To: The Anatomical Record Part A

Immunocytochemical Localization of MAPKAPK-2 and Hsp25 in the Rat Temporomandibular Joint

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ABSTRACT: One series of our research has shown an intense expression of immunoreaction for heat shock protein 25 (Hsp25) in various cellular elements in the rat temporomandibular joint (TMJ). This protein is the major substrate of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2), which mediates an intracellular stress-activated signaling pathway to stimulate cytosolic actin reorganization under various stresses. The present study was undertaken to examine the localization of MAPKAPK-2 in the rat TMJ by immunocytochemical techniques. Furthermore, confocal microscopy with double staining was employed to demonstrate the co-localization of MAPKAPK-2 and Hsp25.

Immunocytochemistry for MAPKAPK-2 showed an intense immunoreaction in the cytoplasm of the synovial lining cells, the endothelial cells, and the fibroblasts in the synovial membrane of the rat TMJ. Double immunostaining under a confocal microscope succeeded in demonstrating the co-localization of MAPKAPK-2- and Hsp25-immunoreactions in the cytoplasm of fibroblastic type B synoviocytes in the TMJ. On the other hand, the macrophage-like type A cells expressed MAPKAPK-2-immunoreactions but lacked Hsp25-immunoreactivity. The cells in the articular disc and the chondrocytes in the maturative and hypertrophic layer of the mandibular cartilage also showed intense immunoreactions for MAPKAPK-2 and Hsp25. In addition to cytoplasmic localization, MAPKAPK-2-immunoreactions were found in the nucleus of some synovial lining cells, cells in the articular disc, and chondrocytes. Current observations imply that the presence of the phosphorylation of Hsp25 via activated MAPKAPK-2 in the cytoplasm. The MAPKAPK-2 and Hsp25 possibly participate in the induction of cytoskeletal changes to the various cellular elements in rat TMJ under normal conditions.

Key words: temporomandibular joint, synovial membrane, synovial lining cell, MAPKAPK-2, Hsp25

INTRODUCTION

Many mechanical and/or chemical stimuli elicit specific cellular responses through the activation of mitogen-activated protein (MAP) kinase signaling pathways. In particular, the p38 MAP kinase signaling pathway--called the stress-activated signaling pathway--plays important roles in cell division, apoptosis, cell invasion, and the inflammatory response. (Lee et al, 1994; Xia et al, 1995; Simon et al., 1998; Takenaka et al., 1998). MAP kinase-activated protein kinase-2 (MAPKAPK-2), a Ser/Tyr protein kinase, is a major substrate of p38 MAP kinase (Stokoe et al., 1992a). Previous studies have shown the expression of MAPKAPK-2 in various kinds of cells--including smooth muscle cells (Hedges et al., 1999), endothelial cells (Rousseau et al., 1997; Azuma et al., 2001; Dalle-Donne et al., 2001), fibroblasts (Huot et al., 1998), and macrophages (Kotlyarov et al., 2002)--to mediate multiple physiological activities (Kotlyarov et al., 2002). The immunolocalization of MAPKAPK-2 has been reported in the cytoplasm and/or nucleus of cells; the major substrate of MAPKAPK-2 is murine heat shock protein 25 (Hsp25), an analogue to human Hsp27, and CREB both in the cytoplasm and nucleus, respectively (Stokoe et al., 1992b; Landry and Huot, 1995; Tan et al., 1996; Guay et al., 1997).

The temporomandibular joint (TMJ), a bilateral diarthrosis with a synovial membrane, exists under unique and complicated conditions due to loading with occlusal force. The synovial membrane in the TMJ consists of a unique cellular lining--the synovial lining cell layer or synovial intima--and a connective sublining layer. The ultrastructural observations of many researchers affirm the two types of synovial lining cells -- including macrophage-like type A and fibroblast-like type B cells -- in the synovial lining layer of TMJ (for review, see Nozawa-Inoue et al., 2003), as reported in other systemic joints (for review, see Iwanaga et al., 2000). Our studies have shown an intense expression of immunoreaction for Hsp25, which has diverse functions (Ciocca, 1993; Arrigo and Préville, 1999) in various cellular elements--fibroblast-like

type B cells, stromal cells in the articular disc, chondrocytes in certain layers of condylar cartilage--in murine TMJ (Nozawa-Inoue et al., 1999a; Andoh et al., 2001). In particular, fibroblast-like type B cells expressed intense Hsp25 protein and mRNA, indicating that this protein is useful as a specific marker for fibroblast-like type B cells. In addition, immunocytochemistry for Hsp25 revealed two types of unique cytoplasmic processes in fibroblast-like type B cells: one a thick, long process extended towards the articular cavity from the deeper part of the lining layer, and the other a slender process which covers the synovial membrane (Iwanaga et al., 2000; Nio et al., 2002; Nozawa-Inoue et al., 2003). Both of them are exposed directly to the articular cavity to detect various stimuli. Since the activation of Hsp25 by MAPKAPK-2 has been considered to regulate actin filament dynamics for cytoskeletal reorganization and morphological changes (Guay et al., 1997; Huot et al, 1998), we can easily suppose the involvement of Hsp25 and MAPKAPK-2 in the morphological alterations in the cytoplasmic processes of the fibroblast-like type B cells. However, there is as yet no report on the existence of MAPKAPK-2 in the TMJ.

Clinical experiences have shown a drastic increase in the number of patients with temporomandibular disorders (TMD), which is characterized by a triad of symptoms, including joint sounds, pain, and limited mandibular movement (Kuttila et al., 1998). Biochemical analyses of synovial fluids have reported high levels of various cytokine activities including interleukin (IL)-1, 6, 8 and TNFα in patients with TMD (for review, see Kacena et al., 2001), all of which are involved in the progress of inflammation to induce subsequent bone destruction. The involvement of MAPKAPK2 has been suggested in the transcriptional activation of those proinflammatory cytokines (Beyaert et al., 1996; Kotlyarov et al., 1999; Neininger et al., 2002). Since the activities of these cytokines are also suppressed by a p38 kinase inhibitor, the p38/MAPKAPK2 cascade is regarded as a key target for the treatment of rheumatoid arthritis

(RA) (for review, see Lee et al., 2000). However, no information is presently available on the relationship between MAPKAPK-2 and the etiology of TMD.

The present study was therefore undertaken to examine the localization of MAPKAPK-2 in the rat TMJ by immunocytochemical techniques. Furthermore, confocal laser microscopy with double staining using polyclonal antisera against Hsp25 and MAPKAPK-2 was employed to identify the cell type immunoreactive to both MAPKAPK-2 and Hsp25.

MATERIALS AND METHODS

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

Animals and tissue preparation

Four-week-old Wistar rats, weighing approximately 100 g, were selected as experimental animals because the cartilageous layers at this stage can be most clearly divided into four layers in contrast to unclear lamination at other stages. Six male Wistar rats were anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg) and then perfused with a fixative containing 4% paraformaldehyde and 0.025% glutaraldehyde in a 0.067M 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 equilibrated in a 30% sucrose solution for cryoprotection and embedded in OCT compound (Leica, Nussloch, Germany). Serial sagittal sections including the TMJ were cut at a thickness of 35 µm in a cryostat and mounted onto silane-coated glass slides.

Immunocytochemistry for MAPKAPK-2

The cryostat sections were processed for immunocytochemistry for MAPKAPK-2 using the avidin-biotin-complex (ABC) method (Hsu et al., 1981). The sections were reacted overnight at 4˚C with a polyclonal antiserum against MAPKAPK-2 (1: 900; Stressgen Biotechnologies Corp., Victoria, BC, Canada) which recognizes MAPKAPK-2 from the human, mouse, rat, dog, and bovine (manufacture's instruction). The bound primary antibody was then localized using a biotinylated anti-rabbit IgG (Vector Lab. Inc., Burlingame, CA, USA) and subsequently with

ABC conjugated with peroxidase (ABC kit; Vector Lab. Inc.) for 90 min each at room temperature. 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). Immunostained sections without counter-staining were post-fixed in 1% OsO_4 reduced with 1.5% potassium ferrocyanide for 1 h at 4˚C, dehydrated in an ascending series of ethanol, and finally embedded in epoxy resin (Epon 812; Taab, Berkshire, UK). Plastic sections, 1 µm thick, were stained with 0.03% methylene blue.

Double-labeling immunocytochemistry for MAPKAPK-2 and Hsp25

Fluorescent double labeling immunohistochemistry was performed using an avidin-biotin system according to Burton et al. (1997). Briefly, frozen sections were pretreated with 2% avidin and 2% biotin (Blocking kit; Vector Lab. Inc.) and then incubated overnight at 4˚C with a rabbit polyclonal antiserum against Hsp25 (1:5000; Stressgen Biotechnologies Corp.). The bound primary antibody was localized using a biotinylated anti-rabbit IgG, followed by avidin D-fluorescein (1:300; Vector Lab. Inc.) each for 1 h at 37˚C. After the first round of staining, the sections were rinsed with phosphate-buffered saline (PBS), incubated in 5% normal rabbit serum, rinsed with PBS, and then incubated in affinity-purified goat anti-rabbit IgG Fab fragments (10 µg/ml; MP Biomedicals, Inc., Irvine, CA, USA) for 1 h at 37˚C. Then the blocking steps using avidin and biotin were repeated. The sections were incubated in 10% normal goat serum and then reacted with a rabbit polyclonal antiserum against MAPKAPK-2 (same dilution as mentioned above) overnight at 4˚C. After rinsing, the sections were incubated in a biotinylated goat anti-rabbit IgG followed by avidin D-rhodamine (1:250; Vector Lab. Inc.) for 1 h at 37˚C and finally examined in a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

Immunocontrol experiments

Immunocytochemical controls were performed by: 1) an absorption test using primary antibodies preabsorbed with excess MAPKAPK-2 antigen; 2) replacing the primary antiserum with non-immune serum or phosphate-buffered saline; 3) omitting the anti-rabbit IgG. These control sections did not reveal any immunoreaction. The characterization of the antisera and the origin of antigens have been previously reported in the literature (cf. Clifton et al., 1996).

RESULTS

Immunocytochemistry for MAPKAPK-2 demonstrated an intense immunoreaction in various cellular elements in the rat TMJ, including the synovial lining cells, vascular endothelial cells, synovial fibroblasts, stromal cells in the articular disc, and chondrocytes (Figs. 1, 2).

The synovial membrane covers the inner surface of the articular capsule in the TMJ except for the surface of the articular disc and condylar cartilage. Observations showed a few synovial folds protruding into the articular cavity that developed in the postero-superior potion (Fig. 1b). The synovial lining cells differed in arrangement between anterior and posterior portions; they appeared one-to three-layered at the anterior potion (Fig. 1a), and a single flattened cell layer at the synovial folds in the posterior portion (Fig. 1b, c). MAPKAPK-2-immunopositive synovial lining cells were recognized in all portions of the synovial membrane. In the anterior portion, the synovial lining layer consisted of immuno-positive and -negative cells (Fig. 1a). MAPKAPK-2-immunoreactions were found to be diffusely distributed in the cytoplasm and some nuclei of the synovial lining cells. A few immunonegative cells, intensely stained with methylene blue, possessed vacuole-like structures in their cytoplasm. At the surface of the synovial folds in the posterior potion, MAPKAPK-2-immunoreactivity was discernible in the lining cells with long cytoplasmic projections exhibiting their sheet-like coverage of the synovial surface (Fig. 1b, c). Several fibroblasts in the sublining tissue and vascular endothelial cells were also positive for MAPKAPK-2 in their cytoplasm (Fig. 2c).

In confocal laser microscopy, a double-labeling immunohistochemistry using polyclonal antisera to MAPKAPK-2 and Hsp25 more clearly succeeded in distinguishing the synovial lining cells according to their immuostaining patterns: synoviocytes with co-localization of MAPKAPK-2 and Hsp25, and synoviocytes with MAPKAPK-2- but without

Hsp25-immunoreactivites. The former types of synoviocytes extended their long cytoplasmic processes (Fig. 2b, c), indicating that this cell type is categorized as a fibroblast-like type B cell as shown in our previous study (Nozawa-Inoue et al., 1999a). The later type, round in shape, did not have elongated cytoplasmic processes (Fig. 2a, c). Judging from their configuration and immunonegativity for Hsp25 (Nozawa-Inoue et al., 1999a; Andoh et al., 2001), these synoviocytes could be regarded as macrophage-like type A cells. However, we failed to find the synoviocytes with nuclear but without cytoplasmic localization of MAPKAPK-2 existing in the synovial lining layer.

The articular disc also contained MAPKAPK-2 positive cells, which possessed short cytoplasmic projections, appearing stellate in profile (Fig. 2d). Although a majority of positive cells were filled with an immunoreaction in their cytoplasm, some cells had a nuclear localization of MAPKAPK-immunoreactivity. They also co-expressed MAPKAPK-2 and Hsp25 in their cytoplasm (Fig. 2f).

Since the terminology for the cartilaginous layer of the rat condyle has not been well established yet, we divided the articular cartilage into four layers based on the classification by Bloom and Fawcett (1975): fibrous, proliferative cell, maturative cell, and hypertrophic cell layers. Immunostaining revealed the localization of MAPKAPK-2-immunoreactivity in many chondrocytes in the proliferative, maturative, and hypertrophic cell layers, but only in a few cells in the fibrous layer (Fig. 2g). However, Hsp25-immunoreactivity did not show a similar pattern with MAPKAPK-2; only maturative and hypertrophic cell layers contained Hsp25 positive chondrocytes (Fig. 2h). Thus, the co-localization of MAPKAPK-2 and Hsp25 was identified in chondrocytes in two layers--maturative and hypertrophic cell layers--and not in the proliferative cell layer (Fig. 2i).

DISCUSSION

The present immunocytochemical study was able to demonstrate a wide distribution of MAPKAPK-2 in various cellular elements in the rat TMJ as well as the co-localization of MAPKAPK-2 and Hsp25, a cytoplasmic major substrate of MAPKAPK-2. MAPKAPK-2 is a direct substrate of p38 MAP kinase in response to cellular stress such as mechanical stress, heat shock, osmotic stress, UV irradiation, bacterial lipopolysaccharide (LPS), several inflammatory cytokines, and growth factors (for review, see Ono and Han, 2000). This protein regulates actin filament dynamics through the phosphorylation of cytosolic Hsp25 (Landry and Huot, 1995; Guay et al., 1997; Kotlyarov et al., 2002) as well as the biosynthesis of several proinflammatory cytokines (Kotlyarov et al., 1999, 2002; Neininger et al., 2002). Thus, MAPKAPK-2 is a key molecule for inducing morphological and pathological changes against stress in the TMJ. To our knowledge, this is the first report to demonstrate a precise distribution of MAPKAPK-2 and further confirm the co-localization of MAPKAPK-2 and Hsp25 in the normal rat TMJ.

MAPKAPK-2 has several targets both in the nucleus and cytoplasm: the cAMP-response element-binding protein (CREB) (Tan et al., 1996), activating transcription factor-1(ATF1) (Tan et al., 1996), serum response factor (SRF) (Heidenreich et al., 1999), and E47 (Neufeld et al., 2000) are for the nuclear target, while Hsp25/27, lymphocyte-specific protein 1 (LSP1) (Huang et al., 1997), glycogen synthase (Stokoe et al., 1992a), tyrosine hydroxylase (Thomas et al., 1997), and 5-lipoxygenase (Werz et al., 2000) are for the cytoplasmic target. In cultured cells, activated p38 enters the nucleus after stimulation, and phosphorylates MAPKAPK-2 at different regulatory sites (Ben-Levy et al., 1995; Engel et al., 1995). Once the MAPKAPK-2 masks a nuclear localization signal (NLS) in the C-terminal part of the molecule by phosphorylation, it is rapidly exported to cytoplasm to phosphorylate

cytosolic targets (Ben-Levy et al., 1998; Engel et al., 1998). Therefore, the present immunocytochemical data strongly suggest the presence of the phosphorylation of Hsp25 via activated MAPKAPK-2 in the cytoplasm of various cellular elements in rat TMJ under normal conditions. The fibroblast-like type B cells, in particular, exhibited intense MAPKAPK-2- and Hsp25-immunoreactivity in their cytoplasm. Phosphorylation of Hsp25 by MAPKAPK-2 stabilizes and increases actin filament formation, resulting in cytoskeletal changes (Hedges et al., 1999). Azuma et al. (2001) demonstrated the activation of MAPKAPK-2, and Hsp25 regulates vascular endothelial cell reorientation, elongation, and stress fiber formation in response to shear stress. Due to direct exposure of the fibroblast-like type B cells to the articular cavity, the shear stress of synovial fluid caused by condylar movement may induce cytoskeletal changes through this pathway. The functional significance of MAPKAPK2 and Hsp25 in the stromal cells of the articular disc also may be explained by cytoskeletal changes because the articular disc receives great stress from mechanical forces during jaw movement.

In addition, this signal transduction pathway involving MAPKAPK-2 and Hsp25 has been reported to mediate cell adhesion to the basement membrane collagen type IV serving to modulate adhesion molecules in human breast carcinoma cells (Paine et al., 2000). By the use of immunocytochemistry for laminin, a glycoprotein in the basement membrane, we found the investment of a discontinuous basement membrane around the fibroblast-like type B cells (Nozawa-Inoue et al., 1999b). It is likely that cytoskeletal changes via the MAPKAPK-2/Hsp25 pathway enable the type B cells to attach to this discontinuous basement membrane.

Immunocytochemistry with DAB-development showed the synovial lining cell layer to consist of MAPKAPK-2-positive and -negative cells. The immunonegative cells possessed vacuole-like structures suggesting macrophage-like type A cells. However, confocal

microscopic observation clearly demonstrated both types of synovial lining cells, including type A and B cells immunoreactive for MAPKAPK-2. Taking all these findings together, it is better to consider the existence of immunonegative macrophage-like type A cells because these cells have two phenotypes with immunological features (for review, see Nozawa-Inoue et al., 2003).

In addition to the synovial lining cells with cytosolic MAPKAPK-2 immunoreactivity, a few synoviocytes had MAPKAPK-2 immunoreactivity in their nuclei in the normal TMJ. To date, MAPKAPK-2 in the nucleus has been discussed from viewpoints of a detrimental function in inflammations. The phosphorylation of p38 MAP kinase is involved in the transcriptional activation of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 in chronic synovitis during RA (Miyazawa et al., 1998; Suzuki et al., 2000) although the participation of MAPKAPK-2 in this inflammatory process has not been established. Mice lacking MAPKAPK-2 show a reduction in the LPS-induced biosynthesis of TNF-α, interferon (IFN)-γ, IL-1, IL-6, and nitric oxide (NO) (Kotlyarov et al., 1999, 2002; Neininger et al., 2002). Nuclear MAPKAPK-2 is considered to regulate the productions of these inflammatory cytokines in the nucleus at the level of transcription of mRNA (Neininger et al., 2002). This evidence supports the possibility that MAPKAPK-2 plays an important role in the pathophysiology of RA. Since the expression of p38 MAP kinase was observed predominantly in macrophages and fibroblasts of the synovial lining layer in an arthritic condition (Schett et al., 2000), we assume that any inflammatory stimulation such as LPS increases the nuclear MAPKAPK-2 in these synovial lining cells to induce the production of these cytokines. Under normal conditions as shown in this study, activated MAPKAPK-2 in the nucleus might be needed for the activation of the other transcriptional target such as CREB. However, we can not determine whether the nuclear MAPKAPK-2 in this study was in an active or non-active state because the antiserum used in this study can recognize both of them. Further

investigation on the MAPKAPK-2 expression under inflammatory conditions is needed to elucidate an etiology and establish an effective treatment for TMD.

The role of p38 MAP kinase in chondrogenesis and the hypertrophic differentiation of chondrocytes has been well investigated in spite of scanty information on the substrates of p38 MAP kinase which affect chondrocytes (Stanton et al., 2003; Studer et al., 2003). Furthermore, there has been no report demonstrating the expression of MAPKAPK-2 in chondrocytes. In the present study, the proliferative, maturative and hypertrophic cell layers showed intense immunoreactions for MAPKAPK-2, while MAPKAPK-2 and Hsp25 were co-localized only in the maturative and hypertrophic cell layers. This different immunolocalization pattern suggests that MAPKAPK-2 and Hsp25 might not interact in the chondrocytes of the proliferative cell layer. Although it has not been examined whether p38/MAPKAPK-2 is necessary for chondrocyte proliferation, the intense MAPKAPK-2 immunoreactivity in the proliferative chondrocytes implies that MAPKAPK-2 is involved in the regulation of chondrocyte proliferation. This constitutes one subject for a future study.

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Figure legends

Figure 1.

MAPKAPK-2 immunoreactivity in the anterior (**a**) and posterior (**b, c**) portion of the synovial membrane of the rat TMJ. Plastic sections of a pre-embedding method, counter-stained with methylene blue. **a.** The synovial lining layer consists of a one- to three-layered arrangement of MAPKAPK-2 immuno-positive and -negative lining cells. Immunoreactions are localized in the cytoplasm of the synovial cells with round and clear nuclei (arrowheads). Note MAPKAPK-2 immunoreaction in some nuclei (open arrowheads). Arrows indicate immunonegative cells with many vacuoles. **b.** The arrangement of synovial lining cells differs from that in the anterior potion (**a**). A synovial fold consists of loosely-arranged synovial lining cells with high vascularization. The surface of the synovial membrane is partially covered with a sheet-like cytoplasm with MAPKAPK-2 immunoreaction (arrowheads). **c.** Higher magnification of the boxed area in b. MAPKAPK-2 immunoreactivities localize in the cytoplasm (arrowheads) and in the nuclei (open arrowheads) of some synovial lining cells. Several fibroblasts in the sublining tissue (double arrows) and endothelial cells (arrows) are also positive in MAPKAPK-2-immunoreaction. Asterisk: articular cavity. Scale bars = a: 25 µm, b: 80 µm, c: 40 µm.

Figure 2.

Confocal microscopic images of a double stained section by antisera against MAPKAPK-2 (left panel), Hsp25 (middle panel) and a merged image (right panel) in the rat synovial membrane (a-c), articular disc (d-f), and condylar cartilage (g-i). **a-c.** The synovial lining cells colored yellow are observed to extend long cytoplasmic processes (arrows), indicating the

co-localization of MAPKAPK-2 and Hsp25 in their cytoplasm. Some lining cells show only MAPKAPK-2 immunoreactivity (colored red), but no Hsp25-immunoreaction (open arrowheads). **d-f**. Double positive cells (yellow) appear stellate in their profile in the articular disc. Some of them (arrows) contain MAPKAPK-immunoreactivity in their nucleus (red). **g-i.** The rat condylar cartilage is divided into four layers from the surface: the fibrous (F), proliferative (P), maturative (M), and hypertrophic (H) cell layers. The chondrocytes at the maturative and hypertrophic cell layers show the co-localization of these two proteins (yellow). The proliferative cell layer contains only MAPKAPK-2 immunopositive chondrocytes. Arrows show MAPKAPK-2 immunopositive nuclei (red). Asterisk: articular cavity. Scale bars = a-c: 30 µm, d-f: 40 µm, g-i: 60 µm.