To: The Anatomical Record Part A

The Expression of Estrogen Receptor α (ERα) in the Rat Temporomandibular Joint

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ABSTRACT

Numerous epidemiological studies have pointed out a higher frequency of temporomandibular disorder (TMD) in women than in men, suggesting the involvement of a sex hormone such as estrogen in the pathogenesis of TMD. Although estrogen is known to play pivotal roles in osteoarthrosis or rheumatoid arthritis in systemic joints, there have been few reports about the role of estrogen in the temporomandibular joint (TMJ). Its effect is generally mediated by estrogen receptors (ERs), ERα and ERβ, the former which is a predominant type. In this study, we examined the expression of ERα protein and mRNA in the TMJ of adult male rats by immunocytochemistry and *in situ* hybridization histochemistry. Intense ERα-immunoreactivity was localized in the synovial lining cells, stromal cells in the articular disc, and chondrocytes in the TMJ. These ERα-immunopositive synovial lining cells were characteristic of cytoplasmic processes identified with confocal and immuno-electron microscopy, suggesting that they were synovial type B cells. *In situ* hybridization histochemistry confirmed intense signals for ERα in the synovial lining cells and the sublining fibroblasts at mRNA levels. The nuclei of chondrocytes showed an intense immunoreaction for ERα in the area of the maturative and hypertrophic layer of the articular cartilage. In addition to the nuclear localization of ERα, a weak immunoreaction appeared in the cytoplasm of some ERα-positive cells. These findings supported the hypothesis that the TMJ tissue --at least in the male rat-- has the potential to be an estrogen target tissue.

Key words: estrogen receptor α; temporomandibular joint; synovial membrane; immunocytochemistry; *in situ* hybridization

Introduction

Estrogen, a representative steroid hormone, is known to regulate diverse physiological processes of target tissues including reproductive (Galand et al., 1971), cardiovascular (for review, Dimitrova et al., 2002), skeletal (Migliaccio et al., 1992), nervous (for review, Behl, 2002) and connective tissues (Clark, 1992) in both sexes. The biological activities of estrogen are initiated by a binding to the specific receptor proteins, namely estrogen receptors (ER) (for review, Pavao and Traish, 2001). Two main isoforms of ER have been identified to date: ERα and ERβ. The recent discovery of ERβ (Kuiper, 1996) has allowed classical ER to be renamed as ERα. A wide distribution of ERα has been reported in various tissues by immunocytochemistry using specific antibodies, whereas a definitive distribution of ERβ protein remains unclear because specific antibodies to ERβ have not been available (cf. Pavao and Traish, 2001).

The temporomandibular joint (TMJ) is a bilateral diarthrosis between the mandibular condyle and temporal bone. Temporomandibular disorder (TMD) is characterized by a triad of symptoms, including limited mandibular movement, joint sounds, and pain (Dijkgraaf, 1997). These symptomatic variants indicate that TMD is caused by a combination of factors such as occlusion, mental stress, strength, endurance, and hormones; still, the etiology of this disease remains to be fully understood. Several epidemiological studies have reported that the occurrence of the TMD is more prevalent in women than in men (cf. Campbell et al., 1993; LeResche, 1997; Kapila and Xie, 1998), suggesting the involvement of sex hormones such as estrogen in this etiology. Although estrogen plays important roles in the etiology of postmenopausal osteoarthrosis or rheumatoid arthritis in systemic joints (Ushiyama et al., 1995; Khalkhali-Ellis et al., 2000), little information has been available regarding the relationship between the etiology of the TMD and estrogen (for review, Warren and Fried, 2001).

The synovial membrane in the TMJ plays important roles in joint movement because

of the involvement of the synovial lining cells in the synovial fluid metabolism which effects smooth jaw movement. Many ultrastructural investigations have pointed out that the synovial membrane consists of two kinds of synovial lining cells, macrophage-like type A and fibroblastic type B cells (cf. Barland et al., 1962; Graabæk, 1984). The type A cell is characterized by numerous vesicles, vacuoles and lysosomes, while the type B cell possesses the presence of a well-developed rough endoplasmic reticulum and an amount of dense secretory granules in rodents. In addition, recent immunocytochemical and scanning electron microscopic studies (Nozawa-Inoue et al., 1999; Iwanaga et al., 2000; Andoh et al., 2001; Nio et al., 2002) have revealed type B cells develop characteristic cytoplasmic processes which are easily distinguishable between type A and B cells. Type B cells possess thick dendritic processes which extend towards the articular cavity, and partially cover the synovial membrane, while type A cells develop dense filopodia-like surface folds. Therefore, the morphology of cytoplasmic processes is a useful marker for type B cells in the TMJ.

Heat shock proteins (Hsps) protect cells against irreversible damage, when cells are exposed to stressful conditions (Lindeman et al., 1998). According to their molecular weight, Hsps can be largely divided into two groups of large and small Hsps (Hopkins et al., 1998). Hsp25, one of the small Hsps, serves to protect cells against various stimuli such as hyperthermia (Lavoie et al., 1993), oxidative stress (Mehlen et al., 1995), and inflammatory cytokines (Mehlen et al., 1995). In addition, the involvement of Hsp25 in cell differentiation has been reported even under normal conditions (Welsh and Gaestel, 1998). In our previous studies (Nozawa-Inoue et al., 1999; Andoh et al., 2001), various cellular elements of TMJ were shown to be intensely immunoreactive for Hsp25 in the normal rats and mice. In particular, the type B lining cells in the synovial membrane more intensely expressed Hsp25 protein and mRNA, suggesting that Hsp25 is a useful marker for type B synoviocytes. The fact that Hsp25 was originally discovered as an estrogen-regulated protein suggests a relationship between Hsp25 and estrogen (Edwards et al., 1981). Furthermore, biochemical

studies have shown that *hsp25* might be directly up-regulated by estrogen (Porter et al., 1996) due to the estrogen-responsive element in the 5' promoter region. Taken together, these findings easily lead us to a hypothesis that the TMJ is a target tissue for estrogen. However, little information has been available on the localization of ERα in the TMJ. In the present study, therefore, we examined the expression of ERα protein and mRNA in the TMJ of adult male rats by immunocytochemistry and *in situ* hybridization technique using a specific antiserum and cDNA probe, respectively.

MATERIALS AND METHODS

All experiments were performed under the guidelines of the Niigata University Intramural Animal Use and Care Committee.

Animals and tissue preparation

Six male Wistar rats (4-week-old, weighing approximately 100 g) were used in this study to avoid the influence of the sexual cycle in female rats. They were anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg), and perfused with a fixative containing 4% paraformaldehyde and 0.025% glutaraldehyde in a 0.067 M phosphate buffer (pH 7.4). The TMJs were removed *en bloc* and immersed in the same fixative for 12 h. After decalcification with 10% ethylenediamineteraacetic acid disodium (EDTA-2Na) solution for 4 weeks at 4°C, tissue blocks were dehydrated through a series of graded ethanol, cleared in xylene, and embedded in paraffin. Serial paraffin sections were sagittally cut (5 μm thick), mounted onto silane-coated glass slides, and dried overnight. Some paraffin sections were stained with hematoxylin and eosin.

Immunocytochemistry

Deparaffinized sections were processed for immunohistochemistry for ERα using the avidin-biotin-complex (ABC) method (Hsu et al., 1981). After the inhibition of endogenous peroxidase with 0.3% H_2O_2 in absolute methanol for 15 min, the sections were reacted with a polyclonal antiserum against ERα (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) --which recognizes ERα from the mouse, rat, and human-- for 24 h at 4°C. The bound primary antibody was then localized using biotinylated anti-rabbit IgG (Vector Lab. Inc., Burlingame, CA), and subsequently an avidin-peroxidase complex (ABC kit;Vector Lab. Inc.) for 90 min each at room temperature. The final visualization used 0.04% 3,3'-diaminobenzidine tetrahydrochloride and 0.002% H_2O_2 in a 0.05 M Tris-HCl buffer (pH

7.6).

For confocal microscopic observation, deparaffinized sections incubated with the same primary antibody (1:50) were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100; Vector Lab. Inc.) for 1h at room temperature. For the visualization of nuclei, the sections were incubated with DNase-free RNase (100 μg/mL; Sigma- Aldrich Co., St. Louis, MO) in 2 x standard saline citrate (SSC) for 20 min at 37°C, and counter-stained with propidium iodide (1:3000 , Molecular Probes, Inc., Eugene, OR, USA) in 2x SSC for 5 min. The sections were then examined in a confocal laser scanning microscope (LSM 510; Carl Zeiss, Germany).

For immunoelectron microscopy, the localizations of ERα in the rat TMJs were visualized by an immunogold labeling method. Decalcified tissue blocks were sectioned at a 500 μm thickness with a Microslicer (Dosaka EM, Kyoto, Japan), dehydrated with N,N-dimethylformamide, and embedded in glycolmethacrylate (GMA, Nisshin EM Co. Ltd. Tokyo, Japan). Ultrathin GMA sections were prepared in a Richert Ultracut-N ultramicrotome with a diamond knife, and collected on nickel grids. The sections were soaked with 1% bovine serum albumin for 15 min, and reacted with two consecutive incubations with rat anti-ERα monoclonal antibody (1:500, Stressgen Biotechnologies Corp., Victoria, BC, Canada) and gold conjugated rabbit anti-rat IgG (1:100, 10 nm gold particles, Sigma Chemical Co., St. Louis, MO) for 1h each at room temperature. Following a rinsing in 0.01 M phosphate-buffered saline and distilled water, they were stained with lead citrate and examined under a Hitachi H-7000 transmission electron microscope (Hitachi Co Ltd, Tokyo, Japan).

Immunocytochemical controls of the antiserum were performed by: 1) replacing the primary antibody with non-immune serum or PBS; and 2) omitting the anti-rabbit IgG or the avidin-peroxidase complex.

Probe

Proven ERα probe, complementary to 301-346 bp of ERα c DNA (Koike et al. 1987) was used in this study. The oligonucleotide was labeled with ^{35}S-dATP, using terminal deoxyribonucleotidyl transferase (Promega, Madison, WI, USA) at a specific activity of 5 x 10^8 d.p.m. /µg DNA.

***In situ* hybridization**

An additional rats was decapitated under deep anesthesia mentioned above. Immediately after, the synovial membrane of the TMJ were removed and frozen in liquid nitrogen. These specimens were kept in a deep freezer at -80°C prior to use. The in situ hybridization procedure was performed according to Mowa and Iwanaga (2001). Briefly, 20 µm cryostat sections obtained from frozen fresh tissue were mounted onto glass slides pre-coated with 3-amino-propyltriethoxysilane, fixed in 4% paraformaldehyde for 10 min, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. The prepared sections were prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 7.5), 4 x SSC (1 x SSC; 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% sodium dodecyl sulfate (SDS), 200 µg/ml tRNA, 1 mM EDTA and 10% dextran sodium sulfate. Hybridization was performed at 42°C for 10 h. The slides were washed in 2 x SSC containing 0.1% sarkosyl (Nacalai Tesque, Kyoto, Japan) and twice at 55°C in 0.1 x SSC containing 0.1% sarkosyl. The sections were dipped in Kodak NTB2 nuclear track emulsion and exposed.

The specificity of the *in situ* hybridization was checked by the disappearance of signals when an excess dose of corresponding cold oligonucleotides was added to the hybridization fluid. Consistent ERα mRNA signals above background levels were considered positive.

RESULTS

Immunocytochemistry for ERα demonstrated an intense immunoreaction in the various cellular elements --synovial lining cells, vascular endothelial cells, synovial fibroblasts, stromal cells in articular disc and chondrocytes-- in the rat TMJ.

ERα-immunoreaction and mRNA in the synovial membrane of rat TMJ

The inner surface of the articular capsule, but not the articular cartilage nor the articular disc, was covered with a synovial membrane (Fig. 1). This synovial membrane formed a few folds protruding into the articular cavity, particularly in the postero-superior portion (Fig. 1). Immunocytochemistry showed the synovial lining layer consisting of one to three layers of ERα immuno-positive and -negative cells (Fig. 2a, c). In light microscopy, both these synovial lining cells appeared round in profile. Although the arrangement of the synovial lining cells showed a regional difference: it was a comparatively thick cell layer at the anterior portion and the tip of the synovial fold (Fig. 2a), or a single flattened cell layer at the other area (Fig 2c), synovial lining cells with an ERα-immunoreactivity were recognized in all portions of the synovial membrane. Intense ERα-immunoreactions were found in the nuclei of the synovial lining cells, whereas there were weak reactions in their cytoplasm (Fig. 2c).

Confocal laser microscopic observations with ERα-immunostaining and nuclear propidium iodide more clearly demonstrated the immuno-localization of ERα in the synovial lining cells. ERα-positive synovial cells exhibited respectively intense and weak immunoreactions in the nucleus and cytoplasm, but a few cells lacked any nuclear immunoreaction despite the positive reaction in their cytoplasm. Furthermore, comparatively thick cell processes and slender cytoplasmic processes lining the articular cavity were also seen to exhibit ERα-immunoreactivity (Fig. 2b).

The sublining layer of the synovial membrane also contained ERα-immunopositive cells; some endothelial cells and fibroblasts showed an immunoreaction in their nuclei (Fig. 2b,

c). However, immunocontrol sections did not show any specific immunoreaction for ERα in the synovial membrane (Fig. 2d).

At the electron microscopic level, gold particles indicative of ERα-immunoreactivity were scattered in the nucleus, cytoplasm, and plasma membrane of the synovial cells (Fig. 3). These immunopositive cells extended thick, long cytoplasmic processes towards the articular cavity and covered the surface of the synovial membrane (Fig. 3a), suggested that they were type B cells.

In situ hybridization histochemistry showed intense signals for ERα mRNA in the synovial lining layer and the sublining layer (Fig. 4a). The synovial lining layer contained the synovial lining cells with and without for ERα mRNA (Fig. 4a). In control sections incubated with an excess dose of corresponding cold oligonucleotides, any specific signals for ERα mRNA were not recognizable in the synovial membrane (Fig. 4b).

ERα-immunoreactivity in the articular disc

The articular disc also contained ERα-positive cells. Intense immunoreactions were particularly recognized in the nuclei of the articular stromal cells located in the anterior and posterior bands (Fig. 5a, b). Immunoreactive stromal cells were rare in the central portion (Fig. 5c). The positive cells were round in shape or possessed short cytoplasmic projections, appearing stellate in profile (Fig. 5a, b).

ERα-immunoreactivity in the mandibular condyle

Immunostaining revealed localization of the ERα-immunoreactivity in some population of chondrocytes in the mandibular condyle of the rat TMJ (Fig. 6). Since the terminology of the cartilaginous layer of the rat condyle has not been well established to date, we here divided the articular cartilage into the following four layers based on the classification by Bloom and Fawcett (1975), namely, the fibrous, proliferative, maturative and hypertrophic

cell layers. Each cartilaginous layer did not show any uniform ERα-immunoreactivity. ERα-positive chondrocytes were abundant in the maturative and hypertrophic cell layers in the condyle, as compared with the fibrous and proliferative layers (Fig. 6). The nuclei showed more intense ERα-immunoreactions than the cytoplasm in these chondrocytes (Fig. 6). In contrast, a few chondrocytes in the fibrous and proliferative cell layers demonstrated a weak immunoreactivity for ERα (Fig. 6)

DISCUSSION

A wide distribution of ERα-immunoreactions has been reported in the primate dermal fibroblasts (Bentley et al., 1986), human fibroblasts, human endothelial cells (Brandi et al., 1993), mammary gland cells (for review, Pelletier, 2000), chondrocytes form cows, pigs, and human (Claassen et al., 2001), human osteoblasts (Eriksen et al., 1988), and human osteoclasts (Pensler et al., 1990), in addition to the female reproductive tissues, suggesting diverse effects of estrogen on development, growth, and homeostasis (Clark, 1992). Although many epidemiological studies have indicated a higher frequency of TMD in females than in males (cf. Campbell et al., 1993; LeResche, 1997; Kapila and Xie, 1998), there has been controversy over the presence of ERα in the TMJ. Abubaker et al. (1993) found ERα-immunoreactivity in articular disc of the human TMJ, but Cambell et al. (1993) failed to do so in the articular disc of a human TMJ obtained from a TMD patient. Furthermore, some researchers have pointed out sexual dimorphism in ER expression in the baboon TMJ by autoradiography (Aufdemorte, 1986; Milam et al., 1987); no estrogen-binding site exists in males but abundantly so in females. By the use of an immunocytochemistry, the present study was able to demonstrate clearly the distribution of ERα in various kinds of cellular elements including synoviocytes, stromal cells in articular disc, and chondrocytes in the rat TMJ. Furthermore, certain kinds of the synovial lining cells expressed ERα mRNA as demonstrated by *in situ* hybridization histochemistry. To our knowledge, this is the first report to demonstrate a precise distribution of ERα in normal male rats, suggesting that TMJ is a target tissue for estrogen.

The lining layer of synovial membrane contains two kinds of lining cells; macrophage-like type A and fibroblastic type B cells (Barland et al., 1962; Graabæk, 1984). However it was impossible to observe all organellae in the ERα-positive cells due to the use of GMA resin which is disadvantageous for the detail observations, these two types of lining cells are also distinguished by their cytoplasmic processes (Iwanaga et al., 2000; Nio et al., 2002).

Current confocal and immunoelectron microscopic observations suggested that these ERα-immunopositive synovial lining cells are categorized as type B cells because of the characteristic profiles mentioned above. These evidences support the possibility that the synovial lining cells with ERα mRNA are fibroblastic type B cells. This notion is comparable with previous reports on the human cruciate ligament (Liu et al., 1996) and cultured synoviocytes obtained from human RA-patients (Khalkhali-Ellis et al., 2000).

The functional significance of ERα/estrogen in synovial type B cells remains unclear. In our previous reports (Nozawa-Inoue et al., 1999; Andoh et al., 2001), the type B cells in the rat and mouse TMJs showed an immunoreactivity for Hsp25, which was originally discovered as an estrogen-related protein (Edwards et al., 1981). Moreover, the synovial type B cells have been reported to synthesize and secrete collagens, fibronectin (Matsubara et al., 1983; Mapp and Revel, 1985), and glycosaminoglycans including hyaluronan (Roy and Ghadially, 1967). An administration of estrogen induced a decrease in the volume and synthesis of collagen in the rat tendon (Fischer, 1973), periodontal tissue (Dyer et al., 1980), and TMJ discs (Abubaker et al., 1996). Therefore, it is reasonable to consider that estrogen might regulate the metabolism --including collagen synthesis-- via ERα expressed in the synovial type B cells and the stromal cells in the articular disc.

The present observation of condylar cartilage showed ERα-immunoreactivities in chondrocytes both in the maturative and hypertrophic layers, consistent with a report investigating ERα expression in cultured rat mandible (Ng et al., 1999). The effect of estrogen on the bone metabolism has been well established. In contrast, there have been few studies on the function of estrogen on cartilage in the TMJ, or even in other systematic joints (cf. Claassen et al., 2001). Ng et al. (1999) demonstrated that mandibular cartilage cultured with media containing estrogen caused a decrease in the extracellular matrix and thickness. These results suggested that estrogen downregulates in the cartilage metabolism, due to binding with ERα in the maturative and hypertrophic chondrocytes.

It is interesting that ERα-immunoreactions were seen in the cytoplasm and plasma membrane as well as in the nucleus, clearly shown by the present confocal laser microscopy and immunoelectron microscopy. The traditional concept of the biological function of ERα is based on the transcriptional activity regulated by the estrogen response element (ERE) of target genes. In contrast, though still an on-going discussion, an alternative pathway for estrogen action -- non-genomic action of this molecule-- has been predicted under physiological conditions. Cytoplasmic and plasma membrane complexes of estrogen/ ERα appear to participate in signal transduction, resulting in the regulation of cell growth, survival, and migration (for review, Levin, 2002; Razandi et al, 2003). The activity of p38, one of the mitogen-activated protein (MAP) kinase family, is stimulated by estrogen. Upregulation of p38 by estrogen gives rise to activation of the MAPKAPK-2 kinase, and the phosphorylation of Hsp27 in human endothelial cells. Estrogen was shown to utilize this pathway to protect endothelial cells from any metabolic disruption of actin cytoskeleton, hypoxia-induced cell death, and to stimulate angiogenesis (Razandi et al., 2000). Interestingly, our previous immunocytochemical study for Hsp25, a homologue of human Hsp27 (Gaestel et al., 1993), showed cytosolic expression in the synovial type B lining cells, stromal cells in the articular disc, and chondrocytes in the rat TMJ (Nozawa-Inoue et al., 1999); Hsp25 immunoreaction pattern seems comparable with expression pattern of ERα as shown in this study. Thus, it is likely that ERα and Hsp25 co-localize in the cells of the rat TMJ, and serve to cooperate with each other on such a signal transduction.

Our own immunocytochemical observations provided a clue for the putative involvement of ERα on the physiological function of TMJ tissues, at least of male rats. However, Kennedy et al. (1999) reported on the stage-specific expression of ERα-immunoreaction in chondrocytes of femoral growth plates; an intense reaction occurred in immature rats, but none in the aged ones. This finding indicates different controls on the ERα-expression between reproductive and skeletal tissues. Further investigations on age- and

sex-related changes in ERα-expression are needed to clarify the functional significance of estrogen in the TMJ.

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Figure legends

Fig. 1. Sagittal section of the rat TMJ stained with hematoxylin and eosin. The arrow indicates the anterior direction. A few folds of the synovial membrane protrude into the articular cavity, particularly in the postero-superior portion (boxed area). C, mandibular condyle; D, articular disc; T, temporal bone. Scale bar =200 μm

Fig. 2. Immunoreactions for ERα in the rat synovial membrane. **a:** The synovial lining layer (SL) in the anterior portion consists of a one- to three-layered arrangement of ERα immuno-positive and -negative lining cells. **b:** Confocal microscopic images of ERα-immunoreactions (green), counterstained with propidium iodide (red). ERα-positive synovial cells (arrowheads) exhibit intense immunoreactions in the nucleus (yellow) as well as cytoplasm (green). Note the slender cytoplasmic processes of lining cells showing ERα-immunoreactivity (arrows). Some endothelial cells also show ERα-immunoreactivity (double arrows). **c:** Higher magnification of the synovial membrane of the fold. Intense immunoreactions are localized in the nuclei of the synovial cells (arrowheads). Arrows indicate immunoreactions in the fibroblasts in the sublining layer. **d:** Immunocontrol section adjacent to Figure 2c. No specific immunoreaction is seen in the synovial membrane. Asterisk: articular cavity. Scale bars = 50 μm in a, b, 40 μm in c, d.

Fig. 3. Immunoelectron micrographs showing ERα-immunoreaction in the anterior synovial lining cells. **a:** A synovial lining cell (arrow) contains gold particles indicative of ERα-immunoreactivity. The immunopositive cell extends thick cytoplasmic processes towards the articular cavity and covers the surface of synovial membrane (arrowhead). **b:** High power view of boxed area b in Figure 3a. Gold particles are localized in the cytoplasm as well as plasma membrane of the synovial lining cell. An arrowhead indicates cytoplasmic extensions. **c:** Magnified view of boxed area c in Figure 3a. Note ERα-immunolocalization in the nucleus. Asterisk: articular cavity. Scale bars = 2 μm in a, 0.5 μm in b, c.

Fig. 4. Expression of ERα mRNA in the rat synovial membrane. **a**: The synovial lining cells

show intense signals for ERα mRNA (arrowheads). The fibroblasts in the sublining layer also express ERα mRNA (arrows). **b:** No specific signal for mRNA is found in the control section which was incubated with an excess dose of cold oligonucleotides. Counter-stained with hematoxylin. Asterisk: articular cavity. Scale bars = 30 μm in a, b.

Fig. 5. Photomicrographs showing ERα-immunoreactivity in the anterior (a), posterior band (b) and the intermediate zone (c) of the articular disc. **a, b:** ERα-positive cells are round in shape (arrows) or possess short cytoplasmic projections (arrowheads), appearing stellate in profile. **c:** A few round cells in the intermediate zone are immunoreactive for ERα (arrows). Scale bars = 50 μm in a-c.

Fig. 6. Confocal microscopic images of the ERα-immunoreactive chondrocytes (green) in the condylar cartilage, counterstained with propidium iodide (red). The rat condylar cartilage is divided into four layers: the fibrous (F), proliferative (P), maturative (M), and hypertrophic (H) cell layers. The maturative and hypertrophic cell layers contain rich ERα-immunopositive chondrocytes. The nuclei show more intense immunoreactions for ERα as compared with the cytoplasm. A few chondrocytes in the proliferative cell layers exhibit weak immunoreactivity for ERα. Asterisk: articular cavity. Scale bar = 50 μm.