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Expression and Identification of Mutated Osteoprotegerin in Culture Cells and Larvae of Silkworm

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Abstract The mutated osteoprotegerin (OPG-372) gene was inserted into the baculovirus transfer vector pBacPAK8, and the recombinant plasmid was co-transfected with linearized Bm-BacPAK6 virus DNA into BmN cells, then homologous recombination occurred inside the cells. The recombinant virus BmNPV-OPG-372 was screened and identified by Southern blotting. The recombinant human OPG-372 was expressed in cultured cells and the larvae of silkworm by inoculation of recombinant virus. The expression products were run on the SDS-PAGE and their immunoreactivities were determined by Western blotting. It was found that a 42 kD recombinant protein was expressed in BmN cells and a 46 kD one in larvae respectively. The bioactivities of the recombinant proteins were determined by hypocalcemic effect assay in the mice. The results showed that the recombinant proteins had a significant hypocalcemic effect on mice sera.

Key words OPG-372; silkworm baculovirus expression system; hypocalcemic effect

Osteoprotegerin (OPG, or osteoclastogenesis inhibitory factor, OCIF) is a soluble member of the tumor-necrosis factor receptor family discovered in 1997 that can inhibit osteoclastogenesis and prevent bone loss from resorption. Simonet *et al.* [1] reported that recombinant human OPG (rhOPG) was useful for the prevention and treatment of osteoporosis. Yamamoto *et al.* [2] also found that rhOPG could decrease the concentration of serum calcium and had both preventive and therapeutic effects on hypercalcemia. Therefore, rhOPG offers a good prospect as a new biomedicine in the treatment of human bone disease.

The *OPG-372* gene was a mutated OPG cDNA obtained from normal Chinese human liver cell line L02 by RT-PCR. Sequence analysis showed that the *OPG-372* gene had a nonsense mutation at Gln^{394}, so OPG-372 protein was 8 amino acid residues less at the C-terminal than OPG protein [3].

Though rh-OPG-372 had been expressed in the insect cell and yeast [4], there was no report about its expression in silkworm baculovirus expression system. This paper described the expression and bioactivities of the recombinant human OPG-372 produced in BmN cell and fifth instar larvae.

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Materials and Methods

Materials

E. coli TG1, transfer vector pBacPAK8, wild-type virus Bm-BacPAK6 and BmN cell were conserved at the Institute of Biochemistry, Zhejiang University. Recombinant plasmid pBSK-OPG-372 was provided by Prof. Xiang-Fu WU *et al*. of Shanghai Institute of Biochemistry and Cell Biology. The Digoxigenin DNA labelling and detection kit was purchased from Boehringer Mannheim. Rabbit anti-human OPG polyclonal antibodies were obtained from Santa Cruz. Restriction endonucleases, Klenow fragment of DNA polymerase and T4 DNA ligase were obtained from TaKaRa BioTech. TC-100 insect medium and fetal bovine serum were bought from Gibco BRL. ICR mice were provided by the Animal Research Center of Medical College in Zhejiang University. Kit A045 was purchased from Nanjing Jiancheng Bioengineering Institute, Jiangsu Province, China. Salmon calctionin was provided by the

First Affiliated Hospital of Zhejiang University.

Construction of recombinant baculovirus

The entire OPG-372 cDNA sequence (1119 bp) was excised from plasmid pBSK by *Bam*HI and *Xho*I digestion, and inserted into the transfer vector pBacPAK8. The recombinant transfer vector pBacPAK8-OPG-372 was co-transfected with linearized DNA of Bm-BacPAK6 virus into BmN cells. The recombinant virus Bm-BacPAK-OPG-372 was screened, plaque-purified, and identified by Southern blotting using Digoxigenin DNA labelling and detection kit. Pure recombinant virus was prepared for further research.

Expression of recombinant OPG-372 in BmN cell and silkworm

BmN cells (2×10^6 cells/flask) were infected with recombinant virus (MOI=10). The cells were daily harvested by low speed centrifugation (3000 r/min) for 5 min and washed with phosphate buffer solution (PBS, 0.02 M, pH 7.4).

The 5th instar silkworm (Qingsong×Haoyue) larvae were infected with the purified recombinant virus by subcutaneous injection at about 2×10^5 particles per worm in a volume of 0.005 ml. The hemolymph was collected daily and centrifuged at 12,000 r/min for 10 min, the supernatant was prepared for further assays.

SDS-PAGE and Western blotting

The recombinant OPG-372 (rh-OPG-372) expressed in BmN cells or silkworm was separated on 10% SDS-PAGE, and its immunoreactivity was detected by Western blotting using rabbit anti-human OPG polyclonal antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody as the detecting reagents. The procedures of electrophoresis, transfer and detection of immunoreactivity were performed as described previously [5].

Quantity of rh-OPG-372

Total protein concentration was determined by a modified Lowry procedure (Kit A045) using bovine serum albumin as the standard. The rh-OPG-372 was separated on SDS-PAGE, and the percentage of recombinant protein bands was measured by grey scan. Then the quantity of recombinant protein was calculated from the product percentage multiplied by the quantity of total protein concentration.

Hypocalcemic effect assay

Dose-response of hypocalcemic effect Sixty-four male ICR mice (20 g) were fasted for 24 h, and then were randomly divided into 8 groups. Four groups were administered with 0.075, 0.15, 0.3, 0.6 ml infected hemolymph respectively by abdominal cavity injection, in which the doses of rh-OPG-372 were about 0.75, 1.5, 3 and 6 mg/kg, respectively. One group was injected with the total protein of infected BmN cell, the dose of rh-OPG-372 being 6 mg/kg. Two groups were injected with PBS (0.6 ml) and hemolymph infected with BmNPV (0.6 ml) respectively as negative control. The last group injected 100 mU salmon calcitonin (sCT) acted as positive control. The sera of mice were collected from the fundus oculi at 3 h after injection, and the calcium concentration was determined by the method described previously [4,6].

Time-course of hypocalcemic effect Sixty mice (as described above) were randomly divided into 5 groups. Four groups were injected with 0.6 ml infected hemolymph, in which the dose of rh-OPG-372 was about 6 mg/kg. The mice sera were collected from the fundus oculi at 1.5, 3, 5, 7 h after injection for detection, respectively. The same volume of PBS was injected to the last group as negative control.

Statistic analysis

Values of hypocalcemic effect were expressed as the means (8 or 12 replicates) and compared statistically by *t*-test at the 5% with SPSS 10.0 software.

Results

Construction of the recombinant baculovirus BmNPV-OPG-372

A DNA fragment (around 1.1 kb) was excised from recombinant plasmid pBacPAK8 after *Bam*HI and *Xho*I digestion. It could be concluded that OPG-372 cDNA was inserted into transfer vector pBacPAK8 (Fig. 1). The se-

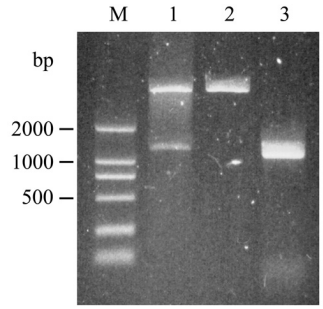


Fig. 1 Identification of pBacPAK8-OPG-372

M, DL2000 marker; 1, pBacPAK8-OPG-372/*Bam*HI + *Xho*I; 2, pBacPAK8/*Bam*HI + *Xho*I; 3, PCR amplification of pBacPAK8-OPG-372.

quence of the inserted OPG-372 cDNA was correct according to DNA sequencing (data was not shown). The purified pBacPAK8-OPG-372 was co-transfected with linear baculovirus Bm-BacPAK6 DNA digested by *Aoc*I. The recombinant virus was then screened, plaque-purified and identified by Southern hybridization (Fig. 2).

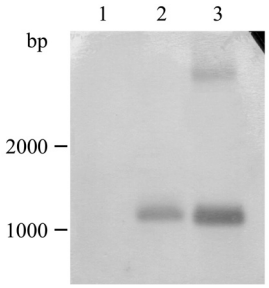


Fig. 2 Southern blotting of Bm-pBacPAK-OPG-372

1, Bm-BacPAK6 DNA/*Bam*HI + *Xho*I; 2, Bm-BacPAK-OPG-372 DNA/*Bam*HI + *Xho*I; 3, pBacPAK8-OPG-372/*Bam*HI + *Xho*I.

Expression of recombinant OPG-372 in BmN cell

The BmN cells were infected with the purified recombinant virus for expression of rh-OPG-372 and collected after 24 h. The results of SDS-PAGE (Fig. 3) and Western blotting analysis indicated that the recombinant protein of rh-OPG-372 was around 42 kD (Fig. 4). The expression level was about 0.08 mg per flask.

Expression of recombinant OPG-372 in silkworm larvae

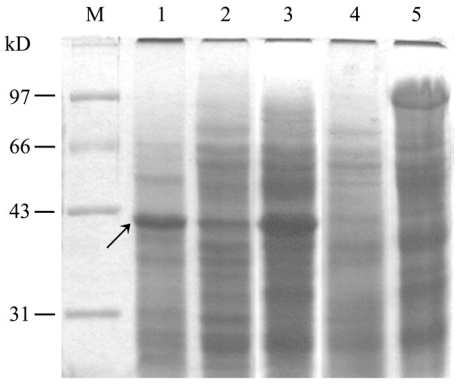


Fig. 3 SDS-PAGE of rh-OPG-372 protein expressed in BmN cells

M, protein molecular weight marker; 1, 2 and 3, BmN cell total protein were infected with Bm-BacPAK-OPG-372, and collected at 96, 72 and 120 h, respectively; 4, BmN cell total protein; 5, BmN cell total protein infected with Bm-BacPAK6. The expressed recombinant protein band was indicated by the arrow.

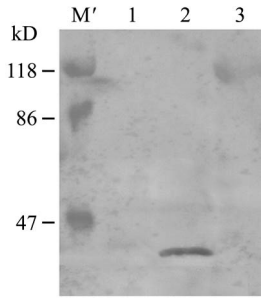


Fig. 4 Western blotting of rh-OPG-372 protein expressed in BmN cells

M', prestain protein molecular weight marker; 1, BmN cell total protein; 2, BmN cell total protein infected with Bm-BacPAK-OPG-372; 3, BmN cell total protein infected with Bm-BacPAK6.

In order to detect rh-OPG-372 expressed in silkworm larvae, the 5th instar larvae were infected with recombinant virus by subcutaneous injection, on the 4th day, the larvae began to loss appetite and the most would die on the 5th day. Hemolymph was harvested on the 4th or 5th day after inoculation. It was indicated that the rh-OPG-372 expressed in silkworm was around 46 kD judged by SDS-PAGE (Fig. 5) and Western blotting (Fig. 6). No signal was found in hemolymph infected with Bm-BacPAK6. The expression level was about 200 mg/L.

Hypocalcemic effect assay

12.5, 25.0, 50.0, 75.0, 100.0, 125.0 and 150.0 mg/L standard concentration calcium were prepared. According to their absorbance at 570 nm, a criteria linear regression

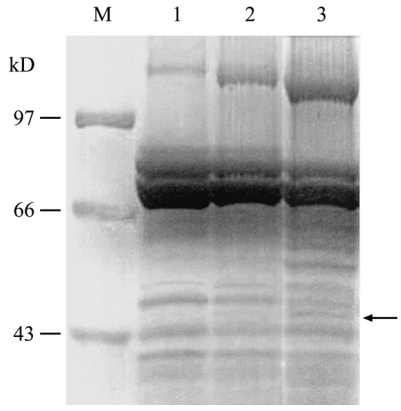


Fig. 5 SDS-PAGE of rh-OPG-372 protein expressed in *B. mori* larvae

M, protein molecular markers; 1, the larvae hemolymph infected with Bm-BacPAK6 and was collected on the 5th day; 2 and 3, the larvae hemolymph infected with Bm-BacPAK-OPG-372 and collected on the 4th and 5th day, respectively. The expressed recombinant protein band was marked by the arrow.

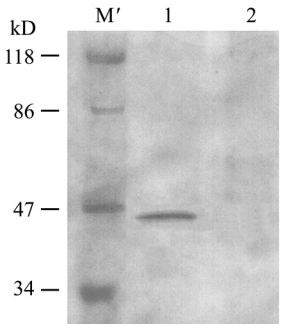


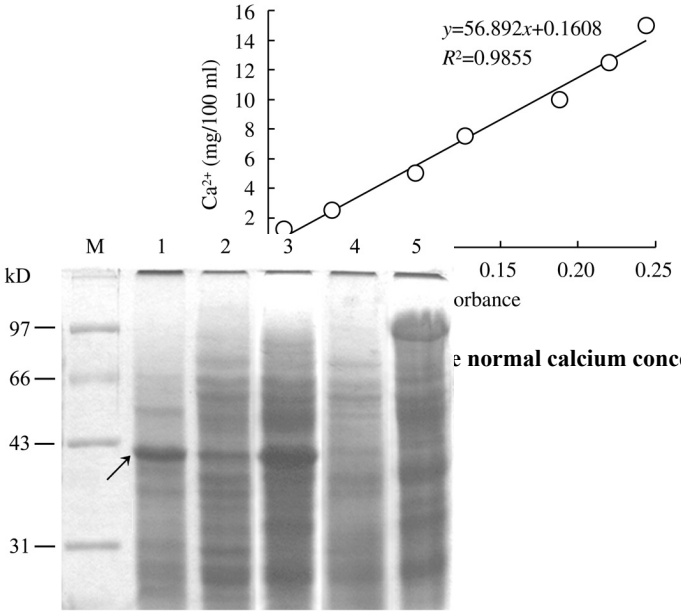
Fig. 6 Western blotting of rh-OPG-372 protein expressed in *B. mori* larvae

M', prestain protein molecular weight marker; 1, the larvae hemolymph infected with Bm-BacPAK-OPG-372; 2, the larvae hemolymph infected with Bm-BacPAK6.

curve for the detection of calcium was obtained (Fig. 7).

Dose-response of hypocalcemic effect The mice were injected with recombinant protein and then sera were collected 3h after injection for detecting the calcium concentration. The results showed that rh-OPG-372 were effective to decrease serum calcium concentration. There was a significant difference between the groups injected with 0.75 mg rh-OPG-372/kg and with hemolymph infected with Bm-BacPAK6 (P<0.01). There was no difference between the latter and the group injected with PBS (P>0.05). And the hypocalcemic effect became obvious with increasing doses of rh-OPG-372 (Fig. 8).

Time-course of hypocalcemic effect Each group of mice was injected with 6.0 mg rh-OPG-372/kg, and the calcium concentration of serum was detected after 1.5, 3, 5, 7 h injection, respectively. The results showed that the hypocalcemic effect was the most significant at 1.5 h. The calcium concentration ascended as time passed, and almost rose to the normal level after 7 h injection (Fig. 9).



e normal calcium concentration

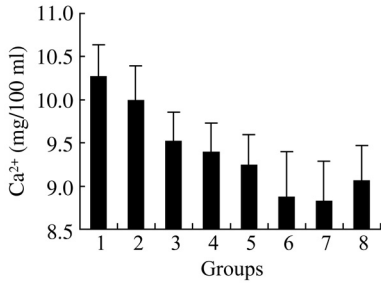


Fig. 8 Dose-response of hypocalcemic effect

1, mice sera Ca^{2+} 3 h after injection with PBS; 2, hemolymph infected with Bm-BacPAK6; 3, 0.75 mg rh-OPG-372/kg; 4, 1.5 mg rh-OPG-372/kg; 5, 3 mg rh-OPG-372/kg; 6, 6 mg rh-OPG-372/kg in larvae; 7, 100 mU sCT; 8, 6 mg rh-OPG-372/kg in cell.

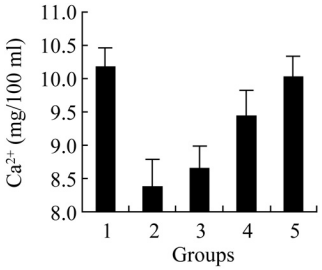


Fig. 9 Time-course of hypocalcemic effect

1, mice sera Ca^{2+} after injection with PBS for 1.5 h; 2–5, mice serum Ca^{2+} after injection with rh-OPG-372 for 1.5, 3, 5 and 7 h, respectively.

Discussion

The silkworm baculovirus expression system is an inexpensive high-level expression system, and this system has been used effectively for the expression of recombinant protein [7]. In this study, the rh-OPG-372 was expressed in BmN cells and silkworm larvae using the baculovirus expression system. The OPG molecular weight of unglycosylated 380-residue mature protein was about 40 kD, and the completely glycosylated protein was about 55–60 kD [1,8]. It was found that a recombinant protein around 42 kD was expressed in BmN cells and 46 kD in larvae, which implied that the recombinant protein expressed in cells and larvae maybe partly glycosylated. Hypocalcemic effect assay indicated that the recombinant protein expressed in cells decreased serum calcium concentration, however, the effect was gentler than that of recombinant protein expressed in larvae.

The morphogenesis and remodeling of bone depends on the integrated activity of osteoblasts that form bone and osteoclasts that resorb bone [9]. OPG was a soluble

protein that inhibited osteoclastic bone resorption both *in vitro* and *in vivo* [1,8,10–12], and decreased the concentration of serum calcium *in vivo* [2,13]. He *et al.* [4] first reported a novel mutated osteoprotegerin gene *OPG-372* and expressed the rh-OPG-372 in the insect cell (Tn-5B1-4) and yeast, and the rh-OPG-372 expressed in the insect cell proved to be effective to decrease serum calcium concentration in the normal mice. In order to make clear whether the rh-OPG-372 expressed in silkworm had the same hypocalcemic effect, mice were injected with the rh-OPG-372 and their sera were collected to detect the effect on calcium concentration. The results indicated that the recombinant protein had a significant hypocalcemic effect, and the effect was more and more obvious as the rh-OPG-372 dose increased. The time-course study showed that the hypocalcemic effect of the rh-OPG-372 became evident within 1.5 h and lasted over 5 h after injection. The rapid hypocalcemic effect, similar to that of calcitonin, could be best explained by assuming that the rh-OPG-372 maybe inhibit the function of mature osteoclasts [2].

In summary, the recombinant human OPG-372 was successfully expressed by silkworm baculovirus expression system, and the recombinant protein was effective to decrease serum calcium concentration in mice and could be a potential drug for prevention and treatment of hypercalcemia. OPG can specifically act on bone tissues, but future study is still necessary to determine the effect of bone resorption inhibition by recombinant OPG-372 expressed in silkworm.

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