The Effects of Endogenous BMP During The Process of BMP-Induced Bone Formation and A Possibility of Clinical Application

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Abstract : The participation of endogenous BMP-2 in the process of ectopic osteoinduction by rhBMP-2 implanted into rat muscles was investigated using immunohistochemistry, immunoelectron microscopy, *in situ* hybridization and real-time RT-PCR. Our results suggested that not only implanted exogenous rhBMP-2 but also endogenous BMP-2 produced by osteogenic and/or chondrogenic cells participates in the rapid progression of ectopic bone formation cascade. Since the statins promote the expression of endogenous BMP-2 and enhance the bone formation, we next examined the stimulative effect of statins on the rhBMP-2-induced osteogenesis, by radiological and biochemical analysis, and histology. These results indicated that subcutaneously injected simvastatin promotes rhBMP-2-induced ectopic bone formation by reducing bone turnover. Simvastatin may become a useful adjuvant therapy for enhancing BMP-2 induced bone formation.

Key words : Bone morphogenetic protein (BMP); Osteoinduction; Immunoelectron microscopy; Bone regeneration; Statins

Introduction

In the ectopic bone formation models induced by rhBMP-2, it has been reported that most of the radiolabelled rhBMP-2 is released from the collagen carrier within 1 day and disappears within 7 days of implantation. Since the bone-formation cascade was then maintained in the absence of exogeneous BMP-2 and mature bone developed up to 3 weeks after implantation, it seems that after the disappearance of implanted rhBMP-2 protein, differentiated chondrocytes/osteogenic cells produces endogenous BMP-2, which plays an important role in the rapid progress of osteogenesis. First, we investigated the participation of endogenous BMP-2 in the process of ectopic osteoinduction.

Since a large amount of rhBMP-2 is necessary to induce a sufficient bone volume in humans, methods of enhancing the bone–inducing activity of rhBMP-2 have been sought. The HMG-CoA reductase inhibitors (statins) are known to enhance the expression of BMP-2 mRNA in osteogenic cells and to stimulate bone formation. In the ectopic bone formation model, statins might promote the production of endogenous BMP-2, derived from osteogenic cells induced by rhBMP-2. In our preliminary study, the effect of oral administered statins on BMP-2-induced ectopic bone were examined, however, there was no stimulative effects. We secondly tried subcutaneous injection of statins to the rat for 20 days after intramuscular implantation of rhBMP-2.

Materials and Methods

Implantation of rhBMP-2

A 5 μg of rhBMP-2 (provided by Yamanouchi Pharmaceutical) was combined with a 3 mg of atelopeptide type I collagen (atelocollagen) solution (Cellmatrix LA®; Nitta Gelatin). The mixture was lyophilized, compressed into a disk form (4 mm in diameter, 1.5 mm in thickness), and implanted into the left calf muscles of 6-week-old male Wistar rats. In the control group, disks made of 3 mg of atelocollagen were only implanted.

Endogenous BMP-2 in the process of ectopic osteoinduction by rhBMP-2

The BMP disks were removed 7, 10, 14 and 21 days after implantation. To evaluate BMP-induced bone formation, we used immunohistochemical staining (LSAB method) and immunoelectron microscopy (post-embedding immunogold method) with anti human BMP-2 monoclonal antibody (provided by Genetics Institute). As well, *in situ* hybridization using rat BMP-2 cDNA probe (HybriProbe® Custom Design TriSeq Kit 100-FITC; BIOGNOSTIK) and real-time RT-PCR using rat BMP-2 primer (Assays on Demand Products, Applied Biosystems) were performed.

The stimulative effect of subcutaneously injected statins

After implantation of BMP disk, the rats were divided into 3 groups. In the treatment 2 groups, each rat was subcutaneously injecteded with either cerivastatin (0.3 mg/kg body weight/day; provided by Bayer) or simvastatin (10 mg/kg body weight/day; provided by Merck, Sharp & Dohme), dissolved in 0.5% carboxymethylcellulose (CMC) solution, given into the cervical back for 20 days. In the control group, only the CMC solution was injected. The BMP disks were removed on day 21 after implantation. To evaluate BMP-induced bone formation, projected and radiopaque area to the X-ray film were measured using NIH Image software, and calcium content, as well as ALP and TRAP activities, was determined. H-E staining was also performed. Statistical analysis was done using Student's *t*-test.

Results

Endogenous BMP-2 in the process of ectopic osteoinduction by rhBMP-2

Immunohistochemistry

On day 7 after implantation, a few chondrocytes expressed endogenous BMP-2. On day 10, a number of mature chondrocytes that expressed BMP-2 were found in the chondroid matrix. On day 14, osteoblast-like and osteoclast-like cells were observed on the surface of the matrix. Many osteocyte-like cells in the chondroid matrix expressed BMP-2. On day 21, most of the chondroid matrix had been replaced by bone and bone marrow was observed. Immunolocalization of BMP-2 was detected in many osteocyte-like cells.

Immunoelectron microscopy

On day 7 after implantation, many irregularly shaped vesicle-like matrices, shown by the aggregated dots of gold particles that indicate immunolocalization of BMP-2, have been released from the mature chondrocytes. On day 14, chondroclasts adjacent to blood vessels, absorbing the calcified chondroid matrix. Gold particles were often detected in the degraded matrix near the ruffled border, in the cytoplasmic vacuoles or the lysosomes of chondroclasts, and in the extracellular matrix facing the blood vessels. On day 21, osteoblasts are seen to have formed an osteoid layer, which often contained matrix vesicles and some gold particles. Gold particles are also seen in the cytoplasmic vacuoles of the osteocytes and in the extracellular matrix.

In situ hybridization

Localization of BMP-2 mRNA on day 21 was observed in fibroblast-like cells in contact with residual atelocollagen fibers and in the osteoblasts arranged on the surface of the bone matrix.

Real-time RT-PCR

Compared with the amount of BMP-2 mRNA seen on day 7 after implantation, the amount of BMP-2 mRNA increased about 2 fold on day 14 and by about 3.8 fold on day 21.

The stimulative effect of subcutaneously injected statins

On radiological analysis, the projected and radiopaque areas in the simvastatin group were significantly larger than those in the control and cerivastatin groups. On biochemical analysis, in the simvastatin group, the calcium content was significantly higher, and both ALP and TRAP activities were significantly lower than those in the control and cerivastatin groups. On histology, new bone formation increased in the simvastatin group compared with that in the control and cerivastatin groups.

Discussion

The matricrine effects of endogenous BMP-2 during endochondral ossification

It would appear that the endogenous BMP-2 secreted from mature chondrocytes is embedded into the calcified chondroid matrix with vesicle-like matrices. Some of it is absorbed by chondroclasts and released to the extracellular matrix through transcytosis, and some of it remains on the resorption surface of the calcified matrix and participates in the osteoblastic differentiation of immature cells (Fig. 1)

Conclusions

These results indicate that not only implanted exogenous rhBMP-2 but also endogenous BMP-2 produced by chondrocytes/osteogenic cells may participate in the rapid progression of the bone formation cascade that occurs during the process of ectopic osteoinduction.

The results also indicate that subcutaneously injected simvastatin promotes rhBMP-2-induced ectopic bone formation by reducing bone turnover. Simvastatin may become a useful adjuvant therapy for enhancing BMP-2 induced bone formation.