positive cells.

CONCLUSIONS: The results of the present study suggest that TNF-α may be involved in apoptosis and/or apoptotic necrosis of chondrocytes as TMJ arthritis progresses from the acute to chronic stage.

J Oral Pathol Med (2006) **35**: 91–8

Keywords: antigen-induced arthritis; apoptosis; rabbit; temporomandibular joint; tumor necrosis factor-α; terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling

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Accepted for publication August 9, 2005

Introduction

Apoptosis was initially reported as a form of programmed cell death involved in many biologic processes, including homeostasis and tissue remodeling during development (1). However, recently it has been shown that in many pathologic conditions, such as inflammation or tumor, abnormal apoptosis is accelerated (2). Chondrocytes have been shown to undergo apoptotic changes in arthritides such as rheumatoid arthritis (RA) and osteoarthritis (OA; 3–5).

Apoptosis is characterized by nuclear DNA fragmentation, nuclear chromatin condensation, and formation of apoptotic bodies. Most studies used the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method to identify DNA fragmentation in apoptotic cells (6). Activated endonuclease cleaves the DNA between nucleosomes and shortens the fragments. The 3′-OH ends of DNA fragments are enzymatically labeled in TUNEL staining.

Antigen-induced arthritis is the closest experimental model of human arthritis (7). Some years ago, we developed a reliable antigen-induced arthritis model in the temporomandibular joint (TMJ) of adult rabbits (8). Our prior investigations revealed that antigen-induced arthritis of the rabbit TMJ has characteristics of both acute and chronic arthritis and that interleukin-1β and tumor necrosis factor (TNF)-α are closely related to changes in mandibular condylar cartilage (8–10). These cytokines, especially TNF-α, modulate apoptosis in various tissues and chondrocytes (10). The aim of this study was to investigate the relationship between TUNEL-positive chondrocytes and TNF-α in this arthritis model.

Materials and methods

Materials

Twenty-one 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. The experiment was performed under the guidelines and permission of the Animal Care Committee of Kyushu Dental College (2002-87).

Arthritis induction and killing protocol

Arthritis was induced as described previously (6). In brief, animals were sensitized by two intracutaneous injections, 2 weeks apart, with 4 mg of ovalbumin (Sigma, St Louis, MO, USA) emulsified in 1 ml of Freund's complete adjuvant (FCA; Sigma). After confirming the establishment of a delayed hypersensitivity reaction, 0.2 ml (20 mg/ml) of ovalbumin in saline was injected into one TMJ. The same volume of saline was injected into the contralateral TMJ as a sham induction.

Animals were killed using an overdose of sodium pentobarbital. Four animals were killed at 1 day, 3 days, 1 week, 3 weeks, and 6 weeks after arthritis induction. One healthy rabbit was killed immediately after purchasing without any treatment as a control.

Immunohistochemistry and histochemistry

Bilateral TMJ samples were taken, fixed in 4% paraformaldehyde for 24 h, decalcified with 10% ethylenediaminetetraacetic acid (EDTA) solution, and embedded in paraffin. Serial 4-μm sagittal sections were cut. After endogenous peroxidase was blocked 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 antirabbit TNF-α at 4°C (10, 11). The sections were rinsed and incubated for 30 min with 15 μg/ml biotinylated rabbit antigoat 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). Diaminobenzidine (DAB Reagent Set, KPL Laboratory, Gaithersburg, MD, USA) was used as a chromogen. Counterstaining was performed with hematoxylin. Negative controls were obtained by replacing the primary antibodies with diluted normal goat serum. All sections were examined by light microscopy.

In situ detection of apoptosis was performed using a TACS^{TM} 2TdT-Blue Label *In situ* Apoptosis Detection kit (Trevigen, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions with minor modification. Sections were treated with proteinase K solution for 15 min at room temperature, and endogenous hydrogen peroxidase activity was quenched with 3% H_2O_2. After rinsing, the DNA was end-labeled with TdT and digoxigenin-11-dUTP. After a 30-min incubation with antidigoxigenin peroxidase, the color was developed with diaminobenzidine tetrahydrochloride. Incubation without TdT served as a negative control.

Assessment of TNF-α and TUNEL-positive cells

For the assessment of TNF-α and TUNEL-positive cells, samples were taken from near the condylar center in four rabbits at each time point. Condylar cartilage was divided into three zones: a fibrous zone, a proliferating zone, and a hypertrophic zone. For each zone, the number of TNF-α or TUNEL-positive cells among 50 cells was counted by light microscopy, and positive ratio was calculated.

Statistics

The TNF-α and TUNEL-positive cells in control, sham, and arthritic joints were evaluated using Mann–Whitney's *U*-test. *P*-value of <0.01 was considered to be significant.

Results

Comparison of immunohistochemistry and histochemistry results between control and sham joints are shown in Table 1.

Control joints

No TNF-α-positive cells were observed in condylar cartilage. Very few TUNEL-positive cells were seen in the hypertrophic zone (Fig. 1).

Table 1 Ratio of TNF-α and TUNEL-positive cells in each zone of control and sham-induced joints

		Sham-induced joints				
	Healthy control	*1 day*	*3 days*	*1 week*	*3 weeks*	*6 weeks*
TNF-α (mean ± SD%)						
Fibrous zone	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Proliferating zone	2 ± 2	4 ± 4	2 ± 1	2 ± 2	2 ± 2	2 ± 2
Hypertrophic zone	3 ± 5	5 ± 4	3 ± 3	3 ± 5	4 ± 4	3 ± 3
TUNEL (mean ± SD%)						
Fibrous zone	2 ± 2	3 ± 3	2 ± 1	2 ± 2	2 ± 1	2 ± 2
Proliferating zone	3 ± 3	30 ± 2*	4 ± 2	4 ± 1	4 ± 2	3 ± 3
Hypertrophic zone	10 ± 5	11 ± 9	11 ± 1	10 ± 4	10 ± 2	11 ± 4

*Statistical significance in comparison between healthy control and sham-induced joints (Mann–Whitney's *U*-test: $P < 0.01$).
Cartilage was evaluated at 1 day, 3 days, 1 week, 3 weeks, and 6 weeks after induction of arthritis. Assays were performed as described in Materials and Methods. Results are expressed as percentage of total TUNEL-positive cells (±SD).
Cartilage was separated four layers from the surface: fibrous zone, proliferating zone, hypertrophic zone, and subcartilage layer. In the present study, fibrous, proliferating, and hypertrophic zones have been evaluated.
TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling.

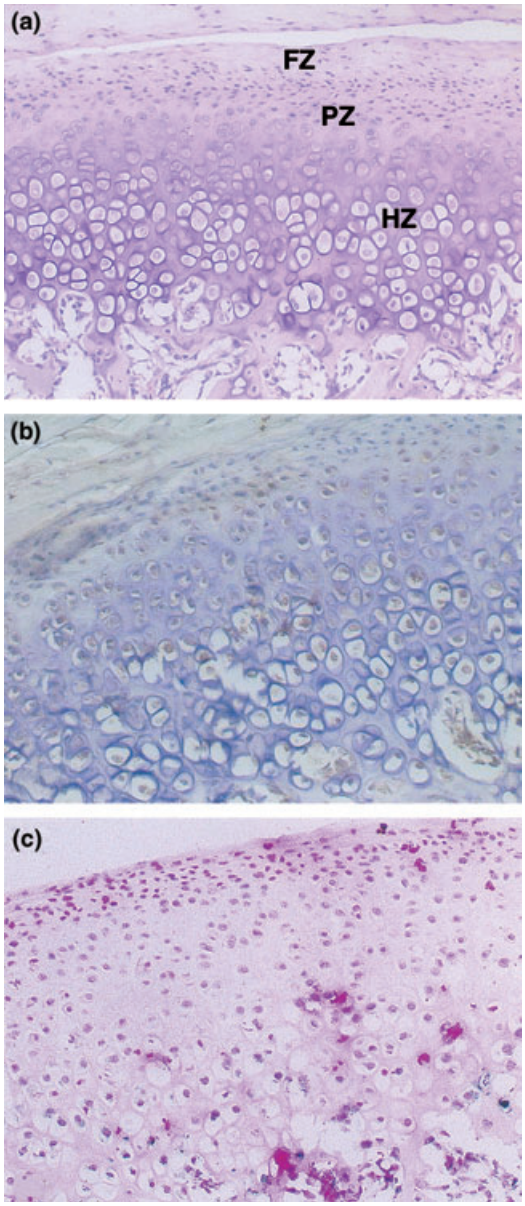

Figure 1 Control joint which is not inducted arthritis. (a) The cartilage layer shows a normal morphology, which is divided into three layers from the surface: fibrous zone (FZ), proliferating zone (PZ), hypertrophic zone [HZ; ×200 hematoxylin and eosin (H & E)]. Immunohistochemical localization of tumor necrosis factor (TNF)-α (b) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL)-positive cells (c) in control joint. (b) No cells are stained with antirabbit TNF-α, so they react negatively for TNF-α by immunohistochemistry (×200). (c) In HZ and subcartilage layer, a few cells are stained with TUNEL, so they react positively for TUNEL by histochemistry (×200).

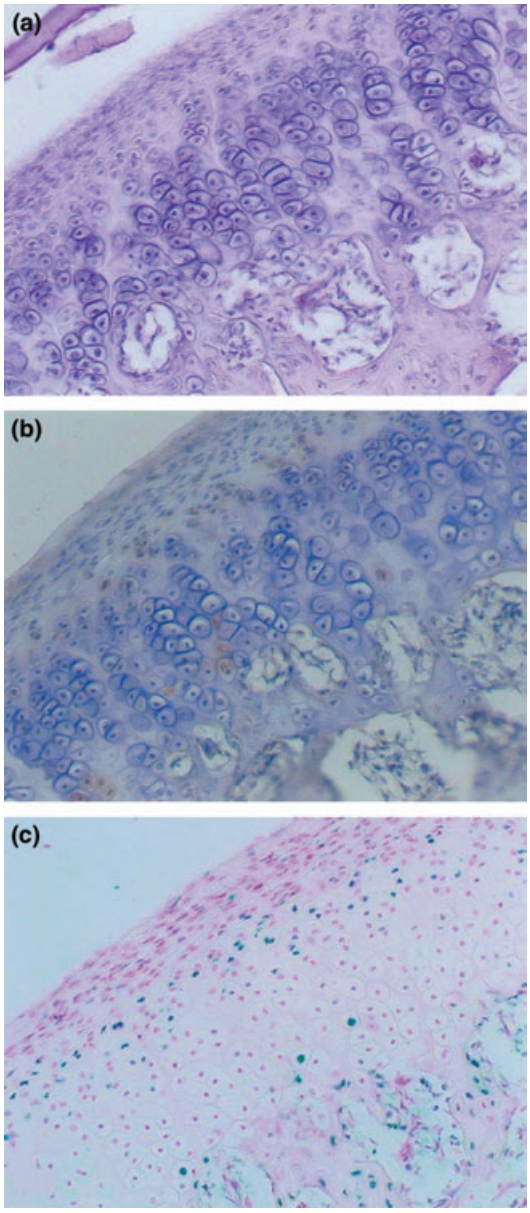

Figure 2 Sham joint 1 day after induction. Immunohistochemical localization of tumor necrosis factor (TNF)-α (b) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL)-positive cells (c) in sham joint. (a) The cartilage layer shows a normal morphology [×200 hematoxylin and eosin (H & E)]. (b) All sections are stained with antirabbit TNF-α and the colors develop with diaminobenzidine (DAB; positive cells are stained light brown). A very few of positive cells are observed in proliferating and hypertrophic zones. Nuclei are counterstained by hematoxylin (×200). (c) TUNEL-positive cells are observed in proliferating and hypertrophic zones (×200).

Sham-induced joints

At 1 day after saline injection, no TNF-α-positive cells were observed in condylar cartilage, and some TUNEL-positive cells were seen in the proliferating zone. No TNF-α or TUNEL-positive cells were observed at any other time points (Fig. 2).

Comparison of immunohistochemistry and histochemistry results between sham and arthritis joints are shown in Table 2.

One day after arthritis induction

In condylar cartilage, there was no observable morphologic change, but there were many TNF-α-positive cells in the proliferating and hypertrophic zones. Some TUNEL-positive cells were observed in the proliferating zone, as seen in the sham-induced joints (Fig. 3).

Table 2 Ratio of TNF-α and TUNEL-positive cells in each zone of arthritis joints

	1 day	*3 days*	*1 week*	*3 weeks*	*6 weeks*
TNF-α (mean ± SD%)					
Fibrous zone	2 ± 2	3 ± 6*	24 ± 7*	20 ± 8*	20 ± 7*
Proliferating zone	73 ± 10*	35 ± 12*	50 ± 6*	48 ± 1*	80 ± 8*
Hypertrophic zone	66 ± 23*	6 ± 5	7 ± 6	52 ± 9*	86 ± 6*
TUNEL (mean ± SD%)					
Fibrous zone	2 ± 3	4 ± 2	22 ± 7*	19 ± 9*	29 ± 14*
Proliferating zone	40 ± 13	49 ± 13*	64 ± 15*	42 ± 8*	77 ± 13*
Hypertrophic zone	4 ± 4	41 ± 11*	52 ± 29*	46 ± 15*	79 ± 13*

*Statistical significance in comparison between sham and arthritis joints at the same experimental period (ANOVA, Mann–Whitney's *U*-test: $P < 0.01$).
Cartilage was evaluated at 1 day, 3 days, 1 week, 3 weeks, and 6 weeks after induction of arthritis. Assays were performed as described in Materials and Methods. Results are expressed as percentage of total TUNEL-positive cells (±SD).
Cartilage was separated four layers from the surface: fibrous zone, proliferating zone, hypertrophic zone, and subcartilage layer. In the present study, fibrous, proliferating, and hypertrophic zones have been evaluated.
TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling.

Three days after arthritis induction
There was no observable morphologic change in the cartilage layer. Some TNF-α-positive cells were observed in the proliferating zone but there were few positive cells in the fibrous and hypertrophic zones. The area showing TUNEL-positive cells extended widely from the proliferating to hypertrophic zones (Fig. 4).

One week after arthritis induction
In condylar cartilage, the zone differentiation became obscure. A few non-nuclear cells were present in the hypertrophic zone. The number of TNF-α-positive chondrocytes increased in the fibrous and proliferating zones but not in the hypertrophic zone. The number of TUNEL-positive chondrocytes also increased in all condylar zones, with the majority located in the proliferating and hypertrophic zones (Fig. 5).

Three weeks after arthritis induction
Thinning of the cartilage layer was observed. The chondrocytes in each layer were disorganized, especially in the hypertrophic zone, and the intercellular collagen network showed signs of disintegration. In the hypertrophic zone, some non-nuclear cells were found. Many TNF-α-positive cells were observed in the hypertrophic zone. The number of TNF-α-positive cells in the fibrous and proliferating zones was approximately the same as at 1 week after arthritis induction. The number of TUNEL-positive cells in every zone of the condyle was about the same as that at 1 week after arthritis induction (Fig. 6).

Six weeks after arthritis induction
The condylar cartilage surface layer had a rough appearance. Marked disorganization of the collagen network between chondrocytes resulted in cluster formation and the obscuring of zone differentiation. In all zones, many TNF-α-positive cells were observed, especially in the clustering areas. TUNEL-positive cells were seen in all condylar cartilage zones. The number of TUNEL-positive cells was greater at 6 weeks after arthritis induction than at any other time period (Fig. 7).

Discussion

Our rabbit model of antigen-induced TMJ arthritis has characteristics of both acute and chronic arthritis (8–10). The period up to 1 week after challenge is considered the acute stage, and this is followed by a chronic stage. Acute stage arthritis is caused by a direct reaction to the injected antigen in the joint cavity; this is similar to several other acute arthritis models using the direct injection of FCA, papain, or lipopolysaccharide (12, 13). Chronic stage arthritis is elicited by continuous stimulation by a small volume of antigen accumulated in the joints, resulting from an antigen–antibody reaction in sensitized animals. This reaction is similar to that of conventional adjuvant-induced polyarthritis, which is widely used as a chronic arthritis model; it does not induce arthritis in TMJs.

Since the late 1980s, apoptosis has been considered to be different from necrosis; apoptosis is physiologic, while necrosis is pathologic (14). However, numerous studies have revealed that apoptosis can also be elicited by toxic injury or disease processes (15). In these non-physiologic conditions, both types of cell death can be encountered following the same initial insult, and the balance between death by apoptosis and necrosis appears to depend on the intensity of the injury and the level of intracellular ATP (16). Majno and Joris (17) pointed out that the term necrosis refers to the changes that take place after cell or tissue death regardless of the pre-lethal process. Apoptosis is one process leading to cell death. Therefore, apoptosis and necrosis do not exist in opposition; necrosis may also occur by the same process that leads to apoptosis. Following these comments, the Cell Death Nomenclature Committee of the Society of Toxicologic Pathologists recommended the use of the terms 'apoptotic' or 'apoptotic necrosis' for cells showing apoptotic cytomorphology. Although TUNEL staining is a widely accepted method for the detection of DNA fragmentation, TUNEL-positive cells are not always undergoing apoptosis (18). We used the term 'apoptotic' for TUNEL-positive cells.

In knee and ankle cartilage in OA and RA, apoptotic chondrocytes significantly increase with disease aggra-

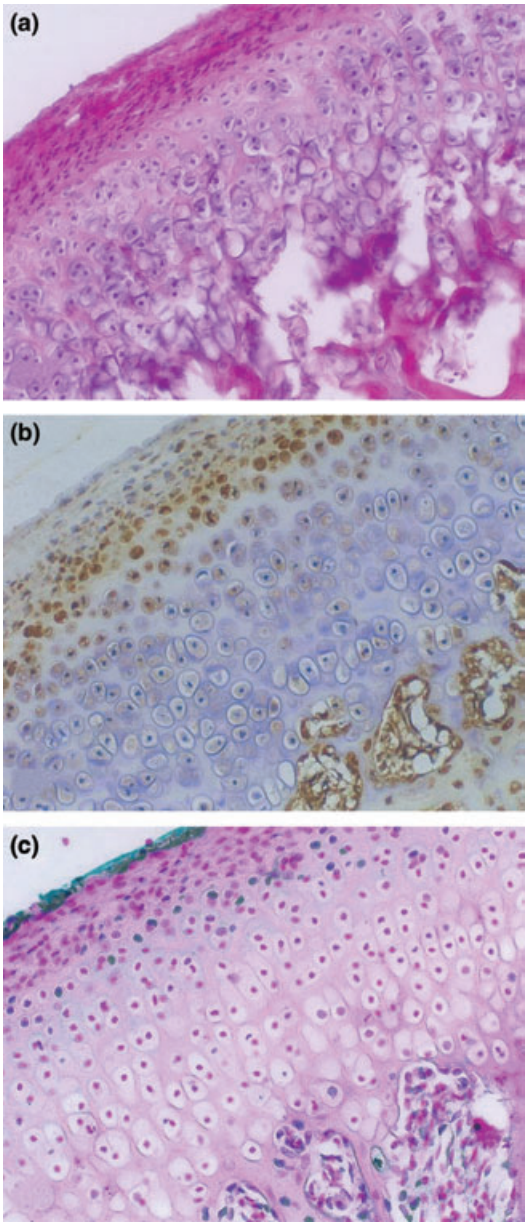

Figure 3 Arthritic joint 1 day after induction. (a) In condylar cartilage, there is no observable change morphologically [×200 hematoxylin and eosin (H & E)]. (b) Tumor necrosis factor (TNF)-α-positive cells are observed in proliferating zone (×200; positive cells are stained light brown). (c) Chondrocytes reacting positively against the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL) are observed in proliferating and hypertrophic zones (×200; positive cells are stained dark green).

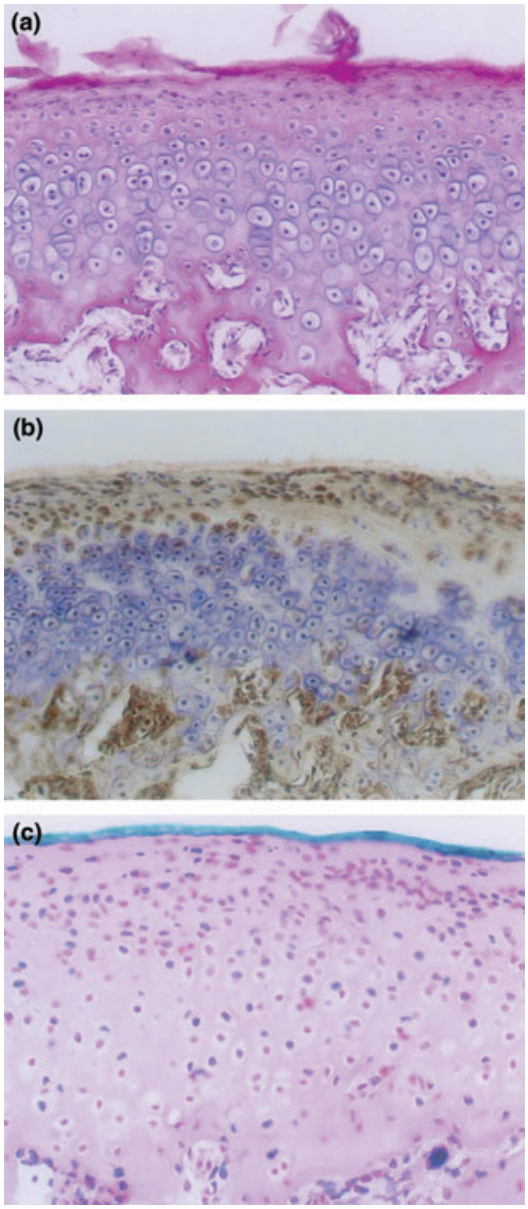

Figure 4 Arthritic joint 3 days after induction. (a) In condylar cartilage, there is no observable change morphologically [×200 hematoxylin and eosin (H & E)]. (b) Tumor necrosis factor (TNF)-α-positive cells are observed in proliferating zone (×200; positive cells are stained light brown). (c) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL)-positive cells were observed from the fibrous, proliferating, and hypertrophic zones (×200; positive cells are stained dark green).

vation. Chondrocyte apoptosis may destroy the cartilage restoration process, eventually resulting in bone–cartilage deformation such as osteophyte formation or cartilage thinning (2, 4, 19–22). We have little knowledge concerning chondrocyte apoptosis in the TMJ, especially in arthritic conditions. Spears et al. (23) recently demonstrated an increase in apoptotic cells in acute TMJ arthritis induced by direct injection of FCA in rats; they detected an increased number of TUNEL-positive cells and the activation of caspase-3 and -8 in cultured TMJ tissue retrieved 2 days after FCA injection. In the present study, we showed an increase number of TUNEL-positive cells in the acute stage of antigen-induced arthritis of rabbit TMJs, consistent with the previous findings for rat arthritis. Gu et al. (24) reported that TUNEL-positive chondrocytes increased at 1–2 weeks after the induction of acute disk displacement in rabbit TMJs. These results suggest that apoptotic changes in the TMJ are closely related to inflammatory and overloading conditions (24).

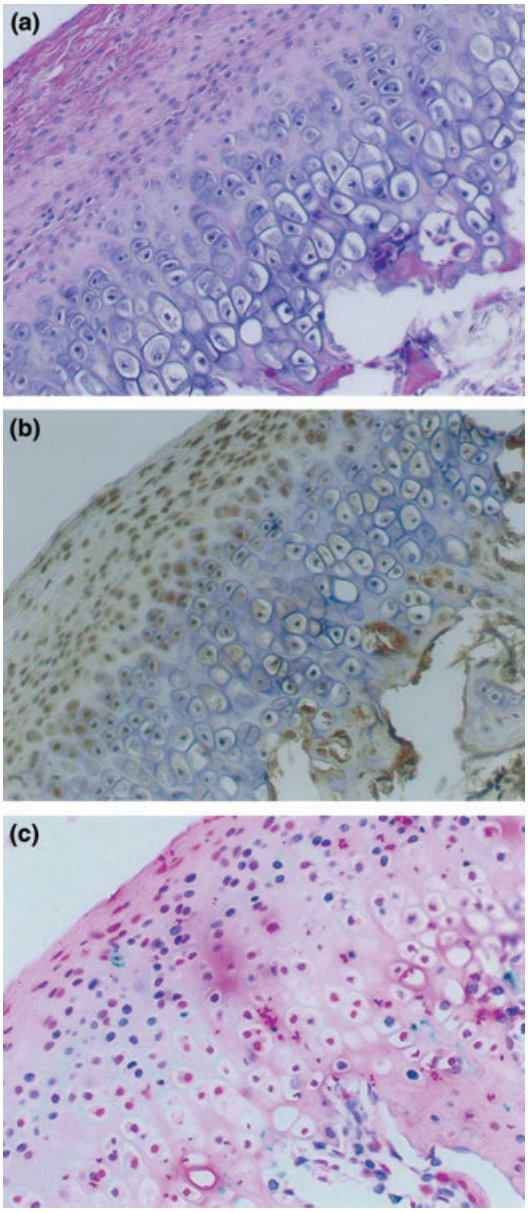

Figure 5 Arthritic joint 1 week after induction. (a) In condylar cartilage, the proliferating zone becomes obscure, and arrangement of chondrocytes are deranged. In hypertrophic zone, non-nucleus cells are observed. (b) Chondrocytes reacting positive for tumor necrosis factor (TNF)-α are identified in proliferating and hypertrophic zones (×200; positive cells are stained light brown). (c) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL)-positive cells are observed in all zones, mainly in proliferating zone (×200; positive cells are stained dark green).

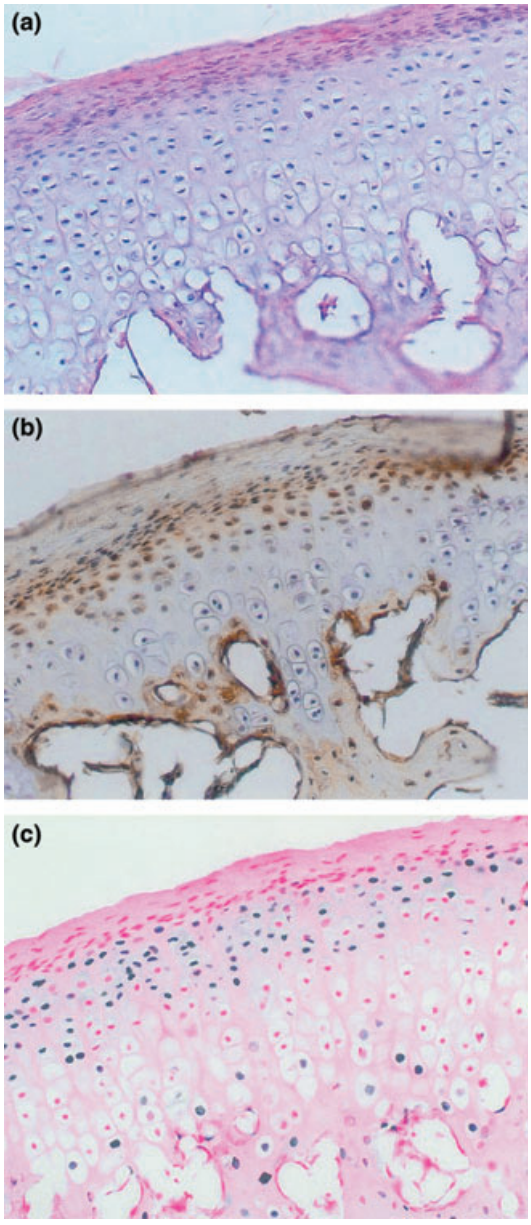

Figure 6 Arthritic joint 3 weeks after induction. (a) In hypertrophic zone, cluster formation is observed. (b) Chondrocytes of the cartilage are disorganized and are positive for tumor necrosis factor (TNF)-α in proliferating zone (×200; positive cells are stained light brown). (c) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL)-positive cells are observed in proliferating and hypertrophic zones, especially in proliferating zone. In hypertrophic zone, there are non-nucleus cells (×200; positive cells are stained dark green).

In adjuvant-induced arthritis, which is widely used as a chronic polyarthritis model, an increased number of TUNEL-positive cells was reported at 3–4 weeks after adjuvant injection (late phase), while few TUNEL-positive cells were observed in early phase arthritis (25). In antigen-induced arthritis, we found a gradual increase in the number of TUNEL-positive cells in chronic stage arthritis. The chronic stage of arthritis in this model is thought to be caused by a mechanism similar to that of adjuvant-induced arthritis (26).

Generally there are two major cascades for apoptosis that of a death ligand and death receptor and the stress reaction to reactive oxygen and nitrogen species. The cascade of a death ligand and receptor is the dominant pathway of apoptosis. Four major death ligands are known: TNF-α, lymphotoxin-α, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL)/Apo2 ligand and TNF-like weak inducer of apoptosis (TWEAK). They all belong to the TNF family, and their receptors belong to the TNF receptor family. After a death ligand

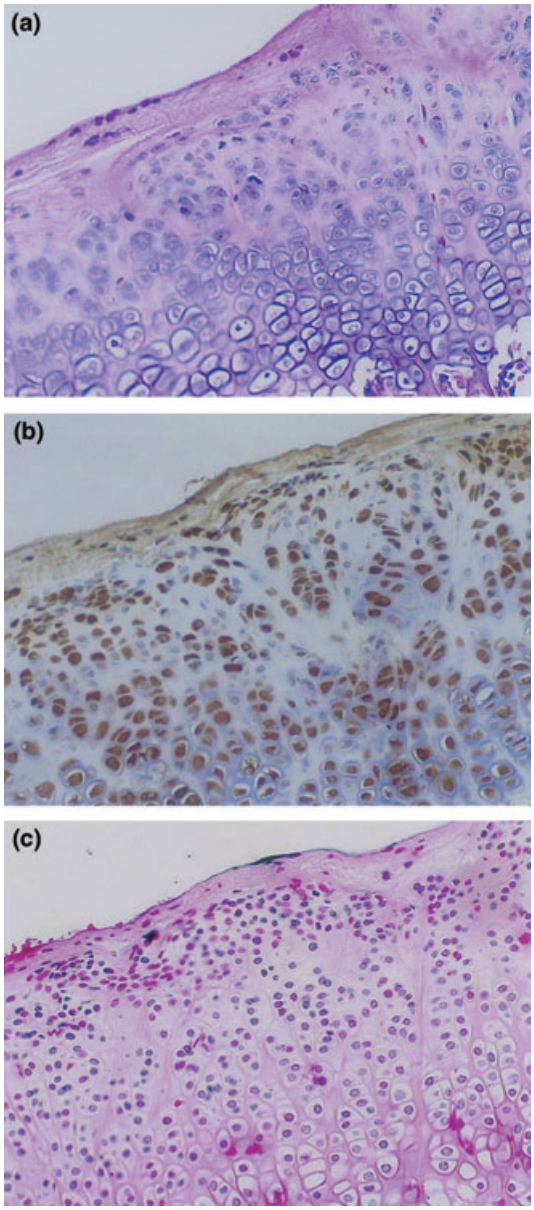

Figure 7 Arthritic joint 6 weeks after induction. (a) The condylar surface becomes rough. Severe cluster formation can be observed in widespread areas. (b) Tumor necrosis factor (TNF)-α-positive chondrocytes are mainly observed in the clustering areas of all zones (×200; positive cells are stained light brown). (c) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL)-positive cells are observed in all condylar cartilage layers(×200; positive cells are stained dark green).

and receptor connect, caspase-8 is activated and the induction of apoptosis is accelerated (26). TNF-α induces apoptosis in a number of cell types and is one of the major proinflammatory cytokines produced mainly by activated mononuclear macrophages and lymphocytes (27, 28). In many joints, including the TMJ, TNF-α is detected in synovial fluid, synovium, and chondrocytes in OA, RA, and temporomandibular disorders (8, 29–33). Cell injury by TNF-α might be caused by synergism of direct apoptosis-inducing ability and of indirect acceleration activity to produce inflammatory cytokines.

Yao et al. (34) showed that chondrocyte apoptosis is present in normal cartilage as well as in OA and RA cells and that it can be induced by stimulation with TNF-α. Aizawa et al. (35) demonstrated that chondrocyte apoptosis can be induced through both Fas and TNF-α receptor-mediated signaling and suggested that chondrocytes are more sensitive to the apoptotic effects of TNF-α. Yao et al. (34) showed that the adenovirus-mediated gene transfer of TRAIL to the rabbit knee could induce apoptosis through the interaction with receptors containing death domains. We have little knowledge regarding the relationship between TNF-α and apoptosis in the TMJ. Recently, apoptotic changes during acute FCA-induced TMJ inflammation was confirmed to be related to increased TNF-α, and the apoptotic changes were suppressed by the addition of anti-TNF-α neutralizing antibodies (23). These findings have not been reported in chronic TMJ arthritis. In the present study, TNF-α-positive chondrocytes seemed to precede TUNEL-positive cells, i.e. the large number of TNF-α-positive cells in early phase acute arthritis decreased in the late phase, while TUNEL-positive cells gradually increased from the late phase of acute arthritis to the early phase of chronic arthritis. In chronic arthritis, TNF-α-positive cells increased again, while TUNEL-positive cells slightly decreased at 3 weeks after arthritis induction and increased again in the late phase of chronic arthritis.

In conclusion, the results of the present study suggest that TNF-α controls apoptosis and/or apoptotic necrosis of chondrocytes in TMJ arthritis progressing from the acute to the chronic stage.

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