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Macrophage colony-stimulating factor promotes the survival of osteoclast precursors by up-regulating Bcl-X_L

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Abbreviations: M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; ODF, osteoclast differentiation factor; OPGL, osteoprotegerin ligand; TRANCE, TNF-related activation-induced cytokine; TRAP, tartrate-resistant acid phosphatase; PBS, phosphate buffered saline; RT, reverse transcription

Abstract

Macrophage colony-stimulating factor (M-CSF) is known as one of the factors essential for osteoclast development. In the present study, we examined effects of M-CSF on the apoptotic pathway of osteoclast precursors and their underlying molecular mechanisms. Osteoclast precursors underwent apoptosis in the absence of M-CSF, even in the presence of receptor activator of NF-κB ligand (RANKL). Active caspase-3 and -9 were detected in the osteoclast precursors and treatments of precursors with their specific inhibitors (Z-DEVD-FMK and Z-LEHD-FMK) decreased the apoptosis. M-CSF decreased apoptosis in a dose-dependent manner with decreasing in active caspases-3 and -9 levels and up-regulating Bcl-X_L. Those effects of M-CSF on inhibiting apoptosis of osteoclasts precursor by regulating anti-apoptotic signals was more effective when combined with RANKL. These results demonstrate that M-CSF acts as a survival factor for the osteoclast precursors. Furthermore, it is believed that the apoptosis of osteoclast precursors may be involved in the activation of caspase-9 and that M-CSF may promote their survival through Bcl-X_L-induced inhibition of caspase-9 activation.

Keywords: apoptosis, bone and bones, caspases, macrophage colony-stimulating factor, osteoclast

Introduction

Osteoclasts are derived from hematopoietic stem cells of the monocyte-macrophage lineage, which are terminally differentiated into multi-nucleated osteoclasts and activated to resorb hard tissue (Roodman, 1999). The differentiation and activation of osteoclasts are supported by osteoblasts and bone marrow stromal cells through expressions of two critical factors, macrophage colony-stimulating factor (M-CSF, also named as CSF-1) and receptor activator NF-κB ligand [RANKL, also named as osteoclast differentiation factor (ODF), Osteoprotegerin ligand (OPGL), and TNF-related activation-induced cytokine (TRANCE)]; Takahashi *et al*., 1999; Tsurukai *et al*., 2000). The osteopetrotic *op/op* mice that produce non-functional M-CSF clarified the role of M-CSF on osteoclast development (Yoshida *et al*., 1990). Adult *op/op* mice showed severe osteopetrosis due to an absence of osteoclasts, and injections of M-CSF were found to ameliorate skeletal sclerosis in these mice (Umeda *et al*., 1996). Further *in vitro* studies revealed that M-CSF affected the formation, chemotaxis and survival of mature osteoclasts (Fuller *et al*., 1993; Amano *et al*., 1998; Jimi *et al*., 1999; Udagawa *et al*., 1999) as well as the proliferation and differentiation of osteoclast precursors (Takahashi *et al*., 1991; Tanaka *et al*., 1993; Tsurukai *et al*., 1998). After the administration of M-CSF in *op/op* mice, the numbers of osteoclasts and osteoclast precursors [tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells] increased, while these cells were not formed from the cells that proliferated in response to M-CSF (Umeda *et al*., 1996). Without M-CSF, most of the peripheral blood mononuclear cells disappeared during the culture process, even in the presence of sRANKL (Quinn *et al*., 1998). Moreover, much has yet to be elucidated on its underlying molecular mechanism. In these osteoclast precursors we surveyed which pathway is involved in the apoptosis of the osteoclast precursors and examined the effects of M-CSF on the pathway. We demonstrated that M-CSF acts as a survival factor for osteoclast precursors, and believe that the apoptosis of osteoclast precursors may be involved in the

activation of caspase-9 and that M-CSF may promote their survival through the Bcl-X_L-induced inhibition of caspase-9 activation.

Materials and Methods

Culture of primary osteoclast precursors

Primary osteoclast precursors were isolated with a slight modification from the methods designed for isolation of avian osteoclasts (Oursler *et al*., 1991). The long bones were aseptically removed from 5-week female mice (ICR) and the soft tissues were removed. The marrow was then flushed out using a 22 gauge needle, and the bones were placed in ice-cold Hank's balanced salt solution (HBSS) and minced into small pieces, which were digested five times with enzyme solution [1 mg/ml type II collagenase (GibcoBRL, Gaithersburg, MD), 0.05% trypsin (GibcoBRL), and 4 mM EDTA (GibcoBRL) in HBSS] for 15 min each time to remove cells other than the osteoclast precursors. After enzyme digestion, the bone pieces were washed three times with HBSS and transferred to a 50 ml conical tube with ice-cold alpha-modified minimal essential medium (alpha-MEM). After 15 min, the bone pieces were shaken vigorously for 1 min to collect the osteoclast precursors and the cell suspension was passed through 40 μm cell strainer (Falcon, Lincoln Park, NJ). After centrifugation at 200 *g*, the cell pellets were resuspended in alpha-MEM containing 10% fetal bovine serum, and cultured in the presence or absence of M-CSF (Peprotech, London, England) or soluble RANKL (sRANKL, Peprotech). In some experiments, caspase-3 inhibitor, Z-DEVD-FMK (Calbiochem, Cambridge, MA) and caspase-9 inhibitor, Z-LEHD- FMK (Calbiochem) was also added to the culture.

TRAP staining

The freshly isolated cells were smeared on a microscopic slide and the cultured cells were fixed and stained for TRAP using a commercial staining kit (No. 387, Sigma Chemical Co, St. Louis, MO) according to the manufacturer's instructions.

Reverse transcription (RT)-PCR

Total RNAs were extracted from freshly isolated or cultured cells in the presence of M-CSF (20 ng/ml) and sRANKL (30 ng/ml) for two or seven days using a RNeasy kit (Qiagen, Hilden, Germany). First strand cDNAs were generated by SuperScript II reverse transcriptase (GibcoBRL) in 20 μl from 1 μg of total RNAs. One μl was subjected to PCR and the cycling condition used was 94°C, 30 s; 62°C, 30 s; and 72°C, 60 s for 30-40 cycles. The primers used for PCR were as follows: 5'-GCTCAGATGAGACTTTG-3' and 5'-ATCAACAATGAGCTGGA-3' for beta 3 integrin; 5'-GTGAAAAGGCGGAATCT-3' and 5'-AGGAACATGTGCTTGTG-3' for calcitonin receptor (CTR); 5'-GGTTGTGGTGGTTGTTGTTG-3' and 5'-TCTAGACAGTGAGCGACATC-3' for c-*kit*; 5'-GAAGAAATATGTGCGCAGGG-3' and 5'-AGACTTGGTGTTCACTAGCA-3' for c-*fms*. The primers used for RANK were as described previously by others (Lean *et al*., 2000). Actin served as a control and its primers were 5'-ATGTGCAAGGCCGGCTTCGCGGGC-3' and 5'-GATGTCCACGTCACACTTCATGAT-3'.

Apoptosis assay

Apoptosis was analyzed by DNA fragmentation assay and cell death detection ELISA. Cellular DNAs were extracted from osteoclast precursors that had been cultured for 24 h in the absence or presence of M-CSF and sRANKL. The floating and attached cells were pooled and incubated in a lysis buffer (200 mM Tris-HCl, pH 8.3; 100 mM EDTA; 1% SDS; 50 μg/ml RNase A; and 50 μg/ml proteinase K). After incubation at 37°C for 4 h in the lysis buffer, the cellular DNAs were extracted from the lysates with phenol/chloroform. Four μg of DNAs was electrophoresed in a 1.5% agarose gel with molecular size markers (a 100-bp DNA ladder, Fermentas, Lithuania). To determine apoptosis quantitatively, we performed the assay using a cell death detection ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. One million cells of the isolated precursors were plated onto a 6-well plate and cultured for 24 h in the absence or presence of M-CSF (2, 20, or 200 ng/ml) or sRANKL (30 ng/ml). After incubation, the plates that contained both floating and attached cells were centrifuged at 200 *g* for 5 min. Cells were lysed with 5 ml of incubation buffer in the kit, and the lysates diluted 1:10 and subjected to ELISA.

Western blot analysis

Isolated osteoclast precursors were cultured for 24 h in the presence or absence of M-CSF (20 ng/ml) and sRANKL (30 ng/ml). The cultured cells, both floating and attached, were washed with ice-cold phosphate-buffered saline (PBS: 150 mM NaCl, 9.1 mM Na_2HPO_4, and 1.7 mM NaH_2PO_4, pH 7.4), and incubated in a lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) on ice for 15 min. Lysates were then passed through a 22-gauge needle and centrifuged at 14,000 *g* at 4°C for 20 min. Twenty μg of proteins were separated on a 10% SDS-polyacrylamide gel under reducing conditions, transferred to a PVDF membrane (Sigma), and blotted with anti-

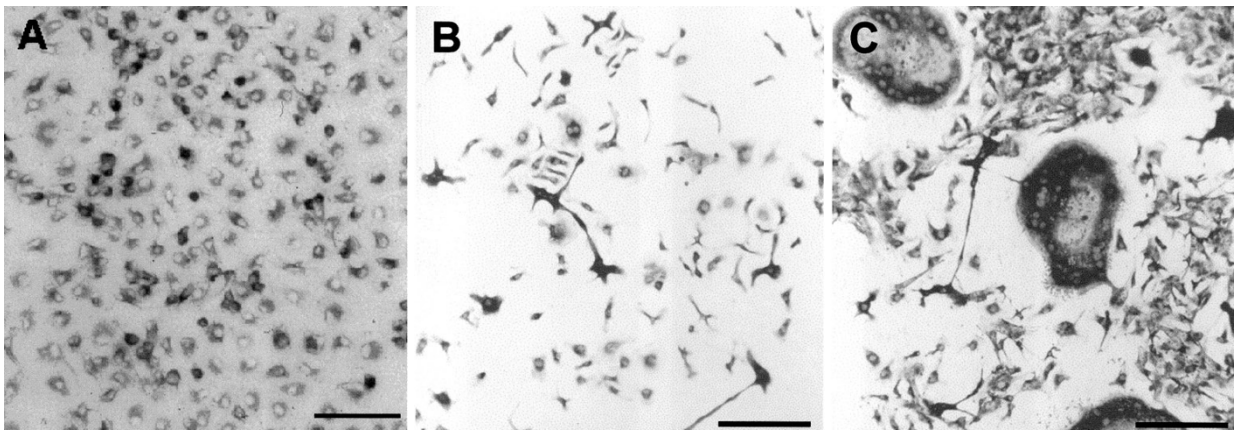

Figure 1. Osteoclast precursors isolated from mouse long bones. TRAP-staining views of freshly isolated cells (A) and cells cultured in the presence of M-CSF (20 ng/ml) and sRANKL (30 ng/ml) for two days (B) and seven days (C). The freshly isolated precursors were stained evenly and weakly for TRAP. Cells cultured for two days remained TRAP-positive and mononuclear and those cultured for seven days were differentiated into multinucleated mature osteoclasts. Bars, 50 μm.

bodies (polyclonal anti-mouse caspase-3, -9, -8, -10, Bcl-2, and Bcl-X_L antibodies purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Alpha-tubulin (its antibody was purchased from Cedarlane, Hornby, Canada) served as a control for the protein loading. The relative densities to alpha-tubulin were analyzed with an Imagemaster (Amersham Pharmacia, Piscataway, NJ).

Results

Isolation of highly pure osteoclast precursors

The purity of the isolated cells was assessed by TRAP staining. About 96% of the isolated cells were mononuclear and TRAP-positive (Figure 1A). Treatment with M-CSF (20 ng/ml) and sRANKL (30 ng/ml) induced differentiation of precursors to mature osteoclast. Two-day cultures with M-CSF and RANKL induced more intensely TRAP-stained cells, but cells remained mononuclear (Figure 1B). Numerous multinucleated TRAP-positive cells were obtained after seven days of culture (Figure 1C). Phenotypes of the freshly isolated osteoclast precursors observed by RT-PCR were β_3-integrin(-)/CTR(-)/c-*kit*(-)/c-*fms*(+)/RANK(+). Transcripts of β_3-integrin and CTR were detected in two-day cultures, and most osteoclast markers were strongly expressed in the seven- day cultures (Figure 2).

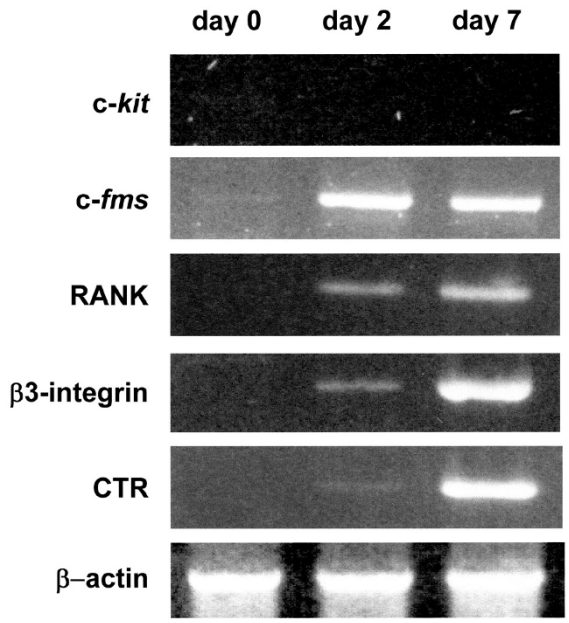

Figure 2. Expressions of osteoclast markers during the differentiation of isolated precursors induced by M-CSF and sRANKL. RT-PCR was performed on freshly isolated cells (day 0) and cells cultured for two (day 2) and seven days (day 7) in the presence of M-CSF (20 ng/ml) and sRANKL (30 ng/ml). PCR products were fractionated in a 1.5% agarose gel and stained with ethidium bromide. A representative result of three independent experiments is shown.

Rescue of osteoclast precursors from apoptotic cell death by M-CSF

DNA extracted from the cells cultured with M-CSF and sRANKL did not show fragmentation, whereas cells cultured in the absence of M-CSF showed the characteristic 'ladder' pattern of DNA fragmentation, consisting of multimers of approximately 180 base pairs (Figure 3A). After treatment with M-CSF (20 ng/ml), the DNA fragmentation pattern disappeared. About 50-60% of the isolated osteoclast precursors underwent apoptotic cell death within 24 h. M-CSF inhibited this apoptotic cell death in a dose-dependent

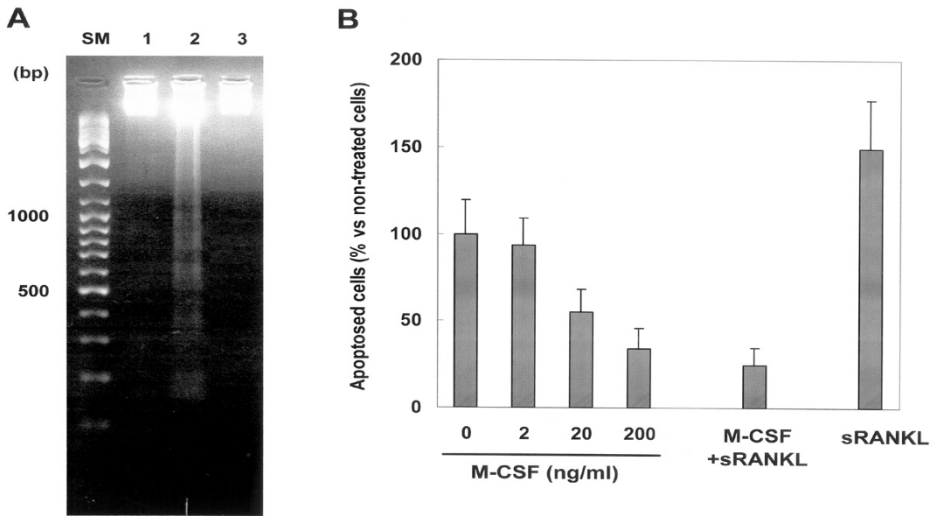

Figure 3. Effect of M-CSF on the apoptosis of osteoclast precursors. (A) Apoptotic features of cells isolated in the absence of M-CSF. Four micrograms of each DNA sample extracted from M-CSF plus sRANKL-treated (lane 1), non-treated (lane 2), and M-CSF-treated (lane 3) cells. Cells without treatment showed a characteristic 'ladder' pattern of DNA fragmentation, while those treated with M-CSF plus sRANKL did not show typical DNA frgmentation pattern. The addition of M-CSF resulted in the disappearance of the DNA fragmentation pattern. (B) Quantitative analysis for apoptosis by cell death detection ELISA. Isolated precursors were cultured for 24 h in the absence or presence of M-CSF (2, 20, 200 ng/ml) or sRANKL (30 ng/ml). Both the floating and the attached cells were pooled and their lysates subjected to ELISA. The results from two independent experiments were pooled and the results shown represent the mean ± SD, n=6. SM, size marker

manner, but could not rescue the cells completely (Figure 3B). Notably, M-CSF and sRANKL (20 and 30 ng/ml, respectively) profoundly inhibited apoptosis, though 30 ng/ml of sRANKL alone increased the apoptotic cell death. In addition to cell death detection ELISA, the number of apoptotic cells that showed typical nuclear fragmentation was counted using Hoechst fluorochrome staining, and similar results were obtained (data not shown). From these results, we conclude that M-CSF may partially rescue osteoclast precursors from apoptotic cell death.

Caspase-9 in the apoptosis of osteoclast precursors and the up-regulation of Bcl-X_L by M-CSF

We examined the apoptotic pathway of osteoclast precursors and the effects of M-CSF on the pathway by Western blot analysis for caspase-3, -9, -8, -10, and Bcl-2, and Bcl-X_L (Figure 4). Caspase-3, one of apoptotic executioners, was first examined. Procaspase-3 was detected at similar levels in all three groups, namely; the non-treated, M-CSF (20 ng/ml)-treated, and the M-CSF (20 ng/ml) plus sRANKL (30 ng/ml)-treated groups, and the level of active caspase-3 was found to be notably lower in the cells treated with M-CSF alone or with M-CSF and sRANKL in combination. We then examined the apoptotic initiators, caspase-8, -9, and -10, which converge on the activation of caspase-3. Procaspase-8 and its active form were not expressed in all groups, but the

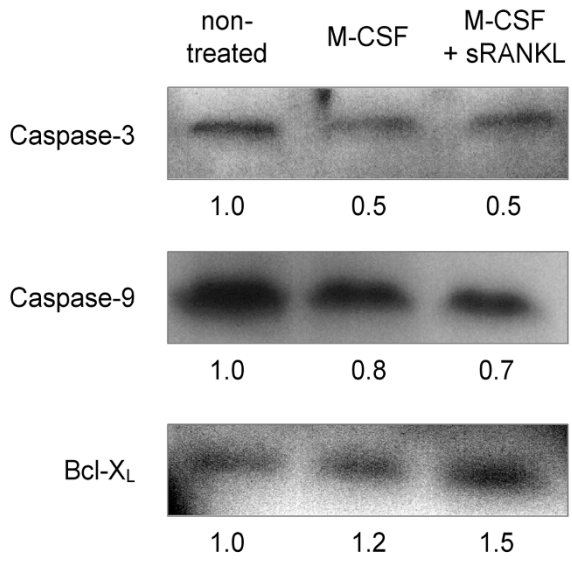

Figure 4. Western blot analysis for caspase-3, caspase-9, and Bcl-X_L in the isolated osteoclast precursors. The isolated precursors were cultured in the presence of M-CSF (20 ng/ml) or sRANKL (30 ng/ml) for 24 h. Floating and attached cells were pooled and their lysates were analyzed by Western blotting. A representative result of two independent experiments is shown. Numbers under the bands indicates the results of densitometric analysis.

active form of caspase-9 was lower in cells treated with M-CSF alone or in combination with sRANKL

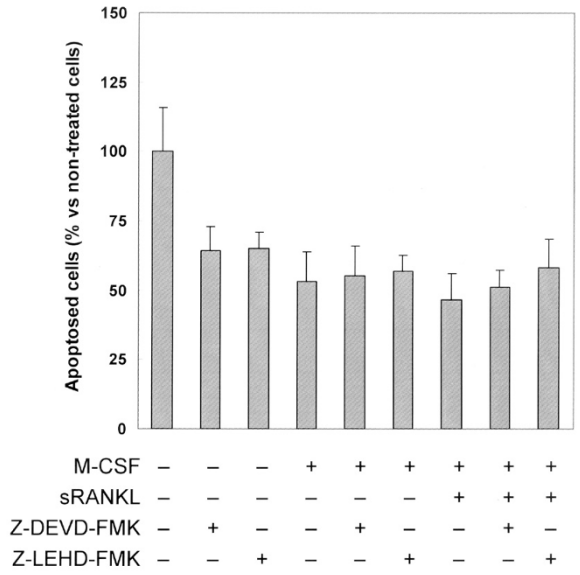

Figure 5. Effects of caspase inhibitors on the apoptosis of osteoclast precursors. The isolated precursors were cultured for 24 h in the presence of caspase-3 inhibitor, Z-DEVD-FMK (100 μM) or caspase-9 inhibitor, Z-LEHD-FMK (100 μM), in addition to M-CSF (20 ng/ml) and sRANKL (30 ng/ml). Floating and attached cells were pooled and their lysates subjected to the ELISA. The results from two independent experiments were combined and the results shown represent the mean±SD, *n*=6.

than in control, while similar amounts of procaspase-9 were expressed in all groups. Procaspase-10 was expressed at similar levels in all groups, but its active form was not detected. Furthermore, caspase-3 and caspase-9 inhibitors partially reversed the apoptosis of osteoclast precursors (Figure 5). Interestingly, apoptosis was inhibited to a similar extent by the caspase inhibitors alone or when combined with M-CSF. These results suggest that withdrawal of M-CSF caused the apoptosis *via* caspase-9 and then caspase-3 activation. Finally, we examined Bcl-2 and Bcl-X_L, the inhibitory modulators of procaspase-9 cleavage. Bcl-X_L was found to be up-regulated by treatment with M-CSF alone and when combined with sRANKL, while Bcl-2 was not detected at any time. M-CSF combined with sRANKL was more effective at regulating anti-apoptotic signals than M-CSF alone.

Discussion

About half of the isolated osteoclast precursors underwent apoptotic cell death within 24 h. This apoptosis is believed to be partly due to M-CSF withdrawal, because treatment with M-CSF inhibited the apoptosis in a dose-dependent manner, however, the inhibitory effect of M-CSF did not lead to complete rescue. Up to 20% of the isolated osteoclast precursors could not be rescued from apoptotic cell death by high doses of M-CSF or M-CSF combined with RANKL. Because the cells were treated with enzymes for isolation of osteoclast precursors, surface protein expression, which is involved in cell adhesion, in cells might have been hampered. In view of the fact that normal cells usually require adhesion to extracellular matrix for survival (Gilmore *et al*., 2000), the apoptosis of isolated precursor cells may have been due in part to the loss of cell adhesion. This possibility is supported by a report that M-CSF did not protect floating osteoclasts from the apoptosis (Sakai *et al*., 2000).

It is known that various forms of cellular stress including the withdrawal of growth factors induce apoptosis by triggering mitochondrial release of cytochrome c, which binds to Apaf1, which in turn self-associates and binds procaspase-9 (Green, 1998; Wolf and Green, 1999). Bcl-X_L inhibits apoptosis by blocking these interactions (Hu *et al*., 1998). Transactivation of the complexed procaspase-9 to active caspase-9 follows, and this caspase-9 then activates downstream caspase-3. We observed that active caspase-3 and caspase-9 were detected in the osteoclast precursors underwent apoptosis, and that caspases-3 and -9 specific inhibitors significantly reversed the apoptosis. These results suggest that activation of caspase-9 and then caspase-3 may be involved in the apoptosis of osteoclast precursors. In mature osteoclasts, the involvement of caspase-3 has been consistently noted, while the involvement of caspase-9 is somewhat controversial (Okahashi *et al*., 1998; Kanaoka *et al*., 2000; Lacey *et al*., 2000). Treatment of M-CSF decreased the levels of active caspase-3 and -9, and up-regulated the expression of Bcl-X_L in osteoclast precursors, suggesting that M-CSF induced cell survival may occur through the Bcl-X_L-affected inhibition of caspase-9 activation. The importance of Bcl-X_L in the survival of osteoclast precursors was observed during the immortalization of osteoclast precursors where Bcl-X_L and SV40 large T antigen were targeted in the osteoclast lineage (Hentunen *et al*., 1998). Furthermore, in macrophages that have common progenitors with osteoclasts, M-CSF induced the expression of Bcl-X_L through Ets2 transcriptional factor (Sevilla *et al*., 1999; Smith *et al*., 2000), which strongly support our suggestion that M-CSF induces Bcl-X_L in osteoclast precursors.

M-CSF combined with sRANKL inhibited the apoptotic cell death more effectively than M-CSF alone. We observed that the freshly isolated precursor cells expressed small amounts of RANK and that this expression of RANK was increased by M-CSF (data not shown). Others have also reported M-CSF-induced RANK expression in osteoclast precursor (Arai *et al*., 1999). These observations suggest that M-CSF

combined with sRANKL may augment the survival of osteoclast precursors, permitting them to respond to the external cue, RANKL, for their differentiation. Interestingly, sRANKL in the absence of M-CSF promoted apoptotic cell death. Recently, Lacey and coworkers (2000) reported similar result in mature osteoclasts, however, little is known on the underlying mechanism.

In summary, we demonstrated that activation of caspase-9 might be involved in the apoptosis of osteoclast precursors. And M-CSF acts as a survival factor for osteoclast precursors through Bcl-X_L-induced inhibition of caspase-9 activation.

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