

## Bumetanide, the specific inhibitor of $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransport, inhibits $1\alpha,25$ -dihydroxyvitamin $\text{D}_3$ -induced osteoclastogenesis in a mouse co-culture system

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The  $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$  cotransporter (NKCC1) is responsible for ion transport across the secretory and absorptive epithelia, the regulation of cell volume, and possibly the modulation of cell growth and development. It has been reported that a variety of cells, including osteoblasts, contain this cotransporter. In this study, the physiological role of NKCC1 in osteoclastogenesis was exploited in a co-culture system. Bumetanide, a specific inhibitor of NKCC1, reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells. In order to investigate the mechanism by which bumetanide inhibits osteoclastogenesis, the mRNA expressions of the receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG) were analysed by RT-PCR. Exposure of osteoblastic cells to a medium containing  $1 \mu\text{M}$  bumetanide reduced RANKL mRNA expression induced by  $10 \text{ nM}$   $1\alpha,25$ -dihydroxyvitamin  $\text{D}_3$  ( $1\alpha,25(\text{OH})_2\text{D}_3$ ), in a dose-dependent manner. In addition, RANKL expression was also analysed with enzyme-linked immunosorbent assay (ELISA) using anti-RANKL antibody. The expression of RANKL was decreased with the increase of bumetanide concentration. In contrast, the expression of OPG mRNA, a novel tumour necrosis factor (TNF) receptor family member was increased in the presence of bumetanide. These results imply that bumetanide inhibits osteoclast differentiation by reducing the RANKL/OPG ratio in osteoblastic cells. However, no significant difference in M-CSF mRNA expression was observed when bumetanide was added. Also, we found that the phosphorylation of c-Jun  $\text{NH}_2$ -terminal kinase (JNK), which regulates the activity of various transcriptional factors, was reduced by bumetanide treatment. Conclusively, these findings suggest that NKCC1 in osteoblasts has a pivotal role in  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis partly via the phosphorylation of JNK. *Experimental Physiology* (2003) **88.5**, 569–574.

Bone remodelling is regulated by the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Both osteoblasts and osteoclasts are regulated by a variety of hormones and local factors (Fuller *et al.* 1998; Lacey *et al.* 1998; Burgess *et al.* 1999; Kong *et al.* 1999). Osteoblasts stem from mesenchymal stem cells, whereas osteoclasts arise by the differentiation of osteoclast precursors of monocyte/macrophage lineage. Osteoblasts and osteoclasts

are required not only for skeletal development, but also for mineral homeostasis and the normal remodelling of bone (Raise, 1998). An imbalance between bone formation and bone resorption causes metabolic bone diseases like osteopetrosis and osteoporosis (Aubin, 1998). Therefore, osteoblasts and osteoclasts are known to be closely related during the process of remodelling (Suda *et al.* 1995; Tsukii *et al.* 1998; Takahashi *et al.* 1999).

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Certain kinds of signalling molecules, such as, the receptor activator of NF- $\kappa$ B ligand (RANKL), osteoprotegerin (OPG) and macrophage colony-stimulating factor (M-CSF), expressed by osteoblasts, are involved in osteoclastogenesis and in osteoclast development. For instance, when osteoblasts/stromal cells are stimulated by osteotropic factors such as parathyroid hormone, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to the receptor activator of NF- $\kappa$ B (RANK; also known as ODF receptor) (Jimi *et al.* 1999). In addition, M-CSF is known to be essential for macrophages to be transformed into osteoclasts, while OPG, a decoy receptor of RANKL, is known to participate in the regulation of osteoclastogenesis (Lacey *et al.* 1998). Specifically, OPG, as a member of the tumour necrosis factor receptor (TNFR) family, inhibits the osteoclastogenesis stimulated by  $1\alpha,25(\text{OH})_2\text{D}_3$ , parathyroid hormone (PTH), or interleukin-11 (IL-11) (Takahashi *et al.* 1999). Consequently, it is believed that RANKL, M-CSF and OPG, which are expressed by osteoblasts, are associated with osteoclastogenesis, and that osteoblasts are a major factor in the bone remodelling process.

With respect to osteoclastogenesis, we have focused on the function of the osteoblast, because it has been reported that the  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransporter (NKCC1) is present in osteoblasts (Whisenant *et al.* 1991). Presumably, in osteoblasts NKCC1 may be involved in the bone remodelling process. In general, NKCC1 is present in numerous and diverse tissues in a wide variety of animal species, and is associated with the regulation of cell volume, the maintenance of ionic gradients across the membrane, and possibly the modulation of cell growth and development (Haas & Forbush, 1998). Apart from some knowledge of the general functions of NKCC1 in a variety of cells, there is no experimental evidence as to whether NKCC1 activity in osteoblasts is related to osteoclastogenesis. Therefore, we hypothesized that NKCC1 in osteoblasts might concern osteoclastogenesis with respect to RANKL, OPG and M-CSF mRNA expression. In this study, we applied an osteoblast/stromal cell co-culture system to evaluate the effect of bumetanide, a specific inhibitor of NKCC1, on  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclast formation, mRNA expression profiles in RANKL, OPG and M-CSF, and changes in total JNK (t-JNK) and phosphorylated JNK (p-JNK).

## METHODS

### Materials

Routine cell culture media were obtained from Gibco BRL (Grand Island, NY, USA). The tartrate-resistant acid phosphatase staining kit and bumetanide were purchased from Sigma Chemical Co. (St Louis, MO, USA). Trizol was purchased from Invitrogen Corp. (Carlsbad, CA, USA), and the ICR mice were from Samtacho Co. (Seoul, Korea). All other chemicals were of the highest grade commercially available.

### *In vitro* osteoclast formation assay

The osteoblast formation assay was carried out as previously reported by Choi *et al.* (2001). Briefly, the osteoblasts were

isolated from 1- to 2-day-old mice which had been killed by cervical dislocation. Thirty to fifty calvariae were digested in 10 ml of an enzyme solution containing 0.2% collagenase (Wako, Japan) and 0.1% dispase (Gibco) for 20 min at 37°C in a shaking water bath. The supernatant was discarded and 10 ml of the enzyme solution was added. After shaking at 37°C for 20 min, the supernatant was collected carefully and transferred to a new tube. This digestion of calvariae by collagenase–dispase was repeated three times. The collected supernatant (30 ml) was placed in a centrifuge at 1500 g for 10 min, to collect the osteoblastic cells. Cells were resuspended in  $\alpha$ -minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and cultured to confluence in 10 cm culture dishes at a concentration of 105 cells per dish. The cells were then detached from the culture dishes using trypsin-EDTA, suspended in  $\alpha$ -MEM with 10% FCS and used for the co-culture as osteoblastic cells.

Femoral and tibial bone marrow cells, as well as calvariae, were collected from 4-week-old mice which had been killed by cervical dislocation. The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavities flushed by slowly injecting media at one end using a 25-gauge needle. The calvariae and bone marrow cells collected were washed and used in the co-culture.

Mouse calvarial cells ( $1 \times 10^4$  cells well<sup>-1</sup>) were co-cultured with bone marrow cells ( $1 \times 10^5$  cells well<sup>-1</sup>) in  $\alpha$ -MEM containing 10% fetal calf serum in 48-well plates (Corning). The culture volume was made up to 400  $\mu$ l per well with  $\alpha$ -MEM supplemented with 10% fetal calf serum (FCS), in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$  (10 nM), with or without bumetanide (1, 10, or 100  $\mu$ M). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. After incubation for 4 days, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, an osteoclast marker enzyme) staining. *In vitro* osteoclast formation assay was repeated four times.

### Viability test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test is based on the principle that tetrazolium salts are reduced by mitochondrial enzymes (succinate dehydrogenase) and this allows the toxicity of viable cells and the level of cellular differentiation to be measured. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg ml<sup>-1</sup> and filtered to remove any insoluble residue. MTT solution was added directly to the assay plates. The cells were subsequently incubated for an additional 4 h at 37°C. The purple formazan crystals that formed were dissolved in DMSO, and the plates were read on a spectrophotometer (MRX; Dynatech Laboratories, USA) at 570 nm. This assay was repeated four times.

### RT-PCR

The expressions of RANKL, OPG, M-CSF and  $\beta$ -actin (as a positive control) were evaluated by RT-PCR using total RNA isolated from murine osteoblastic cells. Total RNA was isolated using Trizol. The primers used were: for RANKL (750 bp), 5'-ATCAGAAGACAGCACTCACT-3' (forward), 5'-ATCTAG-GACATCCATGCTAATGTTC-3' (reverse); for OPG (636 bp), 5'-TGAGTGTGAGGAAGGGCGTTAC-3' (forward), 5'-TTC-CTCGTCTCTCAATCTC-3' (reverse); for M-CSF, 5'-ATG-ACAAGGCCTGCGTCCGA-3' (forward), 5'-AAGCTCTGG-CAGGTGCTCCTG-3' (reverse, 395 bp) and 5'-GCCGCC-TCCACCTGTAGAACA-3' (reverse, 286 bp); and for  $\beta$ -actin (366 bp), 5'-GGACTCCTATGGTGGGTGACGAGG-3' (forward) and 5'-GGGAGCATAGCCCTCGTAGAT-3' (reverse). PCR was repeated four times.

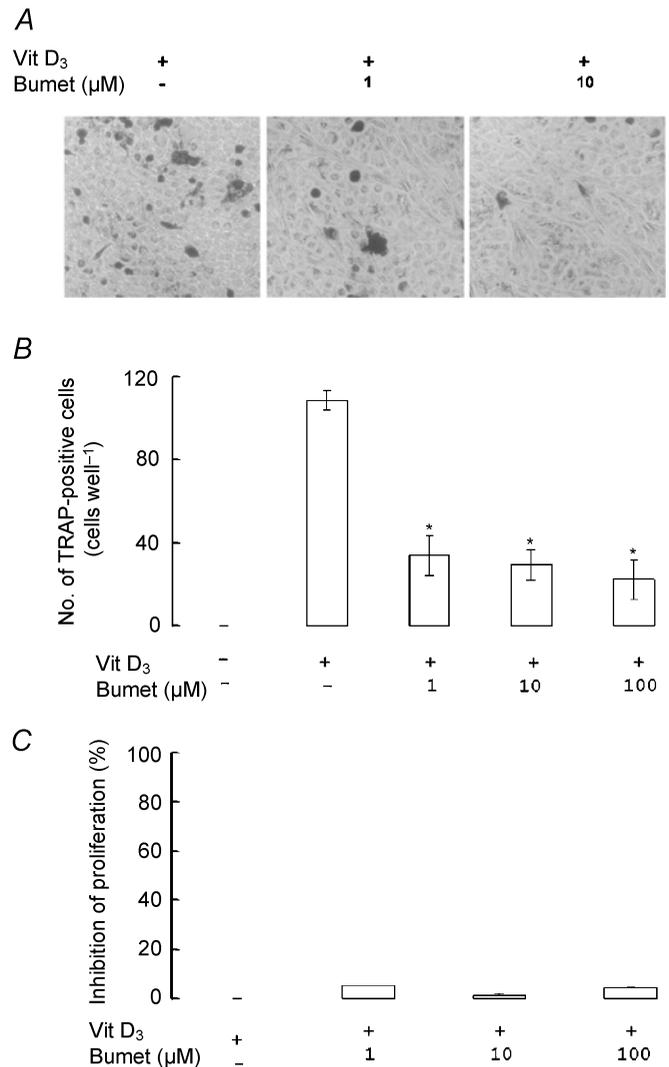
Relative RT-PCR was performed to measure gene expression of RANKL, OPG and  $\beta$ -actin mRNAs. Polymerase chain reactions were performed on a Tgradient 96 PCR machine (Whatman Biometra GmbH, Göttingen, Germany) using ~100 ng of cDNA, 5 pmol of each oligonucleotide primer, 200  $\mu$ M of each dNTP, 1 unit of *Taq* polymerase (Applied Biosystems, CA, USA) and 10  $\times$  *Taq* polymerase buffer in a 50  $\mu$ l volume. The PCR program initially started with a 95 °C denaturation for 5 min, followed by 25–38 cycles of 95 °C for 1 min,  $T_a$  for 1 min ( $T_a$ , annealing temperature: 45.3 °C for RANKL, 47.9 °C for OPG, 56 °C for M-CSF, and 58 °C for  $\beta$ -actin). Linear amplification range for each gene was tested on the adjusted cDNA. The less expressed transcripts of RANKL and OPG required 35 cycles of PCR for detection. For M-CSF and  $\beta$ -actin, 30 and 25 cycles of PCR was performed, respectively. The PCR samples were electrophoresed on 1% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The gels were stained with ethidium bromide (10  $\mu$ g ml<sup>-1</sup>) and photographed on top of a 280 nm UV light box. The quantity and base pair size of the PCR-generated DNA fragments were estimated relative to DNA ladder standards. Densitometry values were measured at each cycle sampling using the TINA software (University of Manchester, UK). RT-PCR values are presented as a ratio of the specified gene's signal in the selected linear amplification cycle divided by the  $\beta$ -actin signal.

#### Western blot

Protein extracts were prepared from osteoblastic cells as follows. Osteoblastic cells were resuspended in pre-warmed cracking buffer (80 mM urea, 0.05% SDS, 0.4% 10 mM Tris-HCl, 1 mM EDTA, 40  $\mu$ g ml<sup>-1</sup> bromophenol blue, 10  $\mu$ l ml<sup>-1</sup>  $\beta$ -mercaptoethanol, 70  $\mu$ l ml<sup>-1</sup> protease inhibitor cocktail (Sigma P8340) and 50  $\mu$ l 100  $\times$  PMSF (100 mM), at 60 °C), and transferred to a tube containing glass beads (425–600  $\mu$ m; Sigma). After disruption with glass beads, the cell re-suspensions were lysed by heating at 70 °C for 10 min followed by vigorous vortexing for 1 min. The lysates were separated from unbroken cells by centrifugation at 13 400 *g* for 5 min. For Western blot, the protein extracts were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). The membrane was incubated in a blocking solution containing 4% bovine serum albumin (BSA) and 10% normal goat serum (NGS) in phosphate-buffered saline containing 0.15% of Tween-20 (PBS-T) at room temperature for 2 h. The blot was then probed with the monoclonal antibody of p-JNK or t-JNK (Clontech) as primary antibody overnight at 4 °C, and detected with horseradish peroxidase-linked goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) and an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). This Western blot analysis was repeated four times.

#### ELISA

Quantikine M Mouse RANK ligand immunoassay kit (R & D systems Inc., Minneapolis, IN, USA) was used to analyse RANKL expression. Briefly, mRANKL was diluted in calibrator diluent RD6-12 solution to make final concentrations of 0, 31.2, 62.5, 125, 250, 500, 1000, and 2000 pg ml<sup>-1</sup>. Assay diluent RD1W and standards (50  $\mu$ l each) were added to each well and incubated for 2 h at room temperature. A plate was provided to record standards and samples assayed. Each well was aspirated and washed, repeating the process four times for a total of five washes. Mouse RANKL conjugate (100  $\mu$ l) was added to each well and incubated for 2 h at room temperature. Aspiration/washing was repeated as described above. Substrate solution (100  $\mu$ l) was added to each well and incubated for 30 min at



**Figure 1**

Inhibition of osteoclast differentiation by bumetanide. Bumetanide was added to osteoblast/bone marrow co-culture with  $1\alpha,25(\text{OH})_2\text{D}_3$ . After incubation for 4 days, cells were stained using the TRAP staining method and TRAP-positive multinucleated cells that had more than three nuclei were counted. *A*, tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts. Osteoblast/bone marrow co-culture was treated with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  and/or 10  $\mu$ M bumetanide (Bumet). Culture conditions were described in Methods. *B*, the number of TRAP-positive multinucleated cells was decreased by bumetanide. \* Significant difference from the  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated level ( $P < 0.05$ ). *C*, bumetanide did not affect the viability of the co-cultured cells up to 100  $\mu$ M concentration. The statistical significances of differences between the groups were determined using Kruskal-Wallis and Bonferroni tests. Data are shown as means  $\pm$  S.E.M.

room temperature in the dark. Stop solution (100  $\mu$ l) was added to each well and mixed by gentle tapping. The optical density of each well was determined within 30 min, using a microplate reader at 450 nm.

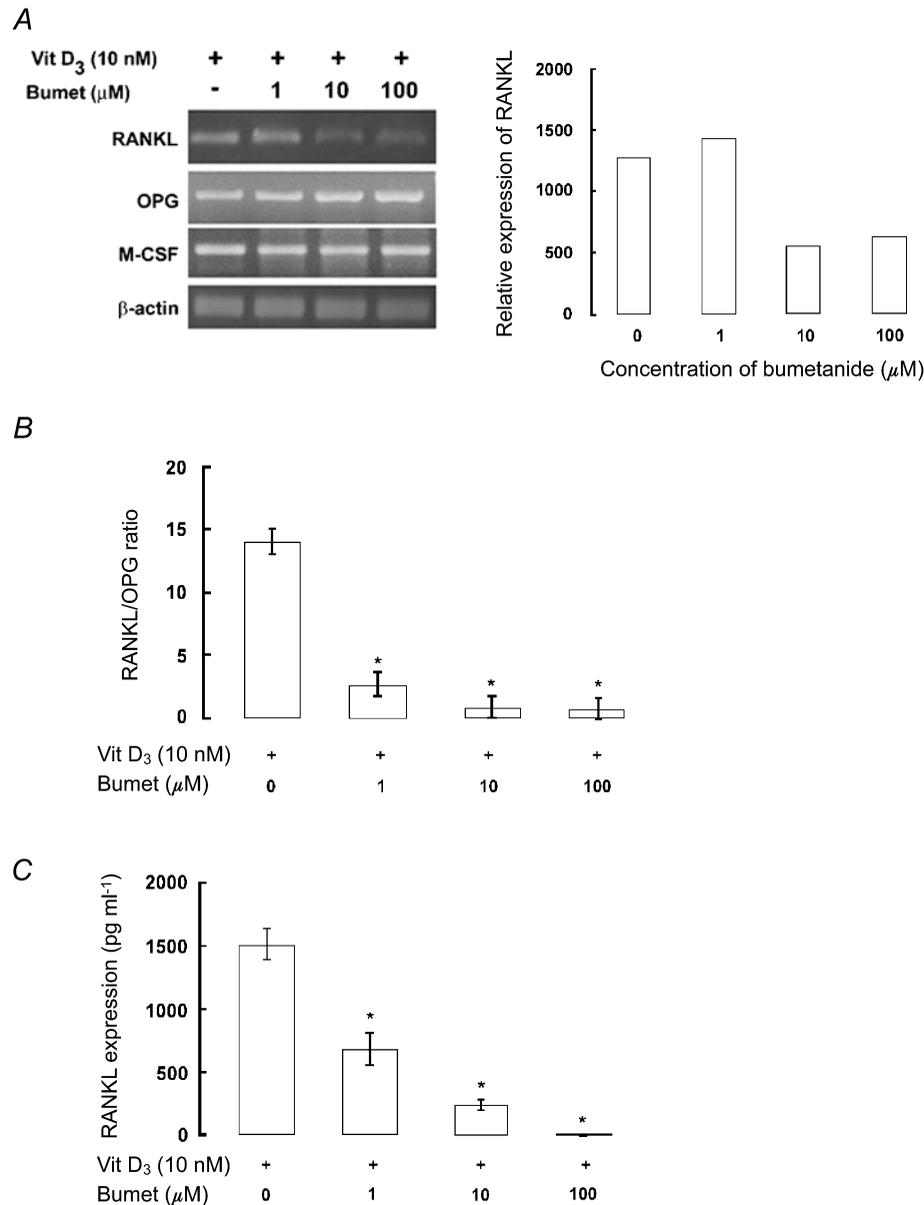
#### Data analysis and statistics

The results are expressed as the mean  $\pm$  S.E.M. The statistical significances of differences between the groups were determined using the Kruskal-Wallis and Bonferroni test. In statistical tests, the  $P$  value  $< 0.05$  was considered to be significant.

## RESULTS

### Bumetanide inhibits $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclast formation

Osteoclastogenesis was induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  in osteoblastic cells/bone marrow co-culture. To inhibit NKCC1 in osteoblasts, 1, 10, or 100  $\mu\text{M}$  of bumetanide was added to co-cultures and incubated at 37°C for 4 days to investigate osteoclast differentiation. When



**Figure 2**

Effect of bumetanide on mRNA expressions of RANKL, OPG and M-CSF in osteoblastic cells. Various concentrations of bumetanide (Bumet) were added to the osteoblast culture with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ . After incubation for 4 days, total RNA was isolated. *A*, expression profile of mRNAs (left panel) and densitometry of RANKL expression (right panel) in osteoblastic cells. *B*, ratio of RANKL and OPG mRNA expression. RNAs were amplified according to the method described in Methods. Relative ratio of RANKL and OPG was calculated based on the density of each band and expressed as mean  $\pm$  S.E.M. *C*, ELISA of RANKL expression. Quantikine M Mouse RANK ligand kit was used to analyse RANKL expression. The assay was carried out according to the manufacturer's instructions. \* Significant difference from the  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated level ( $P < 0.05$ ).

10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  was added to the co-culture,  $111 \pm 4.2$  TRAP-positive multinucleated cells were formed, whereas no TRAP-positive cells were detected in media only. In the presence of bumetanide (10  $\mu\text{M}$ ),  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclast differentiation was reduced (Fig. 1A). The addition of 1, 10 or 100  $\mu\text{M}$  bumetanide reduced the number of TRAP-positive multinucleated cells to  $34 \pm 6.8$ ,  $29 \pm 5.1$ , or  $23 \pm 6.8$  cells per well, respectively (Fig. 1B). However, it might be possible that bumetanide causes cell damage directly without interrupting the normal maturation of osteoclasts. To investigate this possibility we used a viability test. As shown in Fig. 1C, bumetanide did not show a toxic effect when treated at up to 100  $\mu\text{M}$ . These results suggest that the effect of bumetanide on differentiation was caused by a change in the NKCC1 activity, not by its direct toxic effect upon the cells.

#### NKCC1 inhibition caused changes in RANKL and OPG mRNA expression during osteoclastogenesis

As shown in Fig. 2, the expressions of RANKL, OPG and M-CSF in osteoblasts were monitored by RT-PCR in the presence and absence of bumetanide. As the bumetanide concentrations in the cell culture medium were increased, the  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced expression of RANKL mRNA was down-regulated (Fig. 2A). RANKL expression in osteoblasts was inversely proportional to bumetanide concentration. In contrast, the expression of OPG mRNA increased as a function of bumetanide concentration. These findings indicate that bumetanide inhibits osteoclast differentiation by down-regulating the expression of RANKL mRNA and enhancing OPG mRNA expression in osteoblasts. However, the mRNA expression of M-CSF, an osteoclast survival factor, was unchanged throughout the range of bumetanide concentrations (1, 10 and 100  $\mu\text{M}$ ) examined. The ratio of RANKL to OPG mRNA in osteoblast is illustrated in Fig. 2B. As the bumetanide concentration was increased, the ratio of RANKL to OPG mRNA decreased, which means that the inhibition of NKCC1 activity caused changes in the mRNA expression of the signalling molecules, RANKL and OPG, which are closely linked to osteoclastogenesis.

RANKL expression was also analysed with ELISA using anti-RANKL antibody (Fig. 2C). The expression of RANKL was decreased with the increase of bumetanide concentration. In experiments here, OPG expression was not assayed because no remarkable change in OPG mRNA expression was observed compared to that of RANKL expression.

Consequently, bumetanide inhibited osteoclastogenesis, and led to altered RANKL and OPG mRNA expressions. In addition, such changes in the expression levels of signalling molecules were dependent on the bumetanide concentration.

#### Phosphorylation of JNK was suppressed by NKCC1 inhibition

We have demonstrated how the activity of NKCC1 is linked to the RANKL mRNA expression in osteoblastic cells. Because JNK in general is preferentially activated by cytokines (Gravallese *et al.* 2001), and cellular stress and

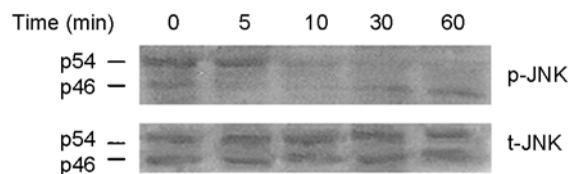
plays a key role in regulating the activity of various transcriptional factors (Anning, 2002), the changes in JNK phosphorylation in osteoblastic cells were examined to investigate the role of NKCC1 on RANKL mRNA expression. Osteoblastic cells were treated with bumetanide and incubated for 60 min. Accordingly, the inhibition of NKCC1 by bumetanide resulted in the inhibition of JNK phosphorylation in a dose-dependent manner, while no significant change in the total expression of JNK (t-JNK) was observed (Fig. 3). These findings suggest that the activity of NKCC1 in osteoblasts is closely associated with RANKL mRNA expression via JNK phosphorylation.

## DISCUSSION

It has been reported that NKCC1 is present in osteoblasts (Whisenant *et al.* 1991). However, it is not known what NKCC1 does during osteoclastogenesis. Therefore, we hypothesized that NKCC1 might be closely involved in osteoclastogenesis, based upon the facts that (1) NKCC1 is present in osteoblasts, (2) NKCC1 is crucial to the regulation of the cell size and volume, which acts as a signal for various cellular functions, and (3) NKCC1 affects the development and growth of the cells such as PC12 and NIH3T3 (Russell, 2000). In the present study, the involvement of NKCC1 in osteoclastogenesis was investigated by inhibiting NKCC1 activity using bumetanide. Interestingly, we found that bumetanide inhibited the osteoclastogenesis (by up to 65% of the control) in a co-culture system (Fig. 1). Bumetanide inhibited  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis.

Nevertheless, it could be argued that the inhibition of osteoclastogenesis by bumetanide might be directly due to the cell damage, and not to the physiological intervention of bumetanide in the normal process of osteoclastogenesis. To rule out the possibility that bumetanide causes non-physiological cell damage, an MTT viability test was performed. The test showed that bumetanide did not exert any harmful effects upon the cells in this co-culture system, which suggests that bumetanide inhibits the formation of TRAP-positive cells, without being toxic to the cells.

Since osteoclast differentiation is mediated by several factors, such as RANKL, OPG and M-CSF (Fuller *et al.*



**Figure 3**

Effect of bumetanide on the phosphorylation of JNK. Osteoblastic cells were treated with 10  $\mu\text{M}$  bumetanide for the indicated time. Anti-phospho-JNK or anti-JNK antibody was used as a primary antibody for the Western blot.

1998; Lacey *et al.* 1998; Burgess *et al.* 1999; Kong *et al.* 1999), we used an osteoblast/stromal cell co-culture system to evaluate whether alterations in NKCC1 activity affect the mRNA expression profiles of RANKL, OPG and M-CSF. RANKL mRNA expression, induced by  $1\alpha,25(\text{OH})_2\text{D}_3$ , was down-regulated upon increasing the bumetanide concentration, and the expression of OPG mRNA increased. In addition, the expression of RANKL was decreased with bumetanide concentration in the process of  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis, being consistent with the decrease in RANKL mRNA expression. These findings indicate that bumetanide inhibits osteoclast differentiation by reducing the RANKL/OPG ratio. On the basis of such findings it might be postulated that NKCC1 activity is in part associated with osteoclastogenesis, that is, by modulating RANKL and OPG expression in osteoblasts.

As previously described, the inhibition of NKCC1 reduced the cellular uptake of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$ , and this results in cellular volume change. O'Donnell *et al.* (1995) demonstrated that endothelial cell volume is also determined by the integrated activities of kinases and phosphatases. In particular, JNK, one of the mitogen-activated protein kinase (MAPK) families, has been reported to be activated by hyperosmolarity and cellular volume changes (O'Donnell *et al.* 1995). These findings suggest that JNK is associated with osteoclastogenesis via osmotic volume change. In the present study, we have shown that bumetanide, which is widely used as a diuretic and as a specific inhibitor of NKCC1, inhibits osteoclastogenesis. Therefore, we need to determine the correlation between JNK and NKCC1 during osteoclastogenesis. Here, Western blot analysis of total and phosphorylated JNK showed that bumetanide did not affect the expression of t-JNK, which remained constant throughout the range of bumetanide concentrations used. However, the activated form of JNK, i.e. p-JNK, reduced as the bumetanide concentration was increased. Understanding the exact nature of down-regulation of osteoclastogenesis by the inhibition of NKCC1 requires further studies at the level of the osteoclast.

In summary, we have provided the first evidence that in osteoblastic cells NKCC1 plays a pivotal role in  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclastogenesis, via the phosphorylation of JNK which regulates the activity of various transcriptional factors.

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