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RESEARCH PAPER

Expression of Recombinant Human BMP-6 in CHO Cell by Fusion of a BMP-6 Mature Peptide to the Signal Peptide and Propeptide of Another Homologue Protein

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Abstract: Bone morphogenetic protein-6 (BMP-6) is a member of transforming growth factors β superfamily with more effective osteogenic activity. In this study, two recombinant plasmids were constructed to produce the recombinant proteins in mammalian expression system. One contained a gene encoding the signal peptide, propeptide, and mature peptide of human BMP-6, namely pcDNA-BMP-6, the other one was the recombinant gene encoding the signal peptide, propeptide of human BMP-2, and the mature peptide of BMP-6, namely pcDNA-BMP2/6. Transient expression in Cos7 cells showed that the pcDNA-BMP2/6 could produce more recombinant protein rhBMP-6 than pcDNA-BMP-6. For stable expression, the Chinese hamster ovary (CHO-*dhfr*$^-$) cells were co-transfected with pcDNA-BMP2/6 and pSV2-*dhfr*, and thereafter selected under the G418 as well as treated with methotrexate for targeting gene amplification. The purified rhBMP-6 with heparin affinity chromatography was shown to be able to possess bone induction activity by measuring the activation of alkaline phosphatase in C2C12 cells.

Key Words: BMP-6; Chinese hamster ovary cell; recombination expression; bone induction

The observation that existence of factors in bone could induce bone formation was decades prior to the key discovery by Urist in 1965. He demonstrated the ability of decalcified bone matrix when ectopically placed could induce bone formation was attributed to the presence of substance in bone matrix that he named bone morphogenetic protein (BMP)[1]. BMPs belong to the transforming growth factors β (TGF-β) superfamily. As many other members of TGF-β superfamily, BMPs are synthesized in cells as large precursors composed of a signal peptide, propeptide, and mature protein. After glycosylation and dimerization by a single interchain disulfide bridge, the precursor was proteolytically cleaved to yield the active dimers. At present, more than 15 different BMPs have been identified with varying degrees of bone inducing activity.

BMP-6 is one of the less characterized member of the BMPs, and plays a critical role in the maturation of hypertrophic cartilage and osteoblast differentiation[2,3]. BMP-6 is induced in different stages of bone fracture and involved in the healing of bone fracture[4]. It is shown either *in vitro* or *in vivo* that BMP-6 compared with the other BMPs is the most consistent and potent osteogentic factor[5,6]. Although BMPs have been successfully isolated from bones, the yields of BMPs were rather low. Meanwhile, their clinical application was very much limited because of the complicated purification procedure and the potential health risk. Alternatively, the use of recombinant genetic technology for the production of large quantities of BMPs has been improved in the efficiency, cost effectiveness, and safety for human application[7,8].

In this study, we constructed two different hBMP-6 mammalian expression vectors, and compared their productivity of rhBMP-6 by transient transfection. One stable

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cell line of expression hBMP-6 was successfully selected in our system. Finally, the purified rhBMP-6 was shown to possess intact bone induction activity.

1 Materials and methods

1.1 Plasmids and strains

Plasmids pSV2-*dhfr* and pcDNA3.1(+) were obtained from ATCC and Invitrogen (USA), respectively. Strain DH5α was grown in Luria Bertani medium, as was Cos7 and C2C12 cells in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Dihydrofloate reductase deficient Chinese hamster ovary cell (CHO-*dhfr*$^-$) was obtained from CTCCCAS (China) grown under nonselective condition, and maintained in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS, hypoxanthine (13.6 mg/L), and thymidine (2.42 mg/L). All cells were maintained in medium containing both 100 u/ml penicillin and 100 μg/mL streptomycin at 37°C incubator with 5% CO_2.

1.2 Enzymes and regents

Enzymes for DNA manipulation were obtained from TaKaRa (China). Reverse transcriptase moloney murine leukemia virus (MMLV), G418, and antigoat IgG-HRP were purchased from Promega (USA). DNA purification kits for agarose gel and plasmid preparation kit were obtained from OMEGA (USA). Lipofectamine 2000 and cell culture medium were obtained from Invitrogen (USA). Goat antihuman BMP-6 antibody was purchased from Santa Cruz Biotech (USA). hBMP-6 ELISA kits were obtained from R&D (USA). Heparin CL-6B agarose was purchased from Pharmacia (USA). Alkaline phosphatase (ALP) activity assays kits and methotrexate (MTX) were obtained from Sigma (USA).

1.3 Primers design and modification

The primers were designed based on human BMP-2 and BMP-6 mRNA sequences. The primers for the signal and propeptide of human BMP-2 (pBMP-2) were as follows: hB2-321F, 5′-gctggatccaccatggtggccgggacccgctgtc-3′ and hB2-1169R: 5′-acgtttttctcttttgtggagaggatg-3′. The forward primers for mature peptide of human BMP-6 (mBMP-6) and unprocessed BMP-6 (BMP-6) were as follows: hB6-1323F, 5′-caacagagtcgtaatca-3′ and hB6-16F, 5′-ccttaagcttccggacgac catgagagata-3′, respectively, and the reverse primer for both fragments was hB6-1721R, 5′-ggccggctcgagttagtggcatccacaag ctct-3′. The primes hB2-1169R and hB6-1323F were specially phosphorylated. The forward and the reverse primers for GAPDH were as follows: 5′-tgaaggtcggtgtgaacggat-3′ and 5′-catgtaggccatgaggtccaccac-3′.

1.4 Construction of recombinant plasmids

The total RNA was extracted from the human placenta using commercial kits (Dingguo, China) following the manufacture's protocol and subjected to oligo-dT primed reverse transcription using MMLV transcriptase. The cDNA encoding the peptide of pBMP-2, mBMP-6, and BMP-6 were amplified using polymerase chain reaction (PCR) with above reverse products as the templates, respectively. The amplified products were purified and recovered from the agarose gel. The cDNAs of BMP-6 was subsequently inserted into a mammalian expressing vector pcDNA3.1(+). The fragment of cDNAs pBMP-2 fused to that of mBMP-6 (namely BMP2/6) was obtained by linkage of the amplified products of pBMP-2 and mBMP-6 by T4 DNA ligase and consequently amplified by primers hB2-321F and hB6-1721R. The cDNAs of BMP2/6 was purified and subsequently cloned into pcDNA3.1(+) vector.

Recombinant plasmids were introduced by the method of heat shock into competent cell of *Escherichia coli* DH5α for characterization of construction, propagation, and maintenance. Clones were identified for correct insert using PCR and restriction analysis. After the initial screening, plasmids of selective clones were subjected to sequencing analysis for the final selection of an expression plasmid carrying sequence that are homologous to the reported sequence using BLAST program.

1.5 Transient expression of pcDNA-BMP-6 and pcDNA-BMP2/6

The recombinant plasmids were isolated using plasmid purification kits following the manufacturer's protocol. The recombinant plasmids (20 μg) were introduced into host cells Cos7 (2×10^6 cells) using electroporation procedure (Bio-Rad Gene Pluser Xcell, Voltage 220 V, pulse length 25 ms, pulse 1, cuvette 4 mm, volume 0.4 mL). The transformants were cultivated in DMEM supplemented with 10% FBS for 24 h, and washed with PBS and cultivated in DMEM for another 24 h. The supernatant was collected for the determination of protein concentration of rhBMP-6. The total RNA was extracted from transfected cells for the detection of rhBMP-6 mRNA expression.

1.6 Establishment of a stable BMP-6 expression cell line

Parental CHO cell that stably expresses rhBMP-6 was produced by co-transfection of pcDNA-BMP2/6 and pSV2-*dhfr* (10:1) into CHO-*dhfr*$^-$ using Lipofectamine 2000. Drug selection was carried out at 48 h after transfection with IMDM containing 10% dialyzed FBS and 0.75 mg/mL G418.

The stable transfection cells were subjected to stepwise increasing concentration of MTX for gene amplification (0.05, 0.2, 0.8, and 1.6 μmol/L). The cells collected at 1.6 μmol/L MTX were trypsinized and plated at 0.8 cells/well in a 96-well plate containing IMDM with 10% dialyzed FBS and corresponding level of MTX, before cells in the 96th well reached confluence, the cells were refed with fresh medium and incubated for one day. The supernatant was completely removed from each well and saved for rhBMP-6 assay; the number of remaining cells in each well was estimated by MTT assay. The relative rhBMP-6 productivity of each clone was obtained by dividing the BMP-6 concentration in the supernatant by the corresponding cell numbers.

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