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Pathological change of articular cartilage in the mandibular head treated with immunosuppressant FK 506

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Summary. While several reports have documented immunosuppressant-induced osteoporosis, the exact mechanism of the pathological change of the joint remains to be clarified. In the present study, we have demonstrated the pathological change of the articular cartilage in the mandibular head of five Sprague-Dawley rats administered with the immunosuppressant FK 506 for 28 days. Three-dimensional micro-computed tomography of the mandibular heads in treated rats showed a significant decrease in trabecular bone volume compared to control rats. Histological observation revealed atrophic change of the articular cartilage. Immunohistological observation using anti-proliferative cell nuclear antibody (PCNA), type I, II, and type X collagen antibodies showed significantly decreased proliferation and differentiation of chondrocytes in the articular cartilage compared with the control group ($p<0.05$). Tartrate-resistant acid phosphatase (TRAP) staining revealed no significant difference in the numbers of osteoclasts at the chondro-osseous junction. Thus, FK 506 administration inhibited chondrogenic cell proliferation and differentiation and might cause osteoporotic change of subcartilage trabecular bone that subsequently forms in the mandibular head.

Key words: Immunosuppressant FK 506, Osteoporosis, Atrophy, Cartilage, Mandibular, Head

Introduction

The development of immunosuppressants has greatly contributed to the success of organ transplantation, by suppressing immunorejection induced in allografts. However, as immunosuppressants cannot be discontinued in post-organ transplantation patients, complications of subsequent lifetime administration are important to determine. It is now well known that immunosuppressant administration can adversely affect bone and mineral metabolism. The immunosuppressant FK 506 has been shown to induce trabecular bone loss in rats (Cvetkovic et al., 1994), as well as to enhance bone resorption in rat femolaris (Fukunaga et al., 2001), suggesting that FK 506 promotes osteoclast activity, which is associated with the development of osteoporosis. While these reports indicate an immunosuppressant effect on bone formation, the exact role of immunosuppressants in bone metabolism remains to be fully elucidated.

The surface of the articular head is covered with articular cartilage. The articular cartilage consists of four zones: the fibrous, proliferative cell, maturative cell, and hypertrophic cell layers. Fibroblasts in the fibrous layer proliferate in the proliferative cell zone, the proliferative cells differentiate into maturative chondrocytes in the maturative cell zone, and chondrocytes differentiate into hypertrophic chondrocytes in the hypertrophic cell layer. Cartilage is subsequently replaced by new bone during the process of endochondral ossification. Studies on cartilage development have extensively documented three distinct collagens produced by chondrocytes in the articular cartilage: fibroblastic cells (chondrocyte precursor cells) express type I collagen; differentiated maturative chondrocytes produce type collagen; and hypertrophic chondrocytes at the chondro-osseous junction synthesize type I collagen (Nimni and Deshmukh, 1973; Aigner et al., 1993; Luder et al., 1998). Several studies have described atrophic change of these articular cartilage layers as a response to cartilage degradation and this change resulted in subchondral bone deformity of the articular head in experimentally induced osteoarthritic models (Furstmann, 1966; Aigner et al., 1993).

We hypothesized that osteoporosis results from osteoporotic and atrophic change of articular cartilage in bone. Here, we set out to confirm or refute our

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hypothesis by investigating the pathological change of articular cartilage induced by immunosuppressant administration. We aimed to observe radiographically, histologically and immunohistologically, the pathological change of articular cartilage in the rat mandibular head post-treatment with FK 506. The mechanism of osteoporotic change of the mandibular head is discussed.

Material and methods

Animals

Six-month-old male Sprague-Dawley (SD) rats (Charles River Co., Osaka, Japan) were maintained on a standard animal diet (Oriental Co., Osaka, Japan) with free access to water and were well acclimatized at 25 °C. Animals were cared for in line with the Guidelines for Animal Research of Okayama University Dental School according to the principles of the Declaration of Helsinki.

Experimental protocol

Animals were divided into experimental and control groups.

Experimental (FK) group: five SD rats were administered 1.0 mg/kg/day of FK 506 (Tacrolismus hydrate; Fujisawa Co., Osaka, Japan) intramuscularly for 28 days.

Control group: five rats were administered physiological saline solution intramuscularly for 28 days.

Following the administration of FK 506 or normal saline solution for 28 days, the mandibular heads were extirpated and prepared for radiographic, histological and immunohistological analyses.

Micro-computed tomography

The 3-dimensional (3D) architecture of trabecular bone of the extirpated mandibular heads was examined using a micro-computed tomography (MCT) system (MCT-CB 100MF; Hitachi Medical Co. Ltd., Tokyo) comprising of an X-ray tube that produces a cone beam detected by a CCD array, and operated at 60 kV, 0.1 mA, with a 170 sec scanning time at x20 magnification. The number of images produced ranged from 150 to 200. By combining the slice images taken at 8 μm, the 3D architecture of the mandibular head was reconstructed. To distinguish bone tissue from soft tissue, a uniform threshold was used for all volumes of interest. The trabecular bone volume fraction (BV/TV) was calculated by the MCT system software package. Statistical analysis of the data was carried out using the Mann-Whitney U-test. Values of $p<0.05$ were considered significant. One specimen from each of the 5 animals in the experimental and control groups was subjected to MCT analysis.

Histological examination

Extirpated specimens were immersed in fixative (4% paraformaldehyde in 0.05 M phosphate buffer, pH 7.4) for 7 days at 4 °C, then decalcified in 5% etylenediaminetetraacetic acid (EDTA) buffered with 0.05 M phosphate (pH 7.4) for 7 to 14 days. After decalcification, specimens were dehydrated with a graded ethanol series and embedded in paraffin before being stained with hematoxylin-eosin for light microscopic observation. Tartrate-resistant acid phosphatase (TRAP) staining was used to detect osteoclasts.

Immunohistological analysis

Specimens were fixed with 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7.4) at 4 °C for 24 h, then decalcified in 5% EDTA buffered with 0.05 phosphate (pH 7.4) for a few days depending on specimen size and degree of calcification present. After dehydration with a graded ethanol series and embedding in paraffin, specimens were cut into 5-μm-thick sections that were subsequently mounted on slides and rehydrated. The slides were immersed in 0.3% hydrogen peroxide solution for 15 min to inhibit endogenous peroxidase, then blocking was done with 10% bovine serum in PBS for 30 min at room temperature. The slides were subsequently prepared for immunohistochemical reaction. Slides were exposed to either mouse anti-proliferative cell nuclear antigen (PCNA) antibody (DAKO, Glostrup, Denmark), rabbit polyclonal anti-type I collagen antibody (Cosmo-Bio, Osaka, Japan), rabbit anti-type II collagen antibody (Cosmo-Bio Osaka, Japan), or rabbit anti-type X collagen antibody (Cosmo-Bio, Osaka, Japan) diluted 1:100 in PBS containing 3% bovine serum albumin (BSA) at 4 °C overnight. After incubation, the slides were exposed to horseradish peroxidase (HRP)-labeled anti-mouse or anti-goat IgG before being incubated for 5 min at room temperature in a medium containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.01% hydrogen peroxide and 0.05 M Tris-HCl (pH 7.6) to visualize immunoreactivity. The sections were counter-stained with methyl green. For a negative control, some slides were incubated with 3% BSA in PBS instead of the primary antibody.

Evaluation of cell number in each cartilage layer and TRAP-positive osteoclasts at chondro-osseous junctions in mandibular head

The numbers of fibroblasts, maturative chondroblasts, hypertrophic chondrocytes, and proliferative cells in articular cartilage, and of osteoclasts at the chondro-osseous junction were counted following staining with anti- type I, II or X collagen antibodies, anti-PCNA antibody, and TRAP, respectively. Statistical analysis of the data was carried out using the Mann-Whitney U-test. Values of $p<0.05$

were considered significant.

Results

Micro-computed tomography

Micro-computed tomography showed the microstructure of trabecular bone in the rat mandibular head. The trabecular thickness of the mandibular condyle in the FK group showed a marked decrease compared with the control group (Fig. 1). BV/TV was significantly decreased in the FK group compared with the control group (FK group: 50.2±2.11; Control: 73.2±2.69%; $p<0.01$) (Fig. 2).

Histological and immunohistological findings

Histological examination revealed that, compared with the control group, the FK group showed remarkable atrophic change throughout the articular cartilage in the mandibular head. In addition, there were fewer whole cells in the FK group. Cartilage became thinner than that of the control group. Subsequent trabecular bone showed remarkable osteoporosis (Fig. 3A,B).

Immunohistochemical observation showed clear divisions between the proliferative, fibrous, maturative, and hypertrophic zones in the articular cartilage. The numbers of fibroblasts in the fibrous layer did not differ significantly between the FK group and control group (Fig. 3C,D). Compared with the control group, there were significantly fewer proliferative cells (Fig. 3E,F), maturative chondrocytes (Fig. 3G,H), and hypertrophic chondrocytes (Fig. 3I,J) in the FK group. TRAP-positive osteoclasts were detected in the chondro-osseous junction of the articular cartilage. The number of osteoclasts at the chondro-osseous junction did not

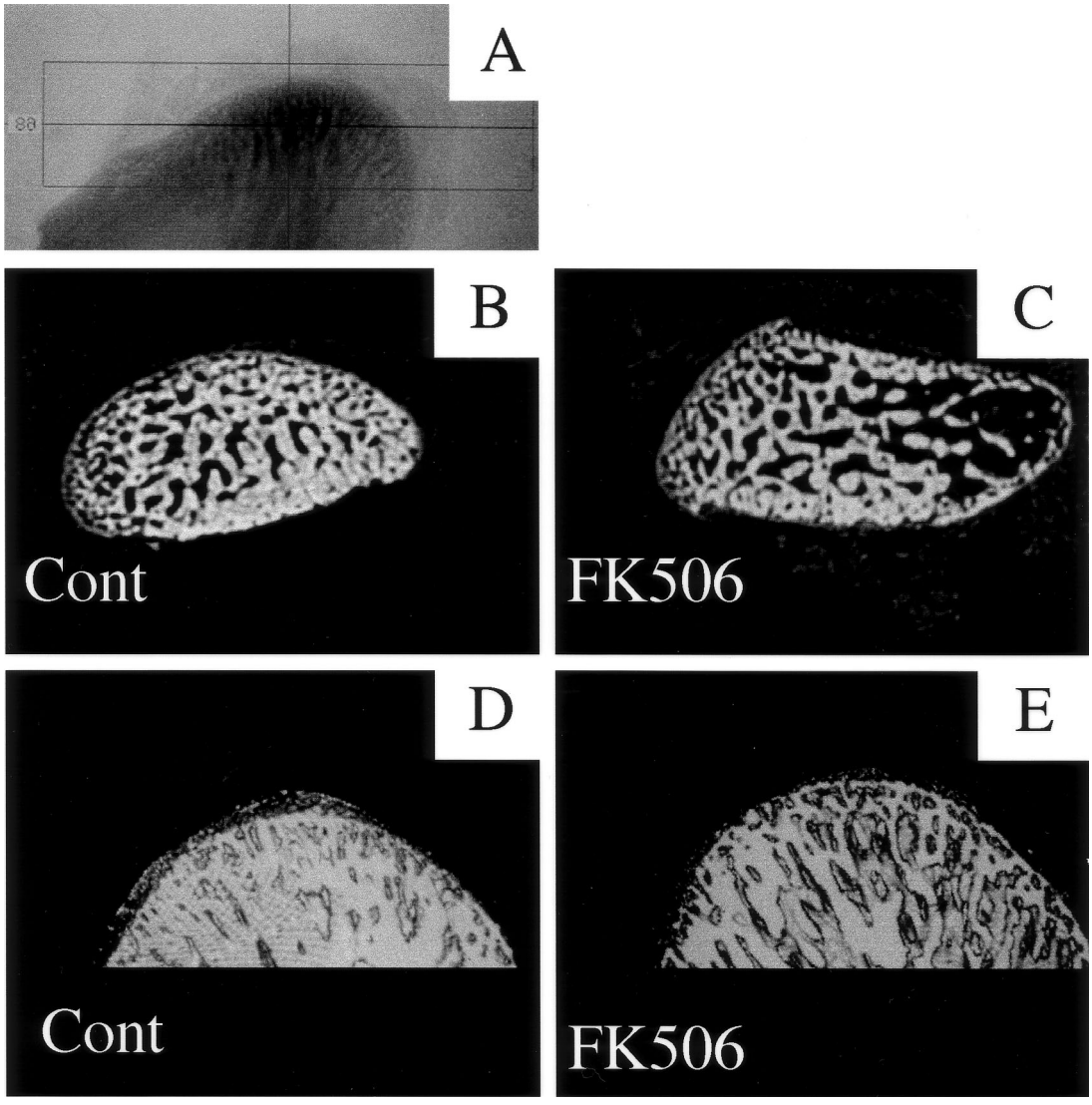

Fig. 1. Three-dimensional micro-computed tomography (3D-MCT) findings of mandibular head in experimental (FK506-treated) group and control group. **A.** Preview of region of interest (ROI) of mandibular head by transmission X-ray. **B.** Axial micro-computed tomography (MCT) of mandibular head in control group shows clearly visible trabecular bone. **C.** Axial MCT of mandibular head in FK group shows atrophic change of trabecular bone when compared with control group. Sagittal view of 3D-CT images of trabecular bone in control group (**D**) and FK group reveals (**E**) greater separation of trabecular bone in FK group.

significantly differ between the two groups (Fig. 3K,L). Figure 4 shows a comparison of numbers of cells between the two groups. Fibroblasts and osteoclasts show no significant difference among the groups, but there are significantly fewer numbers of proliferative, maturative, and hypertrophic chondrocytes in FK group ($p< 0.05$).

Discussion

Although several reports have described immunosuppressant-induced osteoporosis, the influence of these drugs on articular cartilage remains to be clarified. Results of the present study found that FK 506 administration induced osteoporosis in the mandibular head. We speculate that this osteoporotic change is strongly associated with the marked decrease in chondrogenic proliferation and differentiation seen in the articular cartilage.

The traditional target of immunosuppressants is T-lymphocytes (Fukunaga et al., 2001). While it is well established that FK 506 binds to FK 506 binding protein (FKBP) and the resulting complex binds to and inhibits calcineurin, a Ca^{2+}/calmoduline-dependent serine phosphate, the exact mechanism of its action in bone remodeling, as documented by researchers including Sempuku et al. (1996) and Ogawa et al. (1998), remains to be clarified. We chose to use FK 506 in the present study not only because of its known involvement in bone metabolism, but also because it is one of the most widely used drugs to manage the immune response to allografts. We administered 1.0 mg/kg/day of FK 506 to each animal based on previous findings that this dose effectively prevented immunorejection in xenogeneic grafts (Sempuku et al., 1996; Ueno et al., 2002).

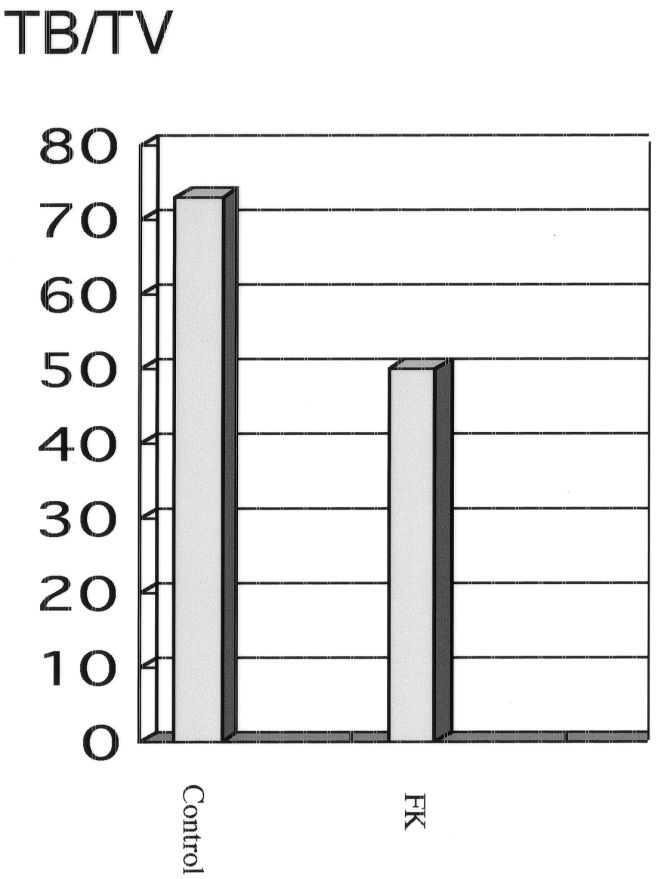

Fig. 2. Change of TB/TV in mandibular head following FK 506 or physiological saline solution administration for 28 days.

In the process of endochondral ossification, osteoblasts are induced into calcified cartilage matrix by vascular invasion and deposited on the calcified cartilage matrix. It is this matrix that subsequently becomes the core of trabecular bone after hypertrophic change (Roberto et al., 2002). Ogawa et al. (1998) used FK 506 to specifically investigate the activity of osteoblasts in bone formation. They measured alkaline phosphatase (ALP) activity and expression of mRNA of osteopontin, and found that FK 506 did not influence osteoblastic proliferation and differentiation in vitro. Thus, it appears that the mechanism of osteoporosis does not directly involve osteoblasts.

Pathological bone resorption is known to lead to osteoporotic change, and involves the digestion of formed bone by activated and proliferated osteoclasts. Fukunaga et al. (2001), in their study on the role of osteoclasts in bone resorption, found that FK 506 administration suppressed the production of osteoclast inhibiting factor (OCIF) in vitro. They suggested that this decrease in OCIF production was responsible for the increased number of osteoclasts that led to osteoporotic change. As TRAP is intensively expressed in osteoclasts and has therefore been used as a marker enzyme of osteoclasts (Minkin, 1982; Cole and Walters, 1987), we used TRAP staining in the present study to detect osteoclasts in the chondro-osseous junction in the articular head. The number of osteoclasts in the mandibular head treated with FK 506 was not significantly different from that of the control group. Thus, osteoclast activity does not seem to be directly related to osteoporotic change induced by FK 506 administration in our model.

To determine the validity of our hypothesis that osteoporosis results from osteoporotic and atrophic change of articular cartilage in bone, we determined immunohistochemically, according to the technique of Ohashi et al. (1997), the extent of chondrogenic cell proliferation and differentiation in the articular cartilage of the mandibular head. Many investigators have demonstrated the presence of extracellular matrix protein synthesized by chondrocytes in articular cartilage (Sandberg and Vuorio, 1987; Von der Mark et al., 1992). Type II collagen is the major collagen of cartilage matrix, its synthesis being observable in the articular cartilage (Aigner et al., 1995; Luder et al., 1998). Also, articular cartilage of the mandibular head produces type I collagen throughout development, possibly due to

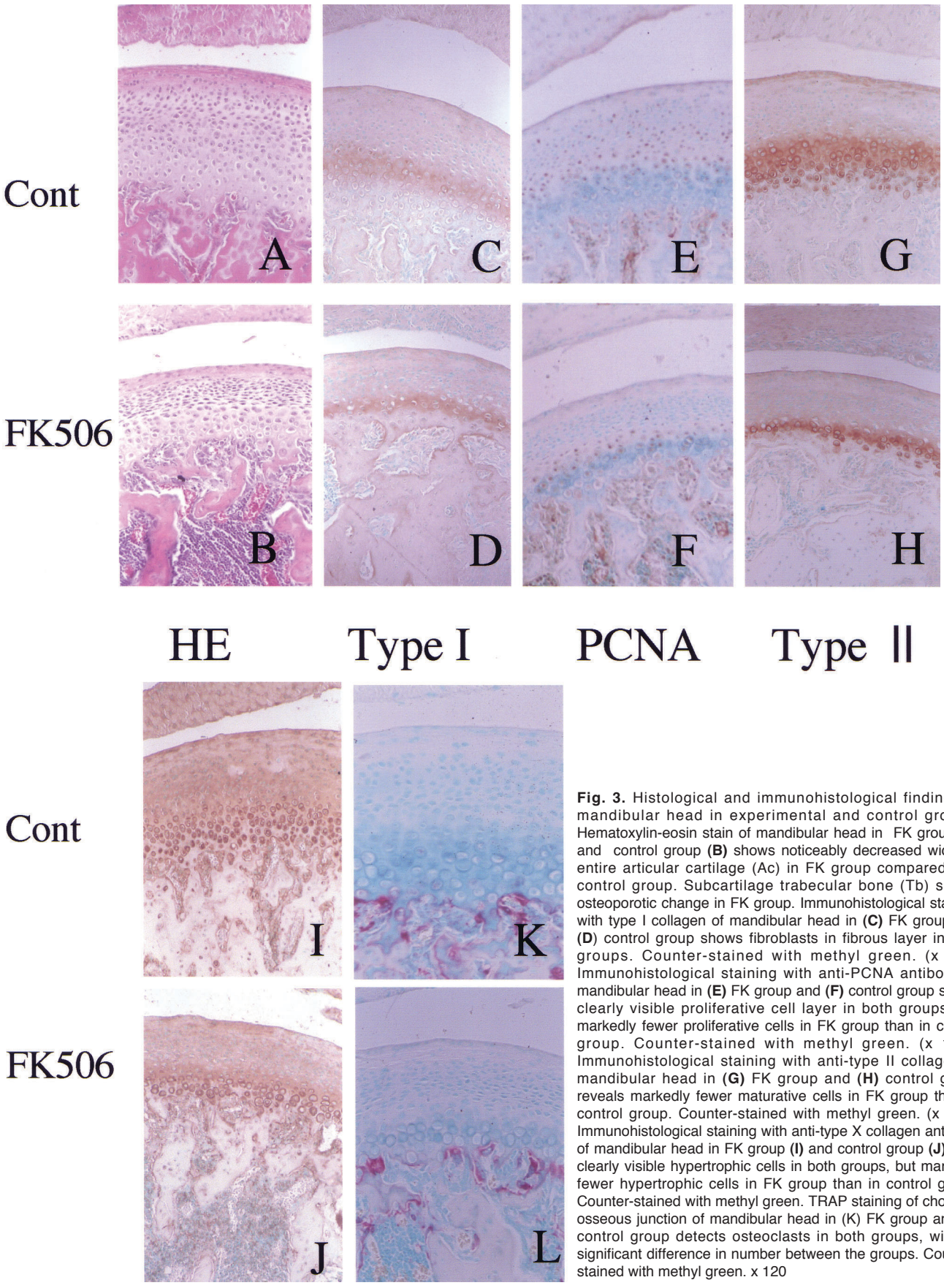

Fig. 3. Histological and immunohistological findings of mandibular head in experimental and control groups. Hematoxylin-eosin stain of mandibular head in FK group **(A)** and control group **(B)** shows noticeably decreased width of entire articular cartilage (Ac) in FK group compared with control group. Subcartilage trabecular bone (Tb) shows osteoporotic change in FK group. Immunohistological staining with type I collagen of mandibular head in **(C)** FK group and **(D)** control group shows fibroblasts in fibrous layer in both groups. Counter-stained with methyl green. (x 120) Immunohistological staining with anti-PCNA antibody of mandibular head in **(E)** FK group and **(F)** control group shows clearly visible proliferative cell layer in both groups, but markedly fewer proliferative cells in FK group than in control group. Counter-stained with methyl green. (x 120). Immunohistological staining with anti-type II collagen of mandibular head in **(G)** FK group and **(H)** control group reveals markedly fewer maturative cells in FK group than in control group. Counter-stained with methyl green. (x 120). Immunohistological staining with anti-type X collagen antibody of mandibular head in FK group **(I)** and control group **(J)**show clearly visible hypertrophic cells in both groups, but markedly fewer hypertrophic cells in FK group than in control group. Counter-stained with methyl green. TRAP staining of chondro-osseous junction of mandibular head in (K) FK group and (L) control group detects osteoclasts in both groups, with no significant difference in number between the groups. Counter-stained with methyl green. x 120

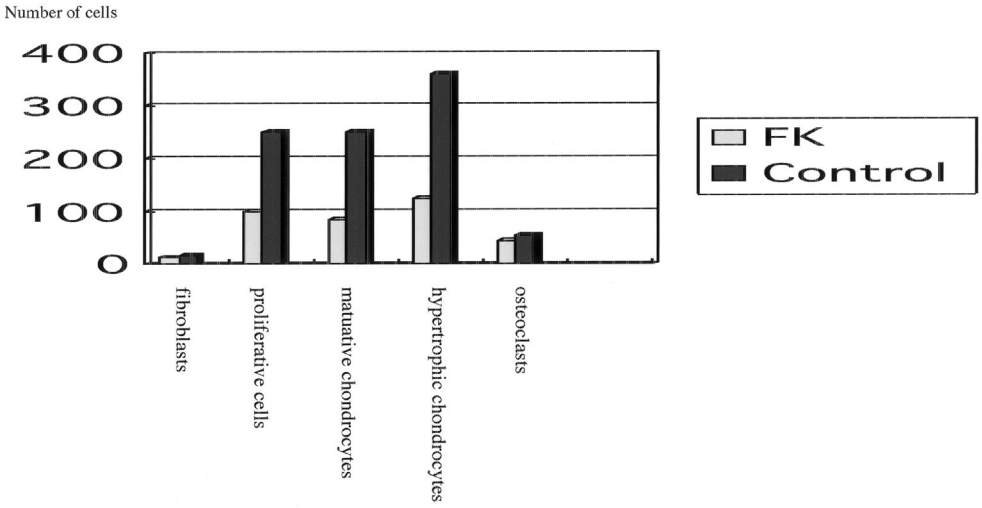

Fig. 4. Change in numbers of fibroblasts, proliferative cells, maturative chondrocytes, hypertrophic chondrocytes and osteoclasts in mandibular head following FK 506 administration for 28 days.

mechanical stress induced by mastication and occlusion (Gay et al., 1976). Type X collagen is known to be present in the hypertrophic cell zone, with some investigators suggesting that type X collagen is involved in the mineralization and ossification of articular cartilage (Chung et al., 1995; Von der Mark et al., 1998). In the present study, immunohistochemical staining of each collagen in the articular cartilage enabled us to observe fibrous cells, proliferative cells, maturative chondrocytes, and hypertrophic chondrocytes in the articular cartilage in the mandibular head. We found that the mandibular head treated with FK 506 had fewer maturative chondrocytes and hypertrophic chondrocytes than the control group, indicating a decrease in chondrogenic cell differentiation. In addition, there were fewer PCNA-positive cells in the mandibular head of the FK group than in the control group. Taken together, these findings suggest that FK 506 administration inhibited chondrogenic cell proliferation and differentiation in the mandibular head, resulting in decreased cartilage formation. Thus, the decrease in cartilage formation induced by FK 506 administration may be responsible for a decrease in subsequent trabecular bone formation in the mandibular head.

Our findings that the immunosuppressant FK 506 might be responsible for osteoporosis of the mandibular head due to structural change of the articular cartilage should be of interest to other oral surgeons and dentists. Patients taking immunosuppressants may be more prone than others to functional failure of the TMJ, such as that caused by osteoarthritis.

In conclusion, we have demonstrated that immunosuppressant administration induces osteoporosis in the mandibular head and suggest that the mechanism of this osteoporosis is related to atrophic change of the articular cartilage in the mandibular head. Further research, in vitro and in vivo, should aim to clarify in greater detail the mechanism of the atrophy of articular cartilage.

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