A novel system for producing human recombinant BMP-2 and study of the growth factor stabilizing conditions

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INTRODUCTION

Bone tissue engineering has been an increasing field of research during the last years. The ideal approach for a regenerative application would consist in the use of cells from the patient, scaffolding materials and differentiation growth factors. **Bone morphogenetic protein-2** (BMP-2) is one such growth factors with a strong ability to induce new bone and cartilage formation and has been used as a powerful osteoinductive component of several late-stage tissue engineering products for bone grafting. In this work, we aimed at obtaining high yields of human recombinant BMP-2 in a stable, pure and biologically active form by use of a new bacteria expression system that circumvents the disadvantages of conventional recombinant protein preparation methods and to perform a study of the stability conditions and the functionality of these peptides *in vitro* in human mesenchymal stem cells and C2C12 murine cell line.

MATERIALS & METHODS

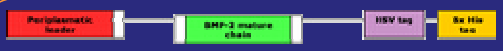


Fig. 1. The sequence coding for mature rhBMP-2 was cloned in a pET-25b vector and expressed in BL21DE3 *E. coli* strain. This vector permitted expression of recombinant protein into periplasm where ambient is permissive to the formation of cysteine bridges of folded protein.

rhBMP-2 was then purified by high affinity chromatography and size exclusion chromatography and tested in C2C12 cell line. This is a well-studied and stable model for testing the *in vitro* biological activity of recombinant BMPs.

Two variants of rhBMP-2 were produced: variant I containing two adjacent sites for protease cleavage in order to eliminate plasmid tags and variant II containing the protein with no additional cleavage sites.

For future bone biomedical applications!

Fig. 2. Expression of recombinant bacteria was performed in a **fermentor** allowing large yields of rhBMP-2, around 110mg/L.

RESULTS & DISCUSSION

Purification of rhBMP-2 by HPLC

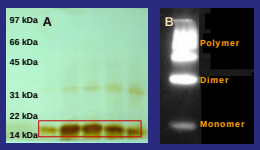
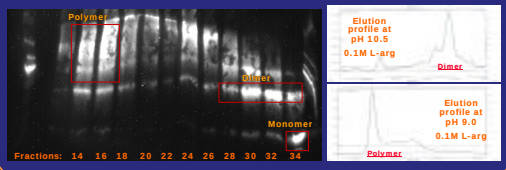


Fig. 3. A) Silver stained reduced SDS-PAGE reveals purification growth factor to up 95%. B) Non-reduced western-blot permitted observe monomer, dimer and polymer fractions. BMP-2 is at pH 8-10. An antibody against the 6x histidine tag was used.

Purification by size exclusion chromatography

Fig. 4. Size exclusion chromatography permitted partial separation of monomer, dimer and polymer fractions, as analysed by Western-blot. Conformations change with pH, buffer and concentration of growth factor.



Effect of L-arginine in solubilization

Table 1. Effect of L-arginine in the solubilization of rhBMP-2

pH 8.5, 0.5M L-arginine	
[total protein] (mg/ml)	% recovery of soluble protein
0.5	**74**
1	55
2	34
3	23

A) Effect of [BMP-2]

B) Effect of pH and [arginine]

1mg/ml total protein			
pH 8.5		0.5M L-arginine	
L-arginine (M)	% recovery of soluble protein	pH	% recovery of soluble protein
0	32	3	5
0.3	44	5	6
0.5	55	7	35
0.7	**63**	8.5	55
1	58	11	**83**

Biological activity assays

MTS cytotoxicity assay

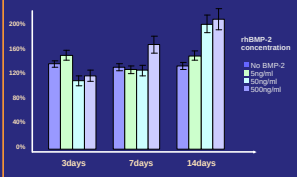


Fig. 5. MTS bioassay revealed no significant cytotoxicity of purified rhBMP-2

ALP bioactivity in human MSCs

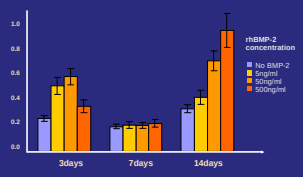


Fig. 6. ALP bioassay revealed an increase in ALP levels with continuous purified 5-500ng/ml rhBMP-2 stimulation

Morphology of human MSCs

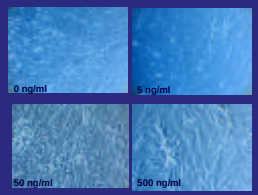


Fig. 7. Addition of 5-500ng/ml rhBMP-2 to human adipose mesenchymal stem cells resulted in changes of morphology. 10 days of cell culture.

von Kossa staining in MSCs

Fig. 8. *von Kossa* bioassay performed in mesenchymal stem cells from bone marrow after continuous stimulation with 500ng/ml purified rhBMP-2 shows evidence of nodule formation. 14 days of cell culture

Morphology of C2C12

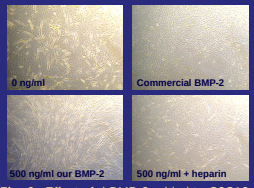
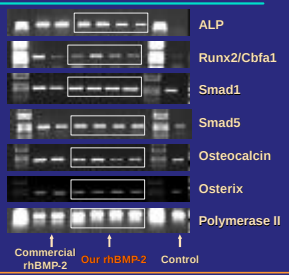


Fig. 9. Effect of rhBMP-2 added to C2C12 after 5 days of cell culture. Changes in morphology are observed but not similar to positive control. With heparin changes are more towards osteoblast-like.

RT-PCR for specific markers



Expression of markers in C2C12

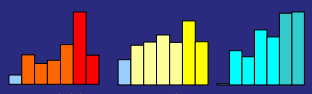
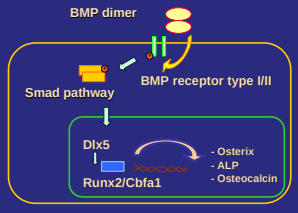


Fig. 10. RT-PCR shows increase of specific markers of osteogenic differentiation (ALP, Smad-5, Smad-1, Runx2) when C2C12 cells were stimulated with 500ng/ml of our rhBMP-2 stabilized at pH 10. 5 days of cell culture

BMP signaling pathway



CONCLUSIONS

- The novel approach described herein shows to be a promising way for obtaining large amounts of partially purified rhBMP-2 which shows evidence of bioactivity, capable of inducing some markers of specific osteogenic (bone) differentiation and showing no relevant cytotoxicity.

ACKNOWLEDGMENTS

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