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КРАТКИЕ СООБЩЕНИЯ

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HIGH LEVEL SECRETORY EXPRESSION OF MURINE OCIL BY CHO CELLS AND ACTION OF OCIL ON OSTEOCLAST DIFFERENTIATION

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Receptor activator of nuclear factor-kB ligand (RANKL), a well-known membrane-bound molecule expressed on osteoblasts and bone marrow stromal cells, is believed to induce osteoclast differentiation and activation by binding to the receptor activator of nuclear factor-kB (RANK), which is expressed on the surface of osteoclast lineage cells. This induction is inhibited by osteoprotegerin (OPG) that is secreted by osteoblast lineage and acts as a decoy receptor of RANKL. Currently the essential role of the OPG/RANKL/RANK system in the process of osteoclast maturation, as well as activation, has been well established, and the majority of bone resorption regulators control osteoclast formation and activation through their effects on this system and especially on the relative expression levels of RANKL and OPG [1].

Osteoclast inhibitory lectin (OCIL) is a novel protein recently identified in mouse osteoblastic cells [2, 3]. OCIL belongs to type II transmembrane molecules with a C-type lectin extracellular domain. Similar to the RANKL/OPG system, OCIL is highly expressed in osteoblasts and chondrocytes. Loss of OCIL leads to osteopenia in adult mice primarily as a result of increased osteoclast formation and/or decreased bone formation [4]. *In vitro* experiments demonstrated that OCIL dose-dependently inhibits multinucleated osteoclast formation from adherent murine spleen cells treated with RANKL and M-CSF. Such an effect relies on the predominant action of OCIL to inhibit the proliferation of mononuclear osteoclast precursors [2, 3, 5]. This strongly suggests that one of the actions of OCIL might be to oppose RANKL in the regulation of osteoclastogenesis. However OCIL has been implicated to act independently of the RANKL/OPG/RANK signaling pathway, because OCIL expression counteracts neither the expression of RANKL nor the RANKL/OPG ratio [2, 3]. Treatment of an osteoblast cell line with OCIL even resulted in an increase of the RANKL expression, but not OPG [6]. However, more recent evidence revealed a mild increase of RANKL in the primary calvarial cells of OCIL deficient mice [4].

The precise mechanism of OCIL action on osteoclastogenesis remains to be clarified. Thus preparation of the recombinant OCIL protein is of great importance for further investigations. Recombinant OCIL was previously prepared using the *Escherichia coli* system with a very low yield of active soluble protein, whereas the preferentially expressed insoluble inclusion bodies could not be refolded [2, 3]. In the current study we have developed a method to get a high expression level of *OCIL* gene in mammalian cells that displayed an improved activity. In this method a secretory eukaryotic expression plasmid, containing eight copies of the *mOCIL* expression cassette, was constructed. The secreted mOCIL protein was able to inhibit osteoclastogenesis *in vitro*. Further investigations suggested that recombinant OCIL may block osteoclastogenesis and osteoclast activity through down-regulation of the RANKL/OPG ratio and expression levels of several osteoclast-specific genes.

EXPERIMENTAL

To construct a secretory eukaryotic expression plasmid containing eight copies of the *mOCIL* expression cassette, we obtained the extracellular cDNA of the murine *OCIL* gene (*mOCIL*) by polymerase chain reaction (PCR) using a plasmid that contains the full length sequence of *mOCIL* cDNA as the template. Primers used were sense primer P1: 5'-*GACGCGGCCCATCACCATCATCACCAT*ACCTATGCTGCTTGC-3', and the antisense primer P2: 5'-ATC*CTCGAG*TTACTAGGAAGGAAAAAAAGGAG-3'. Italicized region in P1 shows the additionally introduced sequence,

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which encodes a 6×His tag (underlined). A XhoI recognition sequence was introduced into the P2 primer. The PCR cycling scheme included 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s in the presence of high-precision Pfu DNA polymerase ("TaKaRa", Japan).

Then a fusion gene named *sig-mOCIL* was amplified by adding a coding sequence for the signal peptide of the murine immunoglobulin kappa chain (Igκ$^+$) upstream of the *mOCIL* PCR product. In brief, the puried *mOCIL* PCR product and a synthesized DNA fragment named P3 were mixed and used as template. The sequence of P3 was 5'-ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTC TGGGTTCCAGGTTCCACTGGT*GACGCGGCCCATCACCAT*-3', in which the underlined region represents the signal peptide coding sequence, and the italicized region indicates the overlapped sequence with the shadowed part of primer P1.

The primers used for gene amplification were primers P2 and P4: 5'-GATAAGCTT*GCCACCATG*GAGACAGACACAC-3', in which the underlined part indicates the recognition sequences of HindIII followed by the highlighted Kozak sequence. PCR amplication comprised of 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min.

To obtain a multicopy insertion of a foreign gene, the eukaryotic expression vector pcDNA3.1(+) was mutated to create a BamHI recognition sequence followed by a EcoRV site behind the BGA polyadenylation sequence by using the Quikchange II XL site-directed mutagenesis kit ("Stratagene", USA). The produced vector contained a pair of isocaudomers at each side of the potential expression cassette, *ie.*, BglII, located upstream of the CMV promoter, and BamHI, located downstream of the transcrition terminator. Then the fusion gene *sig-mOCIL* was digested with HindIII and XhoI, and cloned into the identically treated pcDNA3.1(+) mutant, thus generating the construct harboring a single copy of the *mOCIL* expression cassette. Next the single copy construct was double digested with BglII and EcoRV and the released mOCIL expression cassette was purified and subsequently ligated with the one copy construct treated with BamHI and EcoRV. The newly obtained construct contained two tandem repeats of the forward orientated *mOCIL* expression cassette and the fusion site created by the adhesive ends of BglII and BamHI cleavage sites which could not be digested by either of the two enzymes. The above mentioned procedure was repeated two more times and the construct harboring eight copies of *mOCIL* expression cassette was produced.

In order to determine whether the eight copy construct works more efficiently in expressing recombinant protein than the single copy one, we transfected the constructs and the empty pcDNA3.1(+) mutant vector separately into the chinese hamster cells (CHO) using lipofectamine 2000. The cells stably expressing the constructs were selected in the presence of 600 μg/mL G418 in α-MEM medium containing 10% FBS. The cells were then cultured in CHO-serum free media ("Hyclone", USA) and the supernatants of the cell cultures were collected and concentrated using saturated ammonium sulfate precipitation followed by dialysis against PBS. Protein samples were fractionated by 15% SDS–PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 2 h with 5% nonfat milk, incubated overnight with a rabbit anti-6×His antibody (1:1,000 dilution; Cell Signaling Technology, USA), and then for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive bands were visualized using an ECL Western blotting substrate system. ("Pierce", USA).

Conditioned medium (CM) from CHO cells expressing the eight copy *mOCIL* construct was tested for its ability to inhibit OCL formation in murine bone marrow cultures treated with 10 nM parathyroid hormone related protein (PTHrP). Briefly, 2×10^5 murine bone marrow cells were cultured in the presence or absence of 10 nM PTHrP in a 96-well plate. The cultures were supplemented with 5% (vol/vol) of 48 h CM from CHO cells expressing the single copy *mOCIL* construct, eight copy *mOCIL* construct or the empty vector. Medium was changed at day 3 and day 5 to maintain the concentration of proteins in the medium. At day 7, the cultures were stained for tartrate-resitant acid phophatase (TRAP) activity using a commercially available staining kit ("Sigma", USA). TRAP positive multinucleated cells containing three or more nuclei were scored as osteoclast-like cells.

To identify the molecular mechanism involved in the regulation of osteoclastogenesis, total RNA was extracted from the cell cultures at day 3 with TRIZOL ("Invitrogen", USA) and then converted to cDNA using M-MLV reverse transcriptase ("Promega", USA). Real-time (RT) PCR reactions were performed to study the relative expression levels of *RANKL*, *OPG*, tartrate resistant acid phosphatase (*TRAP*), matrix metallopeptidase 9 (*MMP9*) and cathepsin K (*CTSK*). The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 10 s of denaturation at 95°C, 10 s of annealing at 58°C, and 10 s of extension at 72°C. β-actin was amplified as the internal control and the temperature cycling was the same as described above. *RANKL*, *OPG*, *TRAP*, *MMP9*, or cathepsin K amounts were normalized to that of b-actin in the RT sample. The used primers are listed in the table.

RESULTS AND DISCUSSIONS

It is well accepted that an ideal expression system for obtaining biologically active recombinant proteins are stably transfected mammalian cells. The advantag-

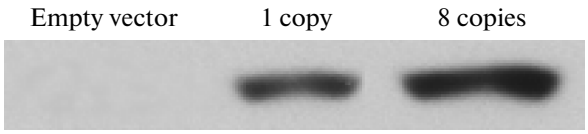

Fig. 1. The expression level of the eight copy construct relative to the single copy one analyzed by Western blotting. The procedures are described in the Experimental section.

es of this system include signal and propeptide cleavage, proper folding, N-linked glycosylation and dimerization, etc. Secretion of recombinant proteins would also favor purification since secreted proteins account for a very small part of the total proteins that are present in the whole cell. Since mouse *OCIL* gene does not encode a signal peptide, it is necessary to add an exogenous signal upstream of *mOCIL* for proper secretion.

A major disadvantage of mammalian cell expression systems is related to its low efficiency in producing recombinant proteins. To overcome this problem, several strategies have been employed. Some researchers confirmed that the presence of introns along with the target cDNA may enhance expression [7]. Gene amplification, through dihydrofolate reductase (dhfr)/methotrexate (MTX) selection, is another conventionally employed method to accomplish high level expression [8]. However, with such a strategy, long period of selection and screening for the high expression clones are required. Moreover, the cell lines obtained through a gene amplification procedure are often unstable when cultured in the absence of MTX. In the presented study we have developed a simple method with enhanced expression efficacy. We modified the eukaryotic expression vector pcDNA3.1(+) by introducing a BamHI reconition sequence and an EcoRV site behind the BGA polyadenylation sequence. The resulting vector contains the recognition sequences for the pair of isocaudomers, ie., BglII and BamHI, at each side of the potential expression cassette. Then the fusion gene sig-mOCIL was firstly cloned into this pcDNA3.1(+) mutant to generate the construct harboring a single copy of the *mOCIL* expression cassette. Next the single copy construct was double digested with BglII and EcoRV to release the *mOCIL* expression cassette, which was then ligated with the parent one copy construct digested with BamHI and EcoRV, thus generating the construct with two copies of the insert. After repeating this procedure for two more times, we obtained the construct harboring eight copies of the *mOCIL* expression cassette. It is possible to get more copies of the insert if more repeats of the procedure are done.

The expression level of the recombinant mOCIL protein secreted by CHO cells was analyzed by western blotting. Compared with CHO cells transfected with the single copy construct, the eight copy construct produced 3-times more of the recombinant mOCIL protein, indicating that the multi-copied construct, made by our strategy, works efficiently in mammalian cells (fig. 1).

Primers used for real-time PCR studies

Gene name	Primer sequence	PCR product, bp
RANKL	Fw: 5'-TCGCTCTGTTCCTGTACTTTC-3'	173
	Rev: 5'- AAAGGCTTGTTTCATCCTCC-3'	
OPG	Fw: 5'- GAGGACCACAATGAACAAGTG-3'	129
	Rev: 5'- AGGAGCTGATGACCAGTTTC-3'	
TRAP	Fw: 5'-GGTATGTGCTGGCTGGAAAC-3'	115
	Rev: 5'-TTGAAGCGCAAACGGTAGTAAG-3'	
MMP9	Fw: 5'-CCTGGAACTCACACGACATC-3'	114
	Rev: 5'-TCACCTCATGGTCCACCTTG-3'	
Cathepsin K	Fw: 5'-ACTGAGAATTATGGCTGTGG-3'	160
	Rev: 5'-TGTACCCTCTGCATTTAGCTG-3'	
β-actin	Fw: 5'-TCAGGTTACTGGTTCGGTCTG-3'	109
	Rev: 5'-ACCAGAGGCATACAGGGACAG-3'	

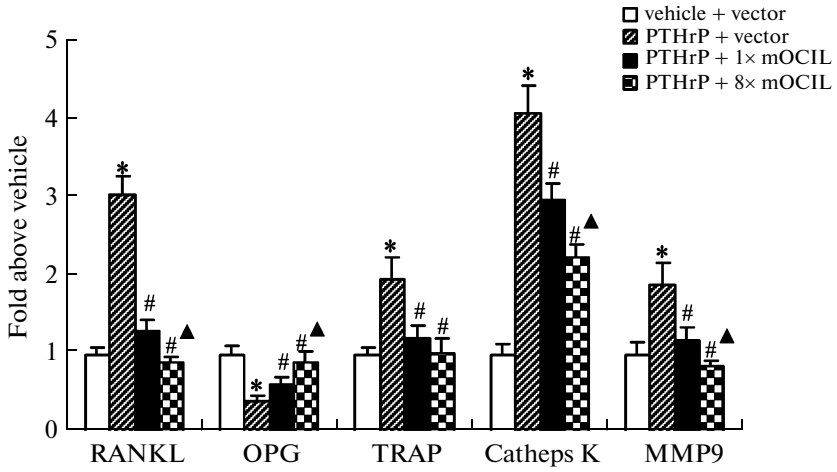

Fig. 2. Effects of the recombinant OCIL protein on the expression of RANKL, OPG, TRAP, cathepsin K and MMP9. *Signicant *vs.* vehicle + vector treatment, $P < 0.05$; # Signicant *vs.* PTHrP + vector treatment, $P < 0.05$; Signicant *vs.* PTHrP + 1× mOCIL treatment, $P < 0.05$.

Next we investigated the bioactivity of the recombinant OCIL by examining its effect on osteoclastogenesis. PTHrP potently stimulated the formation of TRAP-positive multinucleated osteoclast-like cells (OLC) in our culture system. When cultured in the presence of the CM from the CHO cells stably expressing the mOCIL construct, the number of the OLC cells was reduced by 65% (data not shown).

OCIL seems to be capable of acting independently of the RANKL/OPG system in regulating osteoclast formation [2, 3]. This was demonstrated by the inhibition of osteoclastogenesis by OCIL from adherent spleen cultures treated with RANKL and M-CSF. Such cultures lack osteoblasts, and as a consequence, their OPG levels are very low. It was hypothesized that OCIL directly acts through a receptor on osteoclast lineage cells that was not identified yet. OCIL treatment of an osteoblast cell line even resulted in an increase in expression of RANKL/ but not OPG, further supporting the above mentioned hypothesis and indicating the existence of the OCIL receptor on osteoblasts [6]. However, inconsistent with these results, the OCIL-null calvarial cells showed mild increase of RANKL expression [4].

In our present study, using a bone marrow culture system, we found that PTHrP stimulated RANKL expression by 3.1 fold and inhibited OPG expression by 61%, and the CM from multi-copy *mOCIL* transfected CHO cells completely reversed these effects. In contrast, CM from the single copy construct transfected CHO displayed less potency in inhibiting *RANKL* and inducing *OPG* expression. Unlike the spleen cell culture system, bone marrow cells are a mixture comprising the precursor cells of osteoblasts and osteoclasts. The RANKL : OPG ratio was downregulated by OCIL in the osteoblast lineage cells, suggesting that the role of OCIL in regulating osteoclastogenesis may probably involve regulation of the RANKL/OPG system. Moreover, we also examined the expression levels of some other genes including *TRAP*, *MMP9* and cathepsin K. Since TRAP is a well-known marker representing the activity of osteoclasts and the state of bone resorption, MMP9 and cathepsin K are two other specifically and highly expressed proteins in osteoclasts and play important roles in the degradation of extracellular matrix [9–11]. We presumed that these three genes may be involved in the inhibition of osteoclastogenesis by OCIL. As shown in fig. 2, after 3 days of treatment, PTHrP up-regulated mRNA expression of *TRAP*, cathepsin K and *MMP9* by 2, 4.1 and 1.9 times respectively. CM from multi-copy *mOCIL* transfected CHO completely blocked PTHrP-induced expression of TRAP and MMP9 and partially blunted cathepsin K expression by 60%. In contrast, CM from single copy *OCIL* transfected CHO displayed less potency in altering the expression of these genes. Taken together, our study demonstrates that OCIL inhibits osteoclastogenesis in bone marrow cells characterized by down-regulation of TRAP, cathepsin K and MMP9. This role of OCIL involves the regulation of RANKL/OPG in osteoblast lineage cells.

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