Immunohistochemical study of the BMPs and their extracellular antagonists in osteoarthritic human knee joint

Abstract

The osteophytes are bone spurs overgrowing the edge of the articular cartilage during the course of osteoarthritis (OA). The cellular mechanism of their development and growth resembles the intramembranous and endochondral bone development during embryonal and postnatal normal bone development, growth, modeling, remodeling and repair. The role of BMPs in bone development and metabolism is well documented and the members of the BMPs molecular network were recognized as important factors which could modulate new bone development and growth of osteophytes. The purpose of this study is to analyze the coexpression of the most potent osteoinductive members of the BMP family (BMP-2, -4 and -7) and their extracellular antagonists gremlin, noggin, chordin, follistatin in order to establish their role during degenerative process of the synovial joints and growth of osteophyte.

In this study, the BMP-2, BMP-4, BMP-7, gremlin, noggin, chordin, follistatin expressions were analyzed in joint tissues from OA patients and from healthy individuals by immunohistochemistry and Western blot. The immunohistochemistry showed different localization pattern of BMPs and BMP extracellular antagonists expressions in OA vs. normal joint tissues. In osteophyte, BMP-2 was not detected, while BMP-4 and BMP-7 were positive in hypertrophyc chondrocytes and osteocytes at the sites of endochondral bone development. BMP-7 was strongly positive, while BMP-4 was negative in synovial membrane of OA joints. Gremlin, chordin and noggin were found in chondrocytes, osteoblasts of the osteophytic bone and synovial epithelium, while follistatin was found in chondrocytes, blood vessels and synovial epithelium.

Our result demonstrate the significant differences in BMPs and their antagonists expression in normal tissues compare to joint tissue affected by OA and revealed how molecular balance of the local growth factors such as BMPs and their antagonists could be disturbed during degenerative processes. Also, these findings suggest the potent role of these molecular factors in pathogenesis of the OA and/or during growth and development of the human knee joint osteophytes.

INTRODUCTION

Bone morphogenesis is a unique process of bone tissue development, structure forming and maintaining in the whole human biology. This process is not exclusively ongoing during prenatal development and growth, but also it is repeated in adult life during bone modeling, remodeling and healing of the bone fractures. The bone morphogenesis is phe-
nomenon underlying remarkable regenerative capacity of bone and is responsible for great capacity for skeletal adaptation on different biomechanical loading (1). Also, bone morphogenesis occurs as a part of a joint repair attempt process during the degenerative processes of osteoarthritis (OA). During OA, bone morphogenesis lead to development of newly formed bone spurs usually located at the margin of the articular cartilage known as osteophytes (1-3). Therefore, it is accepted to consider an osteophytic bone growth as a repair attempt during the degenerative process of articular cartilage breakdown and destruction of the synovial joints elements. Eventhough being the results of reparative processes in OA affected joints, osteophytes restrict movements in affected joints and could be the cause of an existing pain in patients and therefore they are usually cut off during joint surgery. Still, the osteophytic growth have tendency to repeat after that and cause problems furthermore.

Bone morphogenesis is complex and precisely regulated process modulated by systemic hormones, local growth factors and cytokines generated in the bone cell microenvironment (4). A lot about molecular factors which regulate bone cells proliferation, differentiation, apoptosis and matrix synthesis during bone morphogenesis has been published previously. One of the most important factors involved in complex network of local growth and differentiation factors are the members of the bone morphogenetic proteins family (BMPs) (5-8). They act in an autocrine/paracrine manner and stimulate proliferation of osteoprogenitor stem cells, stimulate and promote differentiation of osteoblast cell lineage and stimulate synthesis of the bone matrix collagenous and noncollagenous proteins (9). Their ability to stimulate bone morphogenesis is called osteoinductivity and therefore BMPs are characterized, beside other function, as osteoinductive (10,11). The most potent osteoinductive BMPs are BMP-2, BMP-4 and BMP-7 (12,13). As the other members of the BMP family, they act through specific cell surface receptors, bone morphogenetic protein receptors (BMPRs) which are phosphorylised upon BMP ligand binding (14,15). Upon that, signal transduction cascade starts with phosphorylation of downstream cytosolic factors called Smad-s. Finally, activated Smads are translocated into the cell nucleus and activate gene transcriptional activity (16). BMP activity is precisely regulated in order to establish the fine balance between diverse functions of different bone cell. Recently, extracellular BMP antagonistic factors were discovered, which bind directly to BMPs with high affinity and prevent BMP interaction with the specific receptors and therefore prevent BMPs effects on cells such as proliferation, differentiation and matrix synthesis. These signaling molecules represent a group of structurally unrelated extracellular secreted proteins (17,18). Follistatin, gremlin, chordin and noggin are such extracellular BMP antagonists and much effort was done recently to explore their localization and function during bone morphogenesis (19-22). Their antagonistic activity became interesting since they can act locally and could prevent excessive bone formation. Therefore they were recognized as potent molecules for pharmacological intervention in diseases with excesses new bone formation such as ectopic bone formation or bone metastatic processes (23, 24). Local application of these molecules could antagonize endogenous osteoinductive signals and hypothetically could prevent osteophyte formation during osteoarthritis. In healthy bone and in healthy joint tissue local osteoinductive factors are in balance with the osteoinhibitory factors and certain feedback mechanism between these factors was proposed to have control of the bone homeostasis. Experimental data demonstrated different temporal and spatial localization of BMPs and their antagonist in normal and OA joint tissues. The role of BMPs in signaling during osteophyte formation has been extensively studied and it was revealed that each BMP has a distinct pattern of distribution in osteophytic and synovial tissues (25-28). Also, to date there are several reports, of the group of authors: Nakase, Tardif and Lories, about BMPs antagonists expression in OA joint tissues (29, 30). However, to date no reports were published about co-expression and localization of the most osteoinductive BMPs (BMP-2, -4 and -7) and their extracellular antagonists: noggin, gremlin, chordin and follistatin in osteophytic tissue and synovial membrane of OA human knee joints. Therefore, in this study we explored the expression of these three most potent osteoinductive BMPs (BMP-2, -4 and -7) and their extracellular antagonists in normal and OA human knee joint.

**MATERIAL AND METHODS**

**Tissue samples**

For the purpose of this study osteophyte tissue, underlying bone tissue and synovial membrane tissue was collected out of knee joints of 20 patients which underwent joint replacement surgery due to severe osteoarthritis (age was in range from 65 to 74 years). Informed consent was obtained from patients included in this study. Normal articular cartilage and subchondral bone were obtained from at an autopsy of individuals with no medical record of degenerative joint or metabolic bone diseases. Mineralized tissue specimens were decalcified in Osteodec solution (during 5 days, at RT), washed in saline, fixed in 4% paraformaldehyde (24 hours) and embedded in paraffin. Using rotational microtome (Leica RM 2155, Leica Instruments GmbH, Germany) tissue samples were cut on 5 μm thick slices, mounted on silinated glass slides and processes for histology and immunohistochemistry. The study was approved by the Ethical Committee of the Faculty of medicine University of Rijeka.

**Immunohistochemistry**

Immunohistochemistry on tissue samples was performed according to manufacturer instruction (Santa Cruz Biotechnology Inc.). The primary antibodies were pur-
chased from Santa Cruz Biotechnology Inc., USA and they were following polyclonal antibodies: goat anti-gremlin antibody (N-20), rabbit anti-noggin antibody (L-18Z), mouse anti-chordin antibody (K-25), mouse anti-follistatin antibody (H-114), mouse anti-BMP-2 (mAHuBMP-2) and anti-BMP-4 (N-16) and goat anti-BMP-7 antibody (T-12). Briefly, tissue slices were deparaffinized in xylene, rehydrated in ethanol and endogenous peroxidase activity was blocked by incubation in 0.3% H2O2/methanol solution. Incubation in 5% normal non-immune serum was done in order to avoid background staining. Incubation with primary antibodies was done in humid chamber (60 minutes at room temperature), washed in PBS and secondary biotinylated antibody was applied during 30 minutes, in humid chamber at room temperature. Thereafter, peroxidase conjugated streptavidin was added and the site of antigen binding was visualized using liquid DAB substrate chromogen. Sections were counterstained with hematoxyline and positive reaction of the antigen-antibody reaction was seen as a dark brown staining. Staining specificity was determined by omission of the primary antibody and substitution of the primary antibody with an autologus pre-immune serum. All control samples showed no antigen staining. Tissue slices were dried and mounted in Histostain, analyzed with an Olympus BX 50 microscope (Olympus, Japan) and photographed using Olympus OM-4 Ti camera. Morphometry was performed by counting the number of immunopositive cells for each antigen studied in this research, at 5 randomly chosen fields of 3 tissue slices 500μm distant from each other. The morphometric data were expressed as a percentage of total cell number that is stained positively for the antigen. The morphometric analysis was done by two independent observers (SZC, LD).

**Western blot analysis**

Tissue samples were homogenized, incubated with Lysis buffer (30 minutes, 4°C) and centrifuged (30 min, 12000 rpm, 4°C). Protein content of each sample was measured by spectrophotometry according to the Bradford method. Electrophoresis was performed under reducing condition on 12% SDS polyacrylamid gel (SDS-PAGE) and then transblotted to nitrocellulose membrane using Transfer-blot semidry transfer cell (BIO-RAD, USA). Membranes were blocked with non-fat bovine milk (RT, 120 min) and after that incubated with primary antibody diluted 1:200 in blocking buffer (RT, 24 hours). After washing in TBS membranes were incubated with secondary antibody described in previous section, diluted 1:2000 (RT, 90 min). At the end, immunoblot was developed using chemiluminiscence and photographed on Agfa film.

**RESULTS**

**Histology of the osteophytes and synovial membrane**

Tissue samples analyzed in this study were marginal osteophytes which are well developed and covered with...
and cartilage cap and underlying immature woven bone tissue. They were characterized as mature forms of osteophytes with a high content of newly formed bone tissue and covering hypertrophic cartilage. Synovial tissue showed no sign of inflammation or fibrosis (data not shown).

**Immunohistochemical localization of the BMP-2, -4 and -7 in osteophyte and synovial membrane**

The results of BMP-2, -4 and -7 immunohistochemical staining showed differences and specificity of localization in normal and osteoarthritic joint tissues. BMP-2 was not detected in osteophytic tissues or in synovial membrane (Figure 1 A, D), while BMP-4 and BMP-7 were positive in the hypertrophic chondrocytes, cells and matrix of the superficial cartilage layer and also in osteocytes of the osteophytic bone tissue (Figure 1 B, C) (Table 1). Epithelium of the synovial membrane was strongly positive only for BMP-7 (Figure 1 f), while BMP-2 was negative in both synovial epithelium and stromal cells (Figure 1 D). Control samples of the normal articular cartilage (Figure 3 A, B, C) and synovial tissues (data not shown) showed no detectable immunohistochemical reaction for BMPs or their inhibitors. Morphometrical analysis revealed that

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**Figure 2.** Immunohistochemical localization of the follistatin (A, E), gremlin (B,F), noggin (C, G) and chordin (D,H) in osteophytes and synovial membrane in OA human knee joint. Follistatin immunostaining showed positive cells in upper layer of osteophyte cartilage cap (A), and epithelium of the synovial membrane (E). Gremlin was found in chondrocytes, osteocytes of the woven bone (B) and synovial epithelium (F). Noggin was positive in chondrocytes (C) and synovial epithelium (G). Chordin was found to be positive in chondrocytes and osteoblasts (D), blood vessels and synovial epithelium (H). Indirect immunoperoxidase stainig, counterstaining with hematoxyline, magnification 200 x.
BMP-4 was positive in 32.15% of the chondrocytes while BMP-7 expression was stronger with 52.55% immunopositive chondrocytes. The stromal cells from OA synovial membrane showed no detectable immunostaining for BMP-2, while BMP-4 had minimal immunostaining (7.05% cells). BMP-7 had the strongest expression found in 97.36% of the epithelial cell and in 90% of the stromal cells (Table 1).

**Immunohistochemical localization of the BMP antagonists in osteophyte and synovial membrane**

The results of immunohistochemical staining for BMP antagonists showed significant differences between different BMP antagonists studied in this analysis and their different localization in osteophytic tissues and synovial membrane. Gremlin was found in chondrocytes, osteoblasts of the woven bone and synovial epithelium (Figure 2. B, F). Chordin was found in chondrocytes and osteoblasts, blood vessels and synovial epithelium (Figure 2. D, H). Follistatin was found in upper layer of osteophyte cartilage cap, blood vessels and synovial epithelium (Figure 2. A, E). Noggin was positive in chondrocytes, osteocytes and synovial epithelium (Figure 2. C, G). Morphometry of the immunohistochemical results revealed that BMP antagonists have the strongest expression in chondrocytes: gremlin in 29.9%, noggin in 29.75%, follistatin in 8.65% and chordin in 12.4% of total chondrocyte number. Gremlin, chordin and follistatin were negative in osteocytes and the only positive BMP antagonist in osteocytes was noggin which showed positivity in 40% of osteocytes. Morphometry of immunohistochemical staining of synovial membrane showed that noggin had maximal expression in epithelial and stromal cells of synovial membrane: 95% and 100% respectively, while chordin, had less intensive expression in stromal cells (68.95%) with maximal expression in epithelial cells. Gremlin and follistatin showed minor expression in epithelial and stromal cells of synovial membrane (Table 1).
Western blot analysis

The results of immunoblotting were consistent with immunohistochemistry. The Western blot analysis showed no detectable signal in normal joint tissue samples (Figure 4, the third and fourth column) and positive signals for BMP-4, BMP-7, gremlin, follistatin, noggin and chordin in osteoarthritic joint tissue samples (Figure 4, the first and second column). The signal for BMP-2 was negative in OA tissue samples (Figure 4, the first line).

DISCUSSION

In this study we have demonstrated existing coexpression of osteoinductive BMPs and their extracellular antagonist, in OA human knee joint tissues. Also we have showed that these factors are differently expressed in different joint tissues osteophyte and synovial membrane. In our previous study we have already showed that among BMPs family members, BMP-2 and BMP-7 are two factors which have strongly specific localization in osteophytes (25). BMP-2 is identified as a molecular factor which is specific for sites of intramembranous bone development, while BMP-7, is strongly associated with cartilage and endochondral bone development sites (19). The present study showed similar results. BMP-2 was negative in osteophytic tissue which is in accordance with the fact that our samples of osteophytes were well developed with minimal amount of fibrous tissues and sites of intramembranous bone development. On the opposite, BMP-7 showed strong expression in osteophytic tissue with specific localization in endochondral sites. This result is in accordance with established chondrogenic role of the BMP-7. Therefore, BMP-7 positive expression in synovial membrane, both in stromal fibroblasts and epithelial cells, is expected and suggests that synovial membrane has important role in joint tissue metabolism, nourishment and growth factors production.

This study demonstrate positive expression of all four studied extracellular BMP antagonists in OA joint tissues, with the highest expression of gremlin and chordin in hypertrophic chondrocytes at the sites of endochondral bone development. In opposite, noggin and chordin showed higher expression in epithelium and stromal cells of the synovial membrane. These data suggest certain diversity in inhibitory role of different BMPs antagonists and could have a significant importance in pathogenesis of OA and osteophyte development.

There are several reports that have demonstrated different expression of the extracellular BMPs antagonists in articular cartilage and synovial tissue during degenerative and inflammatory processes in synovial joints. Nakase et al. have found positive BMP-2 expression in early mesenchimal layers and fibrochondrocytes of the osteophyte which coresponded to intramembranous bone formation sites while BMP-2 was negative in chondrocytes close to bone forming sites i.e. endochondral bone formation sites (30). Tardif et al. have showed different expression of follistatin, chordin and gremlin in osteoarthritic cartilage and synovial fibroblast and suggested that their increased activities appearing in different stages of OA process may reflect different roles in this disease (29). Lories et al. have showed the importance of noggin in destructive and remodeling arthitis (24).

Conclusively, the results of our study confirmed high activity of BMP-4 and -7 during endochondral bone development in osteophytes and in the synovium of OA joints. The results of immunohistochemical detection of chordin, follistatin, noggin and gremlin suggest that these BMP antagonists are up-regulated in both osteophyte and synoval membrane of OA joints, with emphasis on noggin and chordin. The precise role of these molecular factors in the bone morphogenesis has not been elucidated yet, and there are still unanswered questions concerning the balance between the local molecular factors involved in bone morphogenesis during pathological processes of the joint tissues. Different role of chordin, follistatin, noggin and gremlin in molecular network where interaction with other local growth and differentiatational factors are expected, should be taken into consideration for the possible usefulness of these BMP antagonists in prevention of the excessive processes of bone formation in OA and other skeletal disorders.

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