Expression, purification and osteogenic bioactivity of recombinant human BMP-2 produced by Escherichia coli

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ABSTRACT INTRODUCTION:
Among BMP family, BMP-2 is the best characterized molecule and it has the strongest bone-inducing activity. Previously, most rhBMPs have been purified mainly from mammals, like Chinese hamster ovary (CHO) cells. Problems associated with clinical application of CHO-derived recombinant human bone morphogenetic protein are post-translational problems, its low yields and high cost due to the need for use of high doses. To solve this problem, Escherichia coli-derived rhBMP-2 (E-BMP-2) has been examined using the technique of molecular unfolding and refolding as alternative to mammalian cells. The purpose of this study was to develop a highly purified and simplified process for E-BMP-2 production. In addition, we describe the preparation and the biological activity of pure E-BMP-2 via in vitro and in vivo study.

METHODS:
Cloning, Expression and Purification of rhBMP-2
The gene encoding human recombinant BMP-2 was chemically synthesized and inserted into the expression vector which is regulated under T7 promoter. The recombinant E.coli cells harboring the constructed vector were cultivated in LB broth. The expression of rhBMP-2 was induced by addition of 1 mM IPTG. The inclusion body forms of expressed rhBMP-2 were recovered by centrifugation. Collected inclusion bodies were solubilized in 8 M urea and refolded by lowering the concentration of urea. Then rhBMP-2 was purified through several chromatography processes including ion-exchange and hydrophobic interaction columns. Purified rhBMP-2 was identified by SDS-PAGE and immuno-reactive blotting (Western blotting).

Human bone marrow MSC culture and osteogenic differentiation
Human bone marrow mesenchymal stem cells were cultured until five passages in DMEM (low glucose) with 10% fetal calf serum and 1% antibiotic-antimycotics. The treatment groups were as follows: (1) control medium, (2) osteogenic medium, (3) 100 or 500 ng/ml E-BMP-2.

ALP staining
ALP activity was evaluated at 3, 7 and 14 days. The cells were stained by use of fast blue RR salt (Sigma-Aldrich, Bromhdy, Denmark) and 0.25% naphthol AS-MX phosphate alkaline solution buffer (Sigma, Munich, Germany) for 30 min at room temperature.

Alizarin red S staining
The formation of mineralized matrix nodules was determined using Alizarin red S staining. hBMSCs were cultured in 24-well culture plate for 7, 14 and 21 days in the presence or absence of 100 or 500 ng/ml E-BMP-2.

ALP activity assay
To quantify alkaline phosphatase activities of hMSCs were seeded in 24-well culture plate for 7, 14 and 21 days in the presence or absence of 100 or 500 ng/ml E-BMP-2. ALP activity was normalized to the protein concentration of the cell layers.

Calcium assay
To quantify calcium concentration of hMSCs were seeded in 96-well plates and cultured for 3, 7, 14 and 21 days under osteogenic differentiation conditions. Calcium was measured using the QuantiChrom calcium assay kit.

RT-PCR
Total RNA was isolated from hMSCs treated as described above and differentiated towards the osteogenic differentiation for 3 weeks. For amplification of osteogenic markers alkaline phosphatase (ALP), bone sialoprotein (BSP) and collagen I 2 ul of cDNA was used as a template.

In vivo ectopic bone formation
Three Male Sprague-Dawley rat were used in this study. Each rat has six implant sites. Implantation of 10 ug of rhBMP-2 with type I collagen were performed in a muscle pouch of SD-rats abdominal wall for 8 weeks (n = 9). Type I collagen were used as negative controls (n = 9). Thereafter the animals were sacrificed and examined by micro-CT scan (Skyscan 1172, Kontich, Belgium).

RESULTS SECTION:
Analytical detection of rhBMP-2 by SDS-PAGE and Western-blot
The SDS-PAGE results showed that purified rhBMP-2 has the same molecular weight (~26 kDa as a homo-dimer) with that of reference rhBMP-2. The Western blot result revealed the purified BMP-2 is immuno-reactive to anti-BMP-2 antibody and homo-dimer (non-reducing condition) which is composed with 2 molecules of 13 kDa of monomer (reducing condition).

Osteogenic differentiation of BMSCs in response to rhBMP-2
On day 7, the positive reactivity to alkaline phosphatase was abruptly increased in the group treated with E-BMP-2 as compared with the control group. It was also confirmed that there was no significant difference in the positive reactivity to alkaline phosphatase depending on the concentration of E-BMP-2 (100 ng/ml or 500 ng/ml). And, the positive reactivity to Alizarin red S staining was stronger in the group which was treated with E-BMP-2 as compared with the control group.

Measurement of ALP activity
In the group which was treated with E-BMP-2 as compared with the positive control, ALP activity was measured as relatively higher. Besides, as the time elapsed, ALP activity was significantly increased.

Calcium accumulation assay
The amount of calcification was greater in the group treated with E-BMP-2 as compared with the control group. Particularly in the group which was treated with 500ng/ml E-BMP-2, the amount of calcification was increased by more than three times as compared with the control group on day 14.

Changes of gene expression by rhBMP-2 in the BMSC
The expression of ALP mRNA, in particular, on day 7, was of relatively higher degree in the group treated with E-BMP-2 as compared with the control group. The mRNA expression of BSP became notable in the group treated with E-BMP-2 from day 7 on. Until day 21, thereafter, it was persistently present. But there was no significant difference in the mRNA expression of type I collagen between the control group and the group treated with E-BMP-2.

In vivo results
Eight weeks after implantation, no obvious bone or cartilage formation was observed in the all samples of type I collagen. In contrast, in the central area, rhBMP-2-type I collagen displayed definitive bone formation in all of the nine samples. There was a significant difference in the bone formation between the two groups (P = 0.000041)

DISCUSSION:
In the current study, it was examined on in vitro and in vivo experiments whether recombinant human bone morphogenetic protein (rhBMP-2), isolated and purified from E. coli, would be effectively involved in the osteoinduction and osteogenesis. According to the current study, when hMSCs were treated with E-BMP-2 at different concentrations, there were no significant differences in the ALP activity and calcium mineralization between the two groups. These results confirmed that the dose of E-BMP-2 had no significant effect on the osteogenesis in an in vitro setting. Besides, in an intramuscular ectopic bone formation model where the osteoinductivity of E-BMP-2 could be elucidated, the complete bone tissue was formed in all the nine samples to which E-BMP-2 10ug was transplanted with the use of type I collagen with almost no osteoconductivity as a carrier. This was in agreement with the previous reports about CHO cell rhBMP-2. It can therefore be inferred that the bone formation induced by E-BMP-2 in the early stage underwent maturation process until week 8 and this was followed by the remodeling. This bacterial expression system is much more productive than mammalian systems used so far. As described here, a mass production of E-BMP-2 whose osteoinductivity has been demonstrated in high yield would lower the manufacturing cost. This might further contribute to extending the surgical indications for vertebral surgery or non-fusion surgery following the onset of fracture where the autogenous bone grafts are needed.