CHAPTER 1

Expression of rhBMP-2 in *Escherichia coli* and Its Activity in Inducing Bone Formation

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Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-13) superfamily. Recombinant human BMP-2 (rhBMP-2) has been shown to stimulate osteoblast differentiation and bone formation *in vitro* and *in vivo.* For further investigation of the effects of BMP-2 on bone formation *in vivo,* large-scale production of rhBMP-2 is required at low cost and with high yield. In the present studies, we successfully developed an *E. coli* expression system to produce rhBMP-2. *E. coli*-expressed rhBMP-2 has the correct molecular weight and amino acid sequence, in contrast to rhBMP-2 produced by mammalian cells. The biological activities of BMP-2 have been tested *in vitro* in an osteoblast precursor cell line and *in vivo* in different animal models. *E. coli*-expressed rhBMP-2 stimulated osteoblast proliferation and differentiation *in vitro* and induced new bone formation *in vivo.* Our studies demonstrated that *E. coli*-expressed rhBMP-2 is active *in vivo* and has therapeutic potentials in treatment of bone fractures, defects and other conditions associated with bone loss.

Introduction

One of the most important cell systems in the formation, modelling and remodelling of bone tissue is the osteogenic cell lineage. Chondrocytes and osteoblasts are held to belong to this lineage and both cell types are derived from mesenchymal precursor cells. The differentiation of mesenchymal cells

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to bone occurs by one of two routes. Firstly, there is a direct development of bone from the mesenchyme, as in the skull and the face, a process referred to as intramembranous ossification. Secondly, the rest of the skeleton is generated by bone replacement of cartilage via a process of endochondral ossification. Typical examples of endochondral bone formation are seen in long-bone development. The differentiation of mesenchymal cells to mature chondrocytes or osteoblasts is tightly regulated by locally produced bone growth factors, and BMPs are in this respect among the most important regulatory agents.

Osteoinductive factors were discovered when it was demonstrated that pieces of demineralised bone or bone extracts induced new cartilage and bone formation when placed subcutaneously or intramuscularly in rats.[7, 8] The active component was named bone morphogenetic protein (BMP) by Marshall Urist in 1965,[7] but the proteins responsible for bone induction remained unknown until the cloning of human proteins BMPs 2–4 in 1988.[10–12] The purification of BMP was followed using the rat ectopic bone formation assay. This assay involved combination of the protein to be assayed with demineralised rat bone matrix, treated with dissociative agents such as guanidine and urea for removal of the endogenous BMP activity. This combination was then implanted subcutaneously in rats, and after one to two weeks formation of new cartilage and bone was detected by histological methods. Using this approach, BMPs were purified and sequenced.[6, 12] Biochemical characterisation indicated that BMP proteins are dimeric, electrophoresing at 30 kDa under non-reducing conditions and at approximately half this molecular weight after reduction. To further determine which of these proteins are responsible for the osteoinductive activity, the molecular cloning approach was adopted to obtain molecular clones corresponding to each of the proteins present in the purified extract. Based on BMP sequence information, oligonucleotide probes were synthesised and used to screen bovine genomic libraries. Once bovine genes corresponding to the proteins were obtained, they were used to obtain human cDNA clones encoding each protein within the extract. A large number of clones, encoding BMP-1 through BMP-8, were identified by this cloning strategy.[5, 9, 12] With the exception of

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BMP-1, all of the proteins evince mutual amino acid sequence homology. To date, more than 20 BMP family members have been identified and characterised.

BMPs belong to the transforming growth factor-β (TGF-β) superfamily. They have similar sequences, including seven similarly-spaced cysteine residues located in the mature region of the proteins. Based on sequences derived from the cDNAs, BMPs are synthesised as larger precursor molecules. The mature region of 100–130 amino acid residues is released from a propeptide region which is two to three times larger, by cleavage at an Arg-X-X-Arg sequence. Six out of seven cysteine residues on each BMP subunit form three intrachain disulfide bonds, the remaining one forming an interchain bridge to create the dimer. Many of the BMPs are glycosylated in both the mature and propeptide regions, as determined by both biochemical characterisation and the presence of appropriate carbohydrate addition sites in the presumed amino acid sequence.

The production of pure individual BMPs by recombinant methods has allowed the activities of each molecule to be investigated *in vitro* and *in vivo*. Most recombinant BMPs have been produced using a mammalian cell expression system.[4] These systems have the advantage of facilitating the post-translational modifications involved in BMP synthesis, including glycosylation, dimerisation and cleavage of the precursor protein into the mature form, as well as efficient secretion of the protein from the cell into the culture medium. However, in comparison with expression in *Escherichia coli (E. coli),* the mammalian cell expression system is still costly.

In order to examine the effects of rhBMP-2 on bone formation *in vivo* and to evaluate its efficacy as a therapeutic agent in patients, there is clearly a need for large-scale production of rhBMP-2 at low cost and with high yield. Synthesis of the functional BMP-2 protein depends upon efficient transcription of the BMP-2 gene, translation of the BMP-2 mRNA and post-translational processing of the BMP-2 protein. Failure in any one of these processes may result in the lack of BMP-2 secretion or secretion of a biologically inactive molecule. When expressed in its natural host, BMP-2 undergoes extensive post-translational modifications. Such

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modifications of BMP-2 do not occur in *E. coli*. These processes are critical for the biological activity of the expressed BMP-2 protein. In recent years, we have successfully developed an *E. coli* expression system to produce rhBMP-2, successfully restoring the biological activity of the BMP-2 protein after expression in *E. coli*. Our studies demonstrate that *E. coli*-expressed rhBMP-2 possesses potent activity inductive of osteoblast differentiation and bone formation *in vitro* and *in vivo*.[13]

Cloning and Expression of rhBMP-2 in *E. coli*

Expression of rhBMP-2

The hBMP-2 cDNA encoding the mature peptide of the BMP-2 protein was amplified from RNA extracted from human osteosarcoma U2-0S cells. DNA sequencing revealed a 321-base pair (bp) DNA fragment encoding C-terminal 107-amino acid of hBMP-2 protein. In the recombinant plasmid, pMX-BMP2, BMP-2 expression was under the direct control of a P_LP_R thermoinducible promoter (Fig. 1A). RhBMP-2 expression in the DH5α host cells was induced in incubation at 42°C. SDS-PAGE showed the molecular weight of rhBMP-2 to be about 12 kDa and the expression level of rhBMP-2 more than 15% of the total bacterial proteins after six-hour induction at 42°C.

Purification and renaturation of rhBMP-2

The recombinant human BMP-2 expression in DH5α cells was in the form of inclusion bodies. The bacteria were harvested by centrifugation at 4°C and lysed by sonication in Tris-buffered saline (TBS). The *E. coli* inclusion bodies containing expression products were collected by precipitation of the bacterial lysates by centrifugation and then washed in 2% Triton X-100 buffer. The proteins in the inclusion bodies were dissolved in 8 M urea buffer and the supernatant then fractionated by DEAE-52 ion-exchange

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