JOURNAL
OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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High-Level Expression of Recombinant Human Bone Morphogenetic Protein-4 in Chinese Hamster Ovary Cells

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Abstract Bone morphogenetic protein-4 (BMP-4) is a signaling homodimeric molecule that acts as a morphogen to influence cell fate in a concentration-dependent manner. The limited supply of a pure preparation of BMP-4, due to very low level of their expression *in vivo*, makes it difficult not only to study the biological activities of BMPs, but also to use them as a clinical tool. For a large-scale production of BMP-4, human BMP-4 cDNA was expressed in Chinese hamster ovary (CHO) cells by a recently development vector system, which confers position-independent stable expression of the foreign genes. The CHO cell line expressing recombinant human BMP-4 (rhBMP-4) at the level of 7 µg/ml could be obtained after stepwise selection with methotrexate. This level of expression is about 70 times higher than those previously reported. The partially processed form of BMP-4 as well as mature form could be detected, when the aliquots of culture media were analyzed by Western blot. The glycosylation pattern and biological activity of the rhBMP-4 were determined by glycosidase treatment and the induction rate of alkaline phosphatase in mouse osteoblastic cells.

Key words: BMP-4, Chinese hamster ovary (CHO) cell, recombinant protein expression, stable cell line

Bone morphogenetic protein-4 (BMP-4) is a member of the transforming growth factor beta (TGF-β) family and was originally identified as a protein that was capable of inducing ectopic bone formation; however, recent studies have shown that it participates in the development of nearly all organs and tissues [1]. Because of its multi-functional nature, BMP-4 activity or expression is strictly regulated at many different levels [2]. Outside the cell, BMP-4 activity is regulated by its binding proteins such as chordin and noggin, which act as a BMP-4 antagonist by

blocking the activation of cell surface receptors, and by the protease Tolloid, which cleaves chordin to release active BMP-4 [3]. Inside the cell, BMP signaling can be modulated by Smad6 and Smad7, which block signal transmission from the membrane to the nucleus, and by the Smurf family of ubiquitin-protein ligases targeting BMP receptors and other signal components for proteosomal or lysosomal degradation [4, 5].

The biological activity of BMP-4 is also regulated at the level of proteolytic activation. Similar to all TGF-β family members, BMP-4 is synthesized as an inactive precursor, consisting of signal sequence, propeptide, and mature segment. ProBMP-4 is sequentially cleaved at two sites by a proprotein convertase, such as furin, to yield active, carboxy-terminal mature homodimer [6]. It is initially cleaved at a consensus furin motif (-RSKR-) adjacent to the mature ligand (the S1 site), and this allows for subsequent cleavage at an upstream minimal furin motif (-RISR-) (the S2 site) (Fig. 1A) [7]. Pro- and mature domains of BMP-4 remain noncovalently associated after S1 cleavage, generating a complex that is targeted for rapid proteosomal or lysosomal degradation. However, subsequent cleavage at the S2 site liberates mature BMP-4 from the prodomain, and thus it may escape from the degradation and be secreted [8].

Since BMP-4 is a multifunctional protein and can be used for many purposes, such as a therapeutic agent, the mass production of BMP-4 is very important for the study of the biological role and potential medical use of BMP-4. However, it has been very difficult to supply pure BMP-4 preparation in large scale owing to low level of BMP-4 expression in cells. Recently, a mammalian expression vector containing the human β-globin matrix attachment region has been developed, which may confer position-independent expression of the foreign genes, thereby enhancing the expression level of the recombinant proteins [9]. In the present study, we attempted to develop CHO cell lines that produce a high level of recombinant human BMP-4

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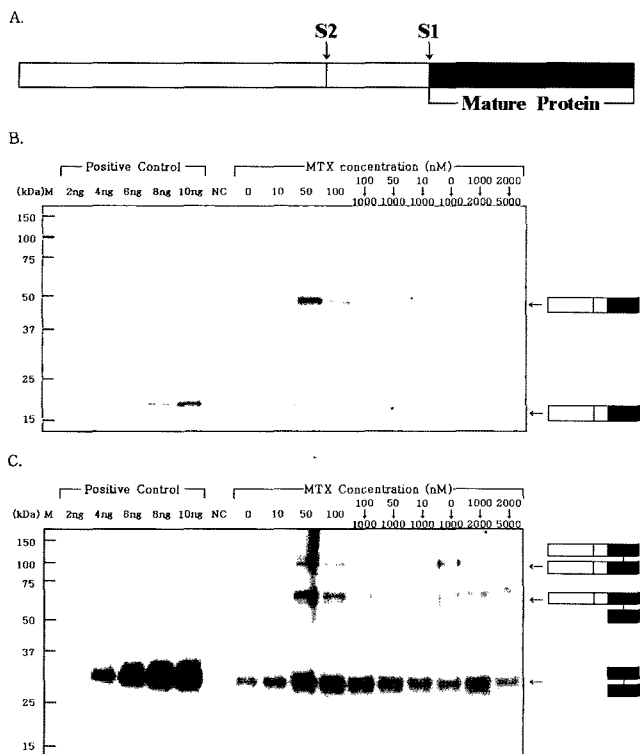Phone: 82-31-201-2437; Fax: 82-31-203-4969;
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A.

B.

C.

Fig. 1. The rhBMP-4 expression in the CHO cells selected with MTX.

(A) Schematic illustration of proBMP-4. The cleavage sites in proBMP-4 are indicated by S1 and S2. The black box represents the mature BMP-4 protein. (B and C) The CHO cells expressing rhBMP-4 were selected with increasing concentration of MTX, as shown on the top of the panels. The culture supernatant (10 μl) was loaded on SDS-PAGE under reduced (B) or non-reduced (C) conditions and subjected to Western blot analysis. The rhBMP-4 purified from the NSO cell line (R&D Systems) was used as a positive control. The culture supernatant of non-transfected CHO cells was used as a negative control (NC). The positions of unprocessed, partially processed, and mature BMP-4 are illustrated schematically to the right of the panels.

(rhBMP-4) protein, using the expression vector containing the human β-globin matrix attachment region.

The recently developed expression vector, pMSV, containing the human β-globin matrix attachment region, was used for the expression of the rhBMP-4 cDNA. The rhBMP-4 expression plasmid, pMBMP4, was constructed by introducing the hBMP-4 cDNA under the SV40 promoter of pMSV [9]. The hBMP-4 cDNA was obtained by RT-PCR from mRNAs of human U-2 osteosarcoma cells expressing hBMP-4. The oligonucleotide primers were designed to amplify the protein coding region of hBMP-4. The sense primer sequence was 5'-CGTCGCTAGCCACCATGATTCCTG-GTAACC-3' and the antisense primer was 5'-TTACGTC-GACTTACTCATCCAGGTACAG-3'. Restriction enzyme sites (NheI and BglII) were engineered into the oligonucleotides in order to facilitate cloning of cDNA into the vector pMSV. Kozak sequence (GCCACC) was also added for efficient start of translation [10]. The amplified hBMP-4

cDNA was digested with NheI and BglII, and the digest was ligated to the vector digested with the same enzymes. The resulting expression plasmid construct, pMBMP4, was completely sequenced to confirm that it contained the same entire coding region of the hBMP-4 protein previously reported [11].

Dihydrofolate reductase (DHFR)-deficient CHO cells (DG44) were co-transfected with the rhBMP-4 expression plasmid pMBMP4 by the liposome-mediated method [12]. The transfected cells were subsequently cultured in nucleotide-free medium to select the DHFR-positive CHO cells. Then, they were subcultured in stepwisely increasing concentration of methotrexate (MTX) to amplify the DHFR gene together with the rhBMP-4 cDNA [13]. Since the direct increase of MTX from 0 nM to 1 μM was impossible for the CHO cells, the cells were first adapted to 10 nM, 50 nM, or 100 nM MTX and then adapted to 1 μM MTX. The levels of the rhBMP-4 expression in the cells adapted to different concentrations of MTX were analyzed by Western blot. The culture supernatant (10 μl) was harvested after 48 h of incubation at 37°C in a 48-well plate containing 200 μl of α-MEM media (1×10⁵ cells/well) and then loaded onto a 10% SDS-polyacrylamide gel. The mouse anti-human
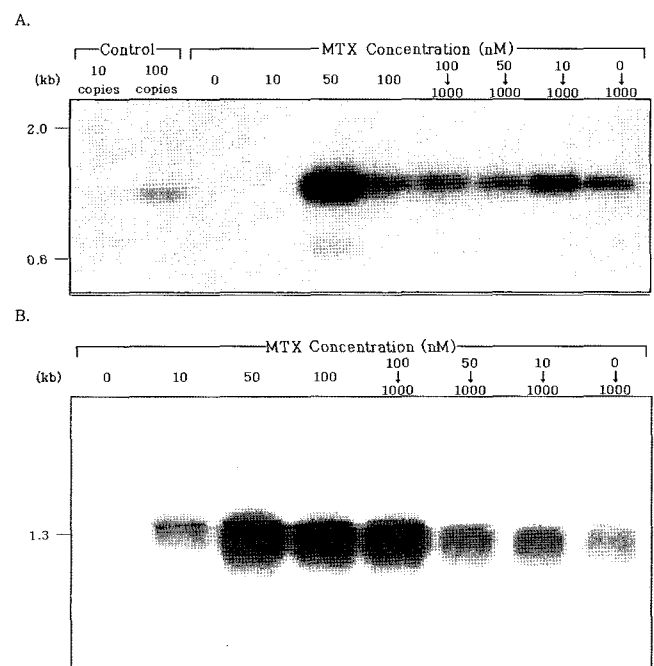
A.

B.

Fig. 2. Southern blot (A) and Northern blot (B) analyses of the CHO cells selected with MTX.

The P³²-labeled rhBMP-4 cDNA was used as a probe. The concentrations of MTX used for the selection are shown on the top of the panels. (A) Genomic DNAs (15 μg) digested with NheI and BglII were electrophoresed on a 0.8% agarose gel and hybridized to the probe. As positive controls, the digested pMBMP4 plasmid DNA corresponding to 10 and 100 copies of the genomic DNA were loaded in Lanes 1 and 2, respectively. (B) The total RNAs (15 μg) were loaded in each lane and hybridized to the probe. The hybridized rhBMP-4 RNAs corresponding to about 1.3 kb can be seen.

BMP-4 polyclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.) and HRP-goat anti-mouse IgG (Zymed, South San Francisco, CA, U.S.A.) were used to detect BMP-4 protein. Under reducing condition, two bands of ~18 kDa and ~52 kDa were detected, corresponding to the mature BMP-4 and proBMP-4 monomer, respectively (Fig. 1B). On the other hand, under nonreducing condition, three major bands of ~36 kDa, ~70 kDa, and ~105 kDa were detected corresponding to the mature BMP-4 dimer, partially processed mature/proBMP-4 dimer, and unprocessed proBMP-4 dimer, respectively (Fig 1C). The partially processed or unprocessed proBMP-4 dimer has not been reported earlier outside the cell, probably because of the low level expression of BMP-4. As shown in Figs. 1B and 1C, the cells adapted to 50 nM MTX showed higher expression of the rhBMP-4 than the cells adapted to 0 nM, 10 nM, or even 100 nM, suggesting that the cells can readily be adapted to direct increase of MTX concentration

up to 50 nM, but not to 100 nM. Further increase of MTX to 1 μM slightly decreased the rhBMP-4 expression, suggesting that the stable cell lines expressing the rhBMP-4 are very difficult to adapt to higher concentration of MTX.

The CHO cells transfected with pMBMP4 were further characterized by Southern and Northern blot analyses. To determine the copy numbers of the rhBMP-4 cDNA, the chromosomal DNAs of the CHO cells adapted at the different concentrations of MTX were analyzed by Southern blotting. Genomic DNAs digested with NheI and BglII were electrophoresed on a 0.8% agrose gel. As shown in Fig. 2A, approximate copy numbers in the CHO cells could be estimated by comparing the density of the DNA band (about 1.2 kb) hybridized with the rhBMP-4 cDNA probe. The CHO cells adapted to 50 nM MTX carried the highest copy numbers, more than 300 copies of the rhBMP-4 cDNA per cell. Northern blot analysis showed that the CHO cells adapted to 50 nM MTX also expressed
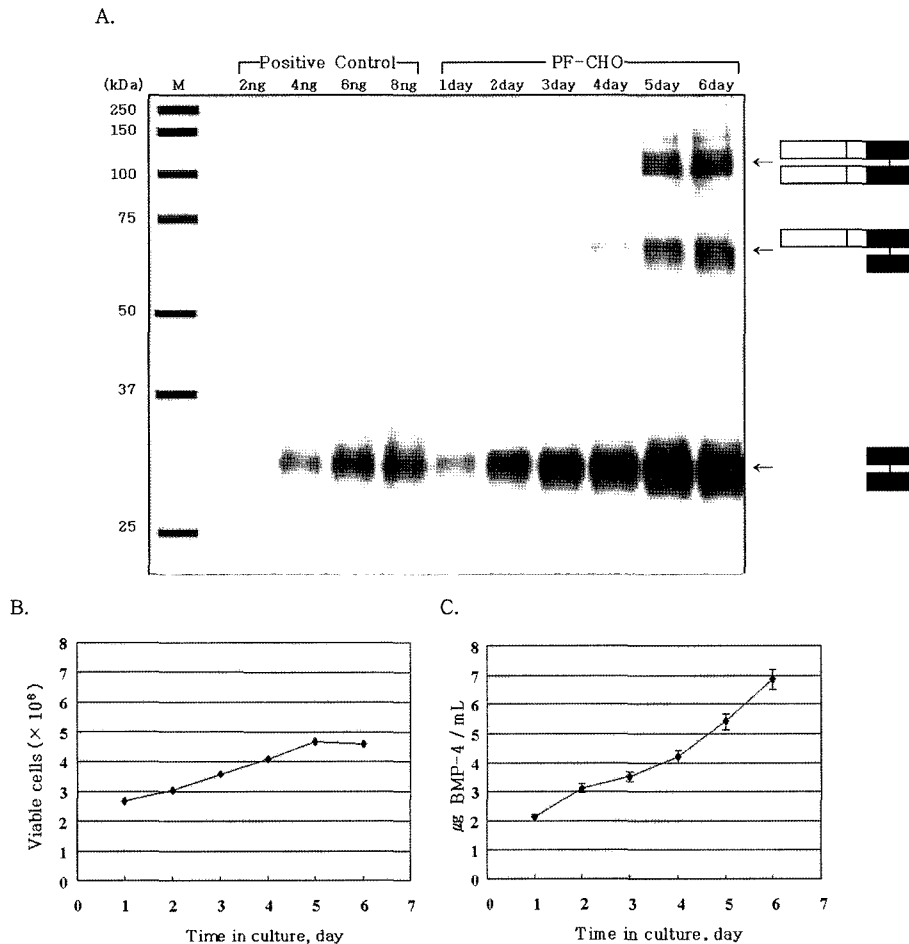

Fig. 3. The rhBMP-4 expression in the CHO cells cultured in the protein-free medium, PF-CHO.
(A) Western blot analysis of the rhBMP-4 in the cell lines grown under PF-CHO. The culture supernatant (10 μl) was loaded on SDS-PAGE and subjected to Western blot analysis, using the antibody against rhBMP-4 (R&D Systems). The rhBMP-4 purified from the NSO cell line (R&D Systems) was used as a positive control. The positions of unprocessed, partially processed, and mature BMP-4 are illustrated schematically to the right of the panels. (B) The total numbers of viable cells grown under PF-CHO. (C) The rhBMP-4 production rate of the cell lines grown under PF-CHO.

the rhBMP-4 mRNA at the highest level (Fig. 2B), thus indicating that the levels of the rhBMP-4 production by the transfected cells correlated well with the amplified gene numbers as well as the mRNA levels of the rhBMP-4.

To examine the applicability of protein-free media such as PF-CHO (HyClon Labs, Logan, VT, U.S.A.) and CD-CHO (Gibco BRL, Grand Island, NY, U.S.A.) for culture of the rhBMP-4-expressing cell line, the cell growth and rhBMP-4 production in these media were compared with those in α-MEM containing 10% serum. A single colony was selected from the CHO cells adapted to 50 nM MTX and used to compare media. It was found that PF-CHO was better than CD-CHO or 10% serum containing α-MEM for cell growth and the rhBMP-4 production (data not shown). As shown in Fig. 3, the total number of viable cells grown under PF-CHO increased until 5 days of culture, and the rhBMP-4 production rate was about 7 μg/ml in 6-day batch culture. Previously, the rhBMP-4 production in mammalian cells has been reported in a few cases, but all the production rates were less than 100 ng/ml [11, 14]. These results suggest that the human β-globin matrix attachment region in the expression vector seems to function nicely to enhance the rhBMP-4 expression, similar to other recombinant proteins reported [9, 15].

To determine the proper folding or glycosylation of the rhBMP-4 produced in the CHO cells, the rhBMP-4 was treated with glycosidases. Thus, the CHO cells expressing rhBMP-4 were seeded into a 6-well plate (3×10^5 cells/ well) and the cells were washed twice with PBS after 24 h of incubation at 37°C and replenished with 1 ml of serum-free media. After 48 h of incubation, the culture supernatant (50 μl) was harvested and treated with 1 unit of endoglycosidase H (Endo H) or peptide N-glycosidase F (PNGase F) for 15 h and analyzed by Western blotting. Carbohydrates that are transferred onto proteins in the endoplasmic reticulumn (ER) are sensitive to Endo H digestion. When further modified in the Golgi, these moieties become Endo H resistant but remain sensitive to PNGase F. Therefore, resistance/PNGase F sensitivity is a hallmark of proteins that are properly folded. As shown in Fig. 4, Endo H-sensitive (asterisks) and Endo H-resistant/PNGase F-sensitive forms of the rhBMP-4 (arrowheads) were detected, indicating that high contents of Endo H-sensitive carbohydrates are retained at one or more glycosylation site(s) on rhBMP-4, which was inaccessible to modifying enzymes in the Golgi. A similar glycosylation pattern was observed previously for BMP-2 and BMP-4 [8, 16].

The activity of the rhBMP-4 produced in the CHO cells was measured by the induction rate of alkaline phosphatase activity in mouse osteoblastic cells (MC3T3-E1 cell). The mouse osteoblastic cells, which were grown for 16 h in 96-well plates, were treated in triplicates with the culture supernatant (3 μl) containing about 20 ng of the rhBMP-4 and incubated for 48 or 72 h. Subsequently, the cells were
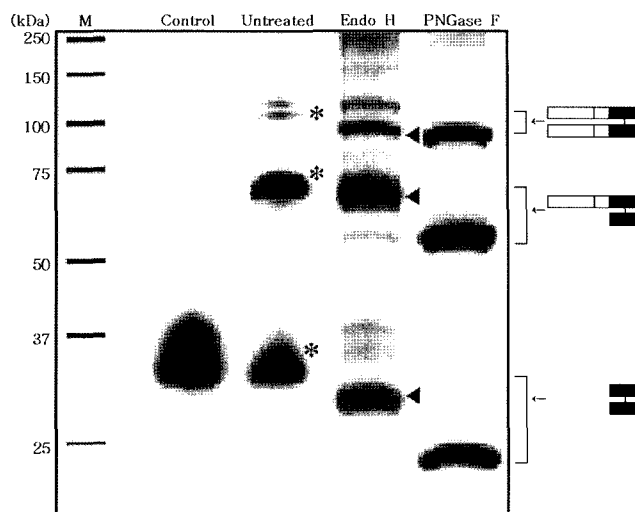

Fig. 4. Glycosylation of the rhBMP-4 expressed in the CHO cell lines.

The culture supernatant (50 μl) of the cell line expressing rhBMP-4 was treated with endoglycosylase H (Endo H) or peptide N-glycosidase F (PNGase F) and subjected to Western blot analysis. Endo H-sensitive and Endo H-resistant/PNGase F-sensitive forms of the rhBMP-4 are indicated by asterisks and arrowheads, respectively. The positions of unprocessed, partially processed, and mature BMP-4 are illustrated schematically to the right of the panels.

washed and lysed by freezing and thawing in 50 mM Tris-HCl, pH 7.5 (100 μl). The lysate (20 μl) was added to 80 μl of 1.2 M diethanolamine (pH 9.8) buffer solution, containing 0.6 mM $MgCl_2$ and 121.6 mM p-nitrophenyl phosphate, to monitor alkaline phosphatase activity. After incubation for 30 min at 37°C, the reaction was stopped by adding 1 N sodium hydroxide (20 μl), and optical density at 405 nm was measured with an ELISA reader. As shown
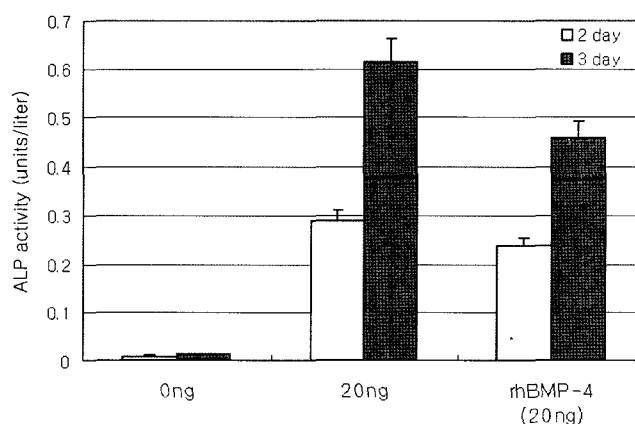

Fig. 5. The biological activity of the rhBMP-4 protein expressed in CHO cells.

The biological activity was measured by the ability to induce alkaline phosphatase in mouse osteoblastic cells. As a positive control, 20 ng of rhBMP-4 (R&D Systems) was used. The standard deviations from mean of three independent assays are indicated by error bars.

in Fig. 5, the rhBMP-4 produced in the CHO cells was able to induce alkaline phosphatase activity in mouse osteoblastic cells; however, it showed slightly lower activity than the positive control (R&D System), which was purified from the NSO cell line. The slightly lower activity of the rhBMP-4 produced in the CHO cells than that of the control might have been due to a lower degree of purity, since the culture supernatant rather than purified protein was used for the assay.

In this study, a stable CHO cell line that expressed at high level of the rhBMP-4 was established using an expression vector containing the human β-globin matrix attachment region. This cell line can be used for the mass production of BMP-4 to study its biological role and to develop as a therapeutic agent.

Acknowledgment

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