Expression of the BMP -2, -4 and -7 and their Antagonists Gremlin, Chordin, Noggin and Follistatin during Ectopic Osteogenesis

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ABSTRACT

Molecular network of the osteogenic BMPs and extracellular inhibitors maintains homeostasis of the skeletal tissues. It is important to determine relationship between BMP-2, -4 and -7 and their inhibitors: gremlin, follistatin, chordin and noggin, during normal osteogenesis. To determine their expression pattern we conducted investigation by inducing ectopic bone formation in rats. The results shown that levels of the BMP-2 and BMP-4 expression in chondrocytes are similar to noggin and follistatin. The latter BMPs and inhibitors have increased levels of the expression at day 14th of the osteogenesis, which suggests their important roles in early phases of the chondrogenesis. Gremlin and chordin have shown increased levels of expression in late phase of chondrogenesis, which suggests their important role in regulation of the osteogenesis initiation. In this study, BMPs and inhibitors have the highest levels of the expression at 21st day in the osteocytes, which suggests their strong interactions in osteogenesis.

Key words: ectopic osteogenesis, BMP-2, BMP-4, BMP-7, gremlin, chordin, noggin, follistatin.

Introduction

Physiological mechanisms of the osteogenesis such as bone growth, development and fracture healing or pathological mechanisms such as development of the osteophyte in osteoarthritis are known in many details. However, discovery of extracellular inhibitors, has driven much attention to understanding their roles and interactions with BMP-2, BMP-4, and BMP-7, especially since BMP-2 and BMP-7 have been applied in therapy of some clinical conditions. BMPs activities are modulated during osteogenesis by several unrelated secreted proteins collectively known as BMP antagonists, which bind to the BMPs and prevent their binding and signaling through their specific receptors. Those are primary: follistatin, gremlin, chordin and noggin. Studies performed on human joint tissues, that involve BMP antagonists have been reported by several researchers¹⁴. Tardif et al. were the first authors that have indicated to important role of the follistatin i gremlin in pathophysiology of the osteoarthritis³. In the cell culture of the osteoarthritic (OA) chondrocytes afore mentioned authors have noticed strong stimulation of the gremlin gene expression, but downregulation of the follistatin gene expression after BMP-2 and BMP-4 were added. In the same study, spatial distribution of the gremlin, BMP-2 and BMP-4 was similar in middle and upper layers of the OA cartilage, which is similar to result of Nakase. Immunolocalization of the BMP-2 was found in chondrocytes of the OA cartilage as well as in the osteophyte⁵. This data supports the opinion that the follistatin and gremlin are differentially regulated in OA chondrocytes. When the expression of the follistatin, gremlin, chordin and noggin was compared between OA chondrocytes and controls, then significantly higher expression of the follistatin and gremlin was found in OA, chordin expression did not significantly differ between OA and controls, while noggin expression was not detected in OA nor in the controls.

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During early phase of the intramembranous ossification, Kwong et al. have determined that endogenous production of chordin and BMP-2 have increased during differentiation of the mesenchymal cells to bone cells. Also, the suppression of chordin improves early osteogenesis, which was demonstrated by increased BMP-2 bioavailability. Such result confirms earlier findings of Petryk et al. of limited in vitro osteogenesis after exogenous application of the chordin. Investigation on the human long bone fracture repair has detected interaction of the BMP-2 and its inhibitors chordin and noggin. BMP-2 is strongly expressed in hypertrophic chondrocytes, while noggin and chordin were expressed equally in hypertrophic and non-hypertrophy chondrocytes of the areas of endochondral ossification.

Recent researches of Tardif et al. indicate to upregulation of the chordin expression in OA chondrocytes, compared to controls, and increased expression of the gremlin and follistatin, as well as BMP-2 and BMP-4 in experimental model of the OA.

Afore mentioned studies indicate to complex relationships between BMPs and BMP antagonists during process of the osteogenesis. In this study of the experimental model of the ectopic ossification, temporospatial expression of the osteogenic BMPs and their inhibitors, trough process of physiological osteogenesis is evaluated.

Previous research lack data of the temporal and histological distribution of the expression of the BMP’s antagonists during the osteogenesis. Physiological balance of the molecular network of the osteogenic BMPs and extracellular inhibitors maintains homeostasis of the skeletal and joint tissues. Therefore, it is important to determine relationship between BMP-2, BMP-4 and BMP-7 and their specific inhibitors: gremlin, follistatin, chordin and noggin, during normal osteogenesis. Such result is a starting point in better understanding of the relationships between BMPs and their specific inhibitors in degenerative joint diseases.

Materials and Methods

The study was performed on 130 female Wistar rats, 4 weeks of age, weighing 250 g. Experimental animals were kept under standard conditions and fed by standard food for laboratory mice and rats ad libitum. Animals are allowed free movement in plastic cages measuring 50x25x25 cm and were exposed to normal cycle of darkness and light (12h:12h). Study groups comprised 12 animals. Control group underwent sham operation.

This study was approved by the Institutional Ethics Committee.

Experimental procedures

Preparation of demineralized bone matrix

Demineralized bone matrix (DBM) powder was prepared out of the long bones of the posterior limb of 30 rats. Bones were rinsed with saline and the bone marrow along with the peristeum was removed. Bones were dried in a thermostat at 37°C and grinded into powder. Size of the bone particles within powder amounted 70–150 μm. Bone powder was demineralized by use of the 0.5 M hydrochloride acid, washed with water and incubated in 200 mM of the Tris buffer (pH 8.0). Demineralized bone powder was washed with denoised water, alcohol and ether and dried at room temperature.

Implant preparation

For implant preparation, 35 mg of DBM, along with 50 μl of the chondroitin sulfate solution and 50 μl of of the collagen type I, was put into siliconized tube and centrifuged for 15 minutes at 12000 rpm. Drying was done at 37°C for 24 h in the thermostat.

Surgical procedure

Each implant was embedded subcutaneously in the left and right pectoral region. Animals were anesthetized by intraperitoneal injection of 3.6% chorol-hydrate (Sigma-Aldrich, Germany). Procedures were performed under conditions of asepsis and antisepsis. Animals were sacrificed after excessive dose of anesthetic, injected inside the abdominal cavity at 5, 7, 14, 21 and 30 days of implantation.

Methods

Implants

At given time periods, implants were collected from each animal. Implants were decalcified in 0.5 M EDTA for a week. Decalcified implants were fixed in 4% paraformaldehyde for 24 hours and cut at microtome on 5 μm thick tissue slides (Leica RM 2155 Rotary-microtome, Leica Instruments GmbH, Germany). Preparations were dehydrated in alcohols with different concentrations (50%, 70% and 96%). For histological analysis, hemalaun-eosin, toluidine blue and Goldner stainings were done.

Imunohistochemistry

Time and spatial distribution of the BMP-2, BMP-4 and BMP-7 and their antagonists: gremlin, noggin, chordin and follistatin expression were determined by use of the immunohistochemistry. Briefly, immunohistochemical procedure was done according to the protocol described in the literature. For immunohistochemical staining the following primary antibodies were used: goat polyclonal anti-human antibody gremlin-1 (N-20), rabbit polyclonal anti-human noggin antibody (L-182), mouse polyclonal anti-human follistatin antibody (H-114), rabbit polyclonal anti-human antibody chordin (K-25), mouse polyclonal anti-human antibody against BMP-2 (mAHuBMP-2), goat polyclonal antibody anti-human BMP-7 (T-12). All primary antibodies were purchased from Santa Cruz Biotechnology Inc., USA. Secondary antibodies were purchased from the manufacturer Dako (Denmark).

Morphometric analysis

Immunopositive cells were counted by use of the microscope at 40x power magnification (Olympus BX 50).
Microphotographs of the specimens were taken by Olympus OM-4 Ti camera (Olympus, Japan). Immunopositive cells were counted in the tissue area of the implant that contains at least 100 cells of interest. Results were expressed as percentage of the immunopositive cells. Each slide was subjected to a double-blind evaluation.

Statistical analysis

Data are expressed as the $\overline{X} \pm$S.E.M. Repeated Measures ANOVA and Fisher Post-hoc test were used to determine specific differences between groups. p values less than 0.005 were considered significant.

Results

Histomorphological changes in implants at the critical temporal points

In the implants at the 5th day of the experimental protocol, a firm connective fibrous membrane on the surface of the implants was detected. It was consisted of elongated fusiform fibroblasts, with their long axis parallel to the surface of the implants. Between single fibroblasts blood vessels which morphologically correspond to capillaries and arterioles were found. Fibroblasts and blood vessels were also found at the periphery of the implant. Surface of the demineralized bone particles within the implant is subjected to multinuclear cells degradation, observed as lacunae and fissures. Also, fibroblast-like cells infiltrated the central part, without blood vessels.

In the implants at the 7th day of the experimental protocol, similar morphological features to those of the 5th day were characterized by: thick fibrous membrane at the surface, high cellular infiltration between bone particles, numerous blood vessels and increased resorption.

At the 14th day the first signs of the induced chondrogenesis are seen. Chondrocytic nests, consisted of hypertrophic chondrocytes, surrounded with the small amount of the metachromatic extracellular matrix are situated in between the DBM particles. (Figure 1. a, b). Also, cellular

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Fig. 1. A. Histomorphology of the central area of the implant. Chondrocytes (arrows) surrounds the bone particles of the implant (P). The cartilage matrix is minimal. In the particles empty osteocytes lacunae (arrowheads) are visible. Goldner staining. B. Area of the cartilage formation in the implant. Chondrocytes (arrows) are between DBM implant particles (P). Toluidine blue staining. C. Histomorphology of the area of the bone formation within the DBM implant. Spiculas of the new formed trabecular bone (B), marrow spaces (MS) and osteocytes (arrows) are visible. Edges of the particles of the DBM are eroded. Goldner staining. D. Histomorphology of the area of the intramembranous bone formation in the DBM implant. Spiculas of the new formed trabecular bone (B) and marrow spaces (MS) are visible. Bone cellular elements are in the different stages of differentiation; there are visible osteocytes in the lacunae (arrows) and the osteoblasts (arrowheads) on the surface of the new formed bone and on the surface of the marrow spaces. Connective tissue (CT) contains osteoprogenitor cells. Edges of the particles of the DBM are eroded. Goldner staining. Bar=200 μm.
infiltration, bone particle degradation and blood vessels penetration are seen.

At the day 21, some cartilage nests are degraded. Newly formed bone trabeculae, built of immature woven bone tissue are seen on the surface of the implant. Between bone particles, three-dimensional network of the bridging bone trabeculae is seen (Figure 1. c, d).

At the 30th day of the experimental procedure for complete degradation of the implants is present.

**Immunohistochemistry**

The expression of the most potent osteoinductive BMPs (BMP-2, BMP-4, BMP-7) is analyzed.

**Immunolocalization of the BMP-2 and BMP-4**

BMP-2 and BMP-4 were expressed in very similar pattern. At the 5th day, BMP-2 and BMP-4 were mainly localized in the fibroblasts (Figure 2. a, f). At the 7th day, immunolocalisation of the BMP-4 in fibroblasts was stronger than those of the BMP-2 (Figure 2. b, g). At days 14th and 21st, except in fibroblasts, BMP-2 and to less extent, BMP-4, were expressed in hypertrophic chondrocytes (Figure 2. c, h) and in osteoblasts (Figure 2. d, i). At the day 30th, the expression of the BMP-2 and BMP-4 was negative (Figure 2. e, j).

**Immunolocalization of the BMP-7 in the implant**

At the 5th BMP-7 was expressed in fibroblasts and also in the blood vessels layer (Figure 2. k). Contrary to BMP-2 and BMP-4, BMP-7 was not expressed in hypertrophic chondrocytes at the day 14th (Figure 2. m). At the day 21st, strong expression of the BMP-7 was observed in osteoblasts (Figure 2. n). Alike to other BMPs at the 30th day, BMP-7 expression was negative (Figure 2. o).

**Immunolocalization of the BMPs inhibitors in the implant**

The expression of chordin, gremlin, noggin and follistatin was analyzed. Compared to other inhibitors, follistatin was the most intensity expressed inhibitor (Figure 3. a - e). The expression pattern of all inhibitors was the same (Figure 3. f - t). At the days 5th and 7th, in-
Inhibitors were immunolocalized in the membrane around the implants, and also in fibroblasts (Figure 3. a, b). At the day 14th, inhibitors were expressed in the hypertrophic chondrocytes (Figure 3. c), and at the day 21st in osteoblasts (Figure 3. d). At day 30th, inhibitors were expressed in the marrow spaces (Figure 3. j, e, o and t).

Temporal expression of the BMPs and inhibitors

The highest expression of all BMPs and inhibitors was observed in osteocytes (Figure 4). At days 14th and 21st significantly lower expression of BMP-7 was found in hypertrophic chondrocytes (Figure 3. c), and at the day 21st in osteoblasts (Figure 3. d). At day 30th, inhibitors were expressed in the marrow spaces (Figure 3. j, e, o and t).

Contrary, follistatin and noggin showed higher expression level in chondrocytes at day 14, compared to day 21st.

Temporal expression of the BMPs and inhibitors in the chondrocytes

Statistical analyses showed significantly less number of the BMP-7 positive chondrocytes at days 14 and 21, compared to BMP-2 and BMP-4 (p<0.005) (Figure 4). At days 14th and 21st, BMP-2 expression was significantly higher, compared to BMP-4. (p<0.005). Analysis of expression of the BMP inhibitors revealed the lowest expression of the gremlin, at day 14th, compared to noggin and follistatin (p<0.005). At day 21st, the least number of the follistatin positive chondrocytes significantly differed to gremlin, chordin and noggin (p<0.005).

The expression of the BMPs and inhibitors in the fibrocytes

The highest number of the BMP-7 positive fibrocytes was detected on 21st day, which is significantly different to 5th day (p<0.005) (Figure 5). The highest expression of...
the BMP-2 and BMP-4 was detected on days 21 and 30, which significantly differed to days 5th and 7th (p<0.005), respectively.

Compared to other inhibitors, follistatin had the highest expression in fibrocytes. More gremlin positive fibrocytes were found at days 5th, 7th and 14th, which is significantly different to 21st and 30th day (p<0.005), respectively.

Discussion and Conclusion

In our study BMP-7 expression is similar to BMP-2 and BMP-4 in osteocytes, but significantly different in chondrocytes. Namely, BMP-2 and BMP-4 were strongly expressed in chondrocytes than BMP-7. This is in consistent to previous evidence of the BMP-2 and BMP-4 acting as stimulators on the chondrocyte maturation and anabolic cells activity during matrix production. Moreover, rhBMP-2 induces expression of the cartilage and bone markers in vitro, as well as formation of cartilage and bone at ectopic and skeletal sites in vivo. Shu et al. showed that BMP-2 is crucial for chondrocyte proliferation and maturation during endochondral ossification at the growth plate sites of the BMP-2 knock out mice. Our result of the BMP-2 and BMP-4 decreased expressions at day 21 could be explained by the fact that cartilage undergoes resorption, and chondrocytes apoptosis in later stages of chondrogenesis. Even not statistically significant, BMP-7 also showed decreased expression at 21st day. This could be related to previous evidence of the BMP-7 gene and protein expression being dramatically reduced in aged and degenerated cartilage. When synovial derived mesenchimal stem cells in culture are exposed to high dose of BMP-7, chondrogenesis is enhanced. So, BMP-7 plays crucial role in regulation of overall cartilage homeostasis.

Regardless to early or late stages of the osteogenesis, inhibitors were similarly expressed in osteocytes. In contrast, their expression in chondrocytes was different. Noggin and follistatin were highly expressed in chondrocytes of the 14th day of the experiment and their expression decreased at the 21st day. Inverse to noggin and follistatin, was the expression of the gremlin and chordin - the lowest at the 14th day and the highest at the 21st day of the experiment.

Similarly to our model of ectopic osteogenesis are those of Nakamura et al., who have induced new bone formation by exogenously administrated BMP-2, resulting in noggin up regulation. During early stages of the chondrogenesis, noggin expression was localized in non-hypertrophic chondrocytes, which coincides to expressions of the BMP-2 and BMP-4. It is possible that these BMPs stimulate noggin production in the chondrocytes, as a part of autocrine regulation during osteogenesis. Such opinion is further confirmed by Kwon et al., in model of the long bone fracture healing. These authors have further developed theory of the influence of the ratio of the inhibitors and BMPs, on the bone fracture repair, by stating that exogenous osteoiduction or noggin blockade, can improve endochondral ossification. After being exposed to BMP-2, BMP-4 and BMP-6, noggin upregulation could be explained as part of protective mechanism against excessive amount of BMPs. We found that at the 21st day, noggin was localized in hypertrophic chondrocytes and osteocytes. Compared to 14th day noggin expression is lower, as well as expression of the BMP-2 and BMP-4 at 21st day in chondrocytes. As new bone trabeculae form, noggin, similarly to BMPs shows the highest expression localized in woven bone. Such relation of the BMPs and noggin was previously described.

In chondrocytes, noggin and chordin are upregulated by inductors of chondrogenesis such as Indian hedgehog. Noggin blocks the effect of of BMPs by blocking alkaline phosphatase synthesis as well as collagen and non-collagenous proteins synthesis in osteoblast. Noggin also inhibits intramembranous ossification and prevents chondrogenesis during limbs development. Transgenic mice with noggin overexpression have decreased trabecular bone volume and impaired osteoblastic function which leads to severe osteopenia and fractures. Lories showed that noggin haploinsufficiency provides certain protection to articular cartilage, against destruction in model of the induced arthritis, while noggin overexpression caused cartilage more vulnerable in the models of the inflammation driven cartilage destruction, systemic autoimmune arthritis and joint ankylosis. Null mutation of the noggin gene leads to severe hyperplasia of the cartilage and multiple joint fusions and leads to animal death. Haploinsufficiency of the noggin causes carpal and tarsal fusions which resemble to some phenotypes of noggin haploinsufficiency in humans.

Abe et al. revealed that rh-noggin inhibits osteoblast and osteoclast formation in mice bone marrow cell culture and this effect could be prevented by adding exogenous BMP-2. Our findings are in consistence with the majority of cited authors and could additionally support the hypothesis of negative feed back mechanism between BMP-2, BMP-4 and noggin.

Regarding follistatin, we showed the same expression pattern as noggin characterized by its upregulation in early chondrogenesis and osteogenesis.

Tardif et al. found positive expression of follistatin, gremlin and chordin (but not noggin) in articular cartilage and synovial tissue. Expression of the follistatin and gremlin is upregulated in OA chondrocytes, but not in synovial fibroblasts. In humans and in OA dogs Tardif speculated that follistatin regulation is mediated by proinflammatory factors such as IFN-γ and TNF-α, while gremlin expression is mediated by BMP-2 and -4. Therefore, follistatin may have stronger link with the inflammatory processes in the joint. Tardif also analyzed chordin expression and found that it is also upregulated in OA chondrocytes but it is not clear which factors regulate its expression.

Zhang et al. suggest important roles of the chordin during skeletal growth and endochondral ossification, being inversely related to the chondrocyte maturation. This is consistent with our finding of chordin high expression in chondrocytes at 21st day. Chordin is probably involved in...
inhibiting further chondrogenesis, since bone formation begins.

Kwong et al. found that blockade of the chordin activity increases the rate of the osteogenic differentiation of the human mesenchymal stem cells. Kwong revealed the most intense noggin and chordin expression in non-hypertrophic and hypertrophic chondrocytes, during fracture healing, while BMP-2 expression was found in non-hypertrophic chondrocytes. Also, noggin and chordin expression was detected in osteoblasts.

Wordinger et al. reported that gremlin regulates cell proliferation and stem cell differentiation. Also, gremlin acts directly on endothelial cells to modulate angiogenesis and endothelial cell migration. Canalis showed that gremlin null mice have decreased weight and body size, due to shortened femoral length, a deformation found in young mice, which has diminished in adult animals. It was concluded that gremlin is not only necessary for skeletal development but also for postnatal skeletal homeostasis.

BMP expression in fibrous membrane is the strongest at day 21, which corresponds to beginning of the bone formation. BMP inhibitors showed constant level of expression throughout the experiment, except for gremlin, which was somewhat less expressed at 21st day and 30th day of the experiment. These patterns of expression of osteoinductive proteins and their inhibitors indicate to fibrous membrane as a auto regulator of histogenetic processes in the implant. Specifically, blood vessels of the con-
nective membrane form unique bridge between the implant and the host. Via blood vessels BMPs and their inhibitors influence the events in the implant. Also connective membrane protects the surrounding tissue or the whole host organism out of the extensive ectopic ossification.

The results of our study have shown that level of the BMP-2 and BMP-4 expression in chondrocytes is similar to noggin and follistatin. All these factors have increased levels of the expression at day 14th of the ectopic osteogenesis, which suggests their important roles in early phases of the chondrogenesis. At day 21st we have detected increased levels of gremlin and chordin expression in chondrocytes, which suggests their important role in regulation of the osteogenesis. BMP-2, BMP-4 and BMP-7, as well as all given inhibitors have the highest levels of the expression at 21st day in the osteocytes, which suggests their strong interactions in osteogenesis.

Conclusively, specific interaction between BMPs and their inhibitors during different stages of the chondrogenesis and in osteogenesis is crucial for the process of ectopic bone formation.

REFERENCES


IZRAŽAJ BMP -2, 4 I -7 I NJIHOVIH ANTAGONISTA GREMLIN, HORDIN, NOGIN I FOLISTATIN TJEKOM EKTOPične OSTEOGEZNE

SAŽETAK