Anti-apoptotic Molecule Bcl-2 Regulates the Differentiation, Activation, and Survival of Both Osteoblasts and Osteoclasts*

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The anti-apoptotic molecule Bcl-2 inhibits apoptosis by preventing cytochrome c release from mitochondria. Although several studies have indicated the importance of Bcl-2 in maintaining skeletal integrity, the detailed cellular and molecular mechanisms remain elusive. Bcl-2−/− mice are growth-retarded and exhibit increased bone volume of the primary spongiosa, mainly due to the decreased number and dysfunction of osteoclasts. Osteoblast function is also impaired in Bcl-2−/− mice. Ex vivo studies on osteoblasts and osteoclasts showed that Bcl-2 promoted the differentiation, activation, and survival of both cell types. Because Bcl-2−/− mice die before 6 weeks of age due to renal failure and cannot be compared with adult wild type mice, we generated Bcl-2−/−/Bim−/− mice, in which a single Bim allele was inactivated, and compared them with their Bcl-2−/−/Bim−/+ littermates. Loss of a single Bim allele restored normal osteoclast function in Bcl-2−/− mice but did not restore the impaired function of osteoblasts, and the mice exhibited osteopenia. These data demonstrate that Bcl-2 promotes the differentiation, activity, and survival of both osteoblasts and osteoclasts. The balance between Bcl-2 and Bim regulates osteoclast apoptosis and function, whereas other pro-apoptotic members are important for osteoclasts.

Skeletal tissue homeostasis is the result of a delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts (1–3). Osteoclasts are multinucleated giant cells primarily responsible for bone resorption and die rapidly by apoptosis after finishing their task. The majority of osteoclasts that are assembled at the remodeling site also die by apoptosis. The remaining osteoclasts are converted to lining cells that cover quiescent bone surfaces or are embedded within the mineralized matrix as osteocytes (4). Several studies have revealed that the apoptosis of osteoblasts and osteoclasts is strictly regulated and plays important roles in maintaining skeletal integrity (5–8). However, the molecular events implicated in osteoblast and osteoclast apoptosis have not been fully elucidated.

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Apoptosis is essential for the development of metazoans and is crucial for the maintenance of cellular homeostasis in mammals. Defects in apoptosis are involved in cancer, autoimmune diseases, and degenerative disorders (9). Apoptosis is a form of programmed cell death that is characterized by specific morphological and biochemical features and is tightly regulated by extracellular stimuli and intracellular signaling pathways (10–13). Morphological features characteristic of apoptosis include condensation of the cytoplasm and the nuclei, which leads to marked cell shrinkage. Other hallmarks include internucleosomal cleavage of chromatin into oligonucleosome-sized fragments and blebbing of the plasma membrane. The Bcl-2 family of proteins regulates the “mitochondrial” pathway to apoptosis. Bcl-2 (B-cell lymphoma-2) was originally identified as an oncogene leading to the development of B-cell lymphoma (14, 15). It is mainly expressed in hematopoietic cells, thymus, liver, kidney, neurons, melanocytes, gonads, and bones (16–18). It resides on the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope and inhibits apoptosis by suppressing cytochrome c release from mitochondria (19, 20). The Bcl-2 family consists of more than 30 members, which can be either anti-apoptotic proteins like Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1 or pro-apoptotic (21). The pro-apoptotic family members are divided into two subgroups as follows: the multidomain members (Bax, Bak, etc.) and the Bcl homology 3 domain-only members (Bid, Bad, Bim, Bik, Puma, Noxa, etc.). Although the Bcl homology 3 domain-only family members display tissue-specific distribution patterns, the multidomain pro-apoptotic members are ubiquitously expressed.

On a C57BL/6 genetic background, Bcl-2−/− mice appear normal at birth, but they soon develop polycystic kidney disease, fail to thrive, and most die before reaching 6 weeks of age (18, 22–25). Bcl-2−/− mice are also profoundly lymphopenic and turn gray at the second hair follicle cycle due to the excessive death of melanocyte stem cells (18, 24, 26). In skeletal tissues, Bcl-2 is expressed in osteoblasts, osteoclasts, chondrocytes, and osteocytes (27, 28). Several studies have demonstrated the importance of Bcl-2 in maintaining skeletal integrity (27, 29–31), but the molecular mechanism of action of Bcl-2 in osteoblasts and osteoclasts has not been fully elucidated. In this study, we have analyzed Bcl-2−/− mice and discovered an essential role for the anti-apoptotic Bcl-2 protein in bone-form-
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ing osteoblasts and bone-resorbing osteoclasts, both in vivo and in vitro.

**EXPERIMENTAL PROCEDURES**

*Expression Constructs and Gene Transduction*—For retrovirus construction, full-length cDNA of the mouse Bcl-2 gene was amplified by PCR, subcloned into pCR-TOPO II vectors (Invitrogen), and inserted into pMx vectors (32). 2×10^6 BOSC23 packaging cells were transfected with 2 μg of the vectors using FuGENE (Roche Applied Science). 24 h later, the medium was replaced with fresh α-MEM, 10% FBS, and cells were incubated for an additional 24 h. The supernatant was then collected as retroviral stock after centrifugation at 3,000 rpm for 5 min. 5×10^6 bone marrow macrophages (BMMs) were incubated with 8 ml of retroviral stock for 6 h in the presence of Polybrene (6 μg/ml) and recombinant mouse M-CSF (30 ng/ml). After 6 h of retroviral infection, the medium was changed to α-MEM, 10% FBS, and M-CSF (100 ng/ml), and cells were cultured for an additional 48 h. BMMs were recovered with trypsin, and puromycin-resistant cells were selected by incubation with α-MEM, 10% FBS containing 2 μg/ml puromycin for 2 days and used for further experiments.

*Real Time PCR*—Total RNA was extracted with ISOGEN (Wako Pure Chemical), and an aliquot (1 μg) was reverse-transcribed using a QuantiTect® reverse transcription kit (Qiagen) to produce single-stranded cDNA. PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) using QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer’s instructions. All reactions were performed in triplicate. After data collection, the relative mRNA copy number of a specific gene was calculated with a standard curve generated with serially diluted plasmids containing PCR amplicon sequences, and normalized to rodent total RNA with mouse β-actin serving as an internal control. Standard plasmids were synthesized with a TOPO TA cloning kit (Invitrogen), according to the manufacturer’s instruction.

** Primer Information**—Each primer sequence of mouse target genes is described as follows: NFATc1, 5′-TCCGAGAATCG-AGATCACCT-3′ and 5′-AGGGGTCTGTGTAAGGTTCC-3′; α1 type I collagen, 5′-ACGTCTGGTGAGATTGGTG-3′ and 5′-CAGGGAAGGCTCTTTCTCCT-3′; alkaline phosphatase, 5′-GCTGATCATTTCCACGGTTT-3′ and 5′-CTGGGCTGGATGTGTGGTT-3′; osteocalcin, 5′-AAGCAGGA-GGGCAATTAGGGT-3′ and 5′-TTTGTAGGGCTCCTCAGC-3′; and β-actin, 5′-AGATGGGATCCAGCAACG-3′ and 5′-GCCGAAATGGTTTGTCA-3′.

*Western Blotting*—Cells were washed with ice-cold phosphate-buffered saline, and proteins were extracted at 4°C with TNE buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 2 mM Na_2VO_4, 10 mM NaF, and 10 μg/ml aprotinin). For Western blotting analysis, lysates were subjected to SDS-PAGE with 7.5–15% Tris-glycine gradient gel or 15% Tris-glycine gel and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 6% milk/TBS-T, membranes were incubated with primary antibodies to Bcl-2 (Pharmingen), activated caspase-3 (Cell Signaling Technology), or β-actin (Sigma), followed by horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Promega). Immunoreactive bands were visualized with ECL Plus (Amer sham Biosciences), according to the manufacturer’s instructions. The blots were stripped by incubating for 20 min in stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.7)) at 50°C and reprobed with other antibodies.

*Animals*—The Bcl-2^-/- and Bim^-/+ mice, which were backcrossed with C57BL/6 mice for more than 10 generations, were generated as reported previously (25). Genotyping of the mice was performed as described previously (23). WT and Bcl-2^-/- mice were generated from the intercross between heterozygous mice. All experiments were performed on 2–3-week-old male mice unless otherwise noted, according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

*Radiological Analyses*—Plain radiographs were taken using a soft x-ray apparatus (CMB-2, SOFTEX), and bone mineral density was measured by dual energy x-ray absorptiometry using a bone mineral analyzer (PiXimus Densitometer, GE Medical Systems). Computed tomography scanning of the femurs was performed using a composite x-ray analyzer (NX-CP-C80H-IL, Nittetsu ELEX Co.) and reconstructed into a three-dimensional feature image by the volume-rendering method (VIP-Station, Teijin System Technology).

*Histological Analyses*—For toluidine blue staining, samples were fixed with 70% ethanol, embedded in methyl methacrylate, and sectioned in 6-μm slices. Histomorphometric analysis was performed from 0.15 mm below the growth plate to a 0.6-mm length of the primary spongosia of the proximal tibiae in WT and Bcl-2^-/- mice. For double labeling, mice were injected subcutaneously with 16 mg/kg body weight of calcine at 3 days and 1 day before sacrifice. TRAP-positive cells were stained at pH 5.0 in the presence of L(+)-tartrate acid using naphthol AS-MX phosphate (Sigma) in N,N-dimethyl formamide as the substrate. Apoptotic osteoblasts were detected by the TUNEL method using the In Situ Cell Death Detection kit, POD (Roche Applied Science), on paraffin-embedded sections of 3-week-old mice.

*Generation of Osteoclasts and Survival/Bone Resorption Assay*—Bone marrow cells were obtained from the femur and tibia of 2-week-old C57BL/6 mice, and BMMs were cultured in α-MEM (Invitrogen) containing 10% FBS (Sigma) in the presence of 100 ng/ml M-CSF (R&D Systems) for 2 days. Osteoclasts were generated by stimulating BMMs with 10 ng/ml M-CSF and 100 ng/ml RANKL (Wako Pure Chemical) for an additional 4–5 days or by the co-culture system established by Takahashi et al. (33). In the TRAP solution assay (TRAP activity), osteoclast precursors were placed (4×10^4 cells per well) in a 96-well plate. After RANKL stimulation, the cells were incubated for the indicated periods. After cells were washed with phosphate-buffered saline, they were lysed by adding 150 μl of 1% Triton X-100 in TRAP buffer to each well and incubated at 37°C for 30 min. TRAP activity was measured using the TRAP assay kit (Sigma).
4 °C for 1 h. Cell lysates (30 μl) were transferred to new 96-well plates, and 100 μl of substrate solution, containing 30 mg of p-nitrophenyl phosphate in 10 ml of TRAP buffer, was added to each well and incubated for 1 h at 37 °C. After incubation, 70 μl of 1 M NaOH was added to stop the enzymatic reaction. Absorbance was measured at 405 nm using a microplate reader (model SH-8000 lab; Corona). Survival and bone resorption assays of osteoclasts were carried out as reported previously (34). Briefly, after cytokine withdrawal, osteoclasts were incubated for 12 h. The osteoclast survival rate was represented as the percentage of morphologically intact TRAP-positive multinucleated cells. Actin ring formation was examined using rhodamine-phalloidin staining. In short, cells were first stained for TRAP to identify osteoclasts and then incubated for 30 min with rhodamine-conjugated phalloidin solution (Molecular Probes, Eugene, OR). The actin rings formed by osteoclasts were detected with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). For bone resorption assays, osteoclasts were cultured on dentine slices for 24 h, and the resorption areas were visualized by staining with 1% toluidine blue and measured using an image analysis system (Microanalyzer).

Osteoblastic Cell Cultures and Assays—For osteoblast cultures, calvaria of neonatal mice were digested five times with 0.1% bacterial collagenase and 0.2% dispase in α-MEM, and the last three cell fractions were combined and cultured as primary osteoblasts in α-MEM containing 10% FBS. Apoptotic osteoblasts were detected by TUNEL methods using the In Situ Cell Death Detection kit, POD (Roche Applied Science). Alkaline phosphatase staining was performed as follows. After osteoblasts were inoculated at a density of 1 × 10^5 cells/well in a 12-multwell plate in α-MEM containing 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate (Sigma), cells were fixed with 10% formalin solution for 15 min. Cells were stained by fast blue BB (Sigma) using naphthol AS-MX phosphate (Sigma) in N,N-dimethylformamide as the substrate. Von Kossa staining was performed as follows. After osteoblasts were incubated for 3 weeks, cells were fixed with 100% ethanol for

FIGURE 1. Three-week-old Bcl-2^{−/−} mice have increased primary spongiosa due to both decreased number and dysfunctionality of osteoclasts. A, radiographic analysis of wild type (WT) and Bcl-2^{−/−} mice at 3 weeks of age. The arrows indicate the sclerotic regions at the metaphysis. Bar, 10 mm. B, histological section of tibiae of WT and Bcl-2^{−/−} mice (hematoxylin and eosin, von Kossa, and TRAP staining). The standardized regions of interest in the metaphyseal region are indicated by boxes. C, analysis of tibiae of WT and Bcl-2^{−/−} mice. Cancellous bone volume (BV/TV) is the percent of total marrow area (including trabeculae) occupied by cancellous bone. Trabecular thickness (Tb.Th) is the mean distance across individual trabeculae in micrometers. Trabecular number (Tb.N) per mm is calculated as (BV/TV)/Tb.Th. These variables can be used to evaluate trabecular connectivity. Eroded surface (ES/BS) is the percent of cancellous surface occupied by Howship’s lacunae, with and without osteoclasts. Osteoblast surface (Ob.S/BS) and osteoclast surface (Oc.S/BS) identify the percent of cancellous surface occupied by osteoblasts and osteoclasts, respectively. Osteoid surface (Osteoid S/BS) is the percent of cancellous surface with unmineralized osteoid, with and without osteoblasts. Osteoid thickness (Osteoid Th) is the mean thickness, in micrometers, of the osteoid on cancellous surfaces. E, calcein double labeling of the mineralized matrix of proximal tibia at an interval of 2 days. WT mice (n = 4), Bcl-2^{−/−} mice (n = 4). Mineral appositional rate (MAR) is the rate (in μm/day) at which new bone is being added to cancellous surfaces. Bone formation rates (BFR) are estimates of cancellous bone volume that are being replaced annually. * indicates significantly different, p < 0.05.

4 °C for 1 h. Cell lysates (30 μl) were transferred to new 96-well plates, and 100 μl of substrate solution, containing 30 mg of p-nitrophenyl phosphate in 10 ml of TRAP buffer, was added to
**FIGURE 2. Bcl-2 promotes osteoclast differentiation.** A, flow cytometry of bone marrow cells obtained from WT and Bcl-2−/− mice. Expression of c-Kit and Mac-1 (CD11b) on c-Fms+ bone marrow cells was analyzed by FACS. B, proliferation of M-CSF-dependent osteoclast precursors assessed by MTT assay. C, differentiation of osteoclasts was assessed by TRAP solution assay after osteoclastogenesis was induced by RANKL stimulation. ** indicate significantly different, p < 0.005. D, mRNA expression of Nfatc1 in WT and Bcl-2−/− preosteoclasts after RANKL stimulation as determined by real time PCR. ** and *, significantly different; ***, p < 0.005; *, p < 0.05. E, time course of change in the expression of the NFATc1 protein as examined by Western blotting in total cell lysates from WT and Bcl-2−/− osteoclasts. F, expression of NFATc1 protein in total cell lysates from WT (empty vector) and Bcl-2−/− osteoclasts, which were retrovirally infected with empty vector or pMx Bcl-2-puro. d, day.
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RESULTS

Three-week-old Bcl-2−/− Mice Have Increased Primary Spongiosa Because of Both Decreased Number and Dysfunctionality of Osteoclasts—To elucidate the role of Bcl-2 on bone metabolism, we first analyzed the skeletal tissue of Bcl-2−/− mice. Bcl-2−/− mice were considerably smaller than their WT littermates, and radiographic analysis of the long bones revealed a sclerotic lesion at the metaphysis, consistent with the observation by McGill et al. (31) (Fig. 1A). Histological and histomorphometric analysis revealed that Bcl-2−/− mice had an increased bone volume in the primary spongiosa (Fig. 1B). The number of osteoclasts and the extent of the eroded surface were decreased in Bcl-2−/− mice (Fig. 1B and C). The osteoblast surface was decreased in Bcl-2−/− mice, although

15 min and stained with 5% silver nitrate solution under ultraviolet light for 10 min. For alizarin red S staining, osteoblasts were incubated for 3 weeks, fixed in 10% buffered formalin, and stained for 10 min with 2% alizarin red S (pH 4.0) (Sigma).

Cell Proliferation Assay—Cell proliferation was determined by a modified MTT assay using Cell Count Kit-8 (Dojindo, Kumamoto, Japan) to count live cells according to the supplier’s protocol. Briefly, the cells (osteoclast precursors, 2 × 10⁴ cells per well; osteoblasts, 1 × 10⁴ cells per well) were placed in a 96-well plate. After incubation for the indicated periods, 10 μl of reaction reagent was added to each well, and cells were incubated for an additional 2 h. Absorbance was measured at 450 nm using a microplate reader (model SH-8000 lab; Corona).

Flow Cytometry—The cell staining procedure for flow cytometry was performed as described previously (35). Bone marrow cells were obtained from the femurs and tibiae of WT and Bcl-2−/− mice at 2 weeks of age. To eliminate erythrocytes, dead cells, and debris, density gradient centrifugation was performed with a Lymphocyte® kit (Cedarlane Laboratories Ltd.). The monoclonal antibodies used in immunofluorescence staining were anti-c-Kit-APC (Pharmingen), anti-Mac-1 (CD-11b)-fluorescein isothiocyanate (Pharmingen), and anti-c-Fms (AF598-1; a gift from Toray Industries Inc.). The anti-c-Fms antibody was fluoresceinated with (R)-phycoerythrin anti-rat IgG (Pharmingen). 10⁶ cells were suspended in 100 μl of 5% fetal calf serum/phosphate-buffered saline (washing buffer). Cells were stained with the first antibody, incubated for 30 min on ice, and washed twice with washing buffer. After the secondary antibody was added, the cells were incubated for 30 min on ice. Cells were then washed twice and suspended in washing buffer for fluorescence activated cell sorting (FACS) analysis. The stained cells were analyzed and sorted by FACS Vantage™ (BD Biosciences).

Statistical Analysis—Statistical analyses were performed using a two-tailed unpaired Student’s t test, and each series of experiments was repeated at least three times.
the osteoid surface and osteoid thickness were increased (Fig. 1D). These mice also displayed a decrease in the mineral apposition rate and bone formation rate, indicating a low turnover bone metabolism (Fig. 1, D and E). The mineralized cartilage area was rather diminished in Bcl-2−/− mice (data not shown), excluding the possibility that the increased mineral tissue in the mice is caused by an accumulation of calcified cartilage matrix. These results suggest that the increased primary spongiosa in Bcl-2−/− mice was mainly caused by the decreased number and dysfunctionality of osteoclasts and not by an increase in bone formation.

**Bcl-2 Promotes Osteoclast Differentiation**—The cellular mechanism underlying the reduced bone resorption in Bcl-2−/− mice was investigated in further detail. We first evaluated whether the population of osteoclast precursors in bone marrow cells was different between Bcl-2−/− and WT mice by analyzing the expression of cell surface markers (c-Fms, c-Kit, and CD11b) of putative osteoclast precursors by flow cytometry (35). The proportion of c-Fms+ bone marrow cells was not different between Bcl-2−/− and WT mice (Fig. 2A). Furthermore, the proportion of c-Kit+ and Mac1+ cells in the c-Fms+ population was also comparable (Fig. 2A). We next compared the M-CSF-dependent proliferation of osteoclast precursors between WT and Bcl-2−/− precursors. As shown in Fig. 2B by MTT assay, no significant difference in proliferation was observed between WT and Bcl-2−/− osteoclast precursor cells. By contrast, when the precursor cells were stimulated with soluble RANKL, osteoclastogenesis was delayed in Bcl-2−/− cells, as determined by TRAP activity (Fig. 2C) and NFATc1 expression (Fig. 2, D and E). The delay in the induction of NFATc1 in Bcl-2−/− pre-osteoclasts was abolished by the retroviral reintroduction of Bcl-2 in these cells, although empty vector introduction had no effect (Fig. 2F). These results suggest that delayed osteoclast differentiation observed in Bcl-2−/− cells is due to the impaired induction of NFATc1.

**Bcl-2 Is Necessary for the Bone Resorbing Activity of Osteoclasts**—We next evaluated the bone resorbing activity of fully differentiated osteoclasts by pit formation assay on dentine slices. As shown in Fig. 3A, Bcl-2−/− osteoclasts exhibited an impaired bone resorbing activity compared with WT osteoclasts, and the pit area generated by Bcl-2−/− osteoclasts was reduced by about 60% compared with that generated by WT osteoclasts. Additionally, actin ring formation of Bcl-2−/− osteoclasts was impaired when cultured on dentine slices (Fig. 3C). Previous studies have revealed an essential role of c-Src proto-oncogene product in the cytoskeletal organiz-
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FIGURE 5. Bcl-2 is essential for the later stage differentiation, mineralization, and survival of osteoblasts. A, proliferation of WT and Bcl-2−/− osteoblasts was assessed by MTT assay. Each collection of osteoblasts was cultured in α-MEM, 10% FBS. B, alkaline phosphatase (ALP) and alizarin red S staining of WT and Bcl-2−/− osteoblasts obtained from calvaria. C, mRNA expression of osteoblastic markers as determined by real time PCR. D, TUNEL staining of WT and Bcl-2−/− osteoblasts incubated with osteogenic medium for 3 weeks. E, proportion of TUNEL-positive osteoblasts incubated with osteogenic medium for 3 weeks. ** significantly different, p < 0.005. F, TUNEL staining of the sections from the diaphysis of the tibiae in WT and Bcl-2−/− mice. Arrowheads indicate TUNEL-positive cells. G, Western blotting analysis of cleaved caspase-3 in total cell lysates from WT and Bcl-2−/− osteoblasts after 4 h of serum starvation. Retrovirus vector-mediated overexpression of Bcl-2 reduced the expression of cleaved caspase-3 observed in Bcl-2−/− osteoblasts. Bars, 100 μm.

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Bcl-2 and bone resorbing activity of osteoclasts. To address the possibility that the impaired actin ring formation and bone resorbing activity of Bcl-2−/− osteoclasts was caused by the reduced activity of c-Src, c-Src activity was examined by Western blotting with anti-phospho-Src antibody, which recognizes an active form of c-Src (36, 37). As shown in Fig. 3B, mature osteoclasts differentiated from Bcl-2−/− bone marrow cells (Bcl-2−/−) exhibited reduced expression of phospho-c-Src as
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compared with wild type osteoblasts (WT). Retroviral reintroduction of Bcl-2 restored bone resorbing and c-Src activity of Bcl-2−/− osteoclasts (Fig. 3D).

Bcl-2 Is Required for Osteoclast Survival—We then examined the survival of Bcl-2−/− osteoclasts. Loss of Bcl-2 accelerated the death of osteoclasts in response to cytokine withdrawal, and most Bcl-2−/− cells died after 12 h (Fig. 4, A and B). Bcl-2−/− osteoclasts displayed an increased expression of activated caspase-3, indicating accelerated apoptosis in Bcl-2−/− osteoclasts (Fig. 4C). The increased apoptosis of Bcl-2−/− osteoclasts was completely prevented by the retroviral reintroduction of Bcl-2 (Fig. 4, D and E). These results suggest that the reduced bone resorption observed in Bcl-2−/− mice is caused by the delayed differentiation, reduced activity, and reduced survival of Bcl-2−/− osteoclasts.

Bcl-2 Is Essential for Late Stage of Differentiation, Mineralization, and Survival of Osteoblasts—We then examined the mechanism of abnormal bone formation in Bcl-2−/− mice. Primary osteoblasts were isolated from neonatal calvaria of Bcl-2−/− and WT mice and subjected to proliferation, differentiation, and survival analyses. Proliferation was comparable between WT and Bcl-2−/− osteoblasts, as determined by MTT assay (Fig. 5A). However, alkaline phosphatase activity and mineralization were decreased in Bcl-2−/− osteoblasts (Fig. 5B). Although the mRNA expression of the α1 chain of type I collagen was comparable, the expression of alkaline phosphatase and osteocalcin was significantly reduced in Bcl-2−/− osteoblasts (Fig. 5C). TUNEL staining revealed accelerated apoptosis in Bcl-2−/− osteoblasts (Fig. 5, D and E). As for in vivo analysis, the number of TUNEL-positive osteocytes in Bcl-2−/− mice was 32%, whereas that of WT mice was 7% in the histological sections of tibiae from WT and Bcl-2−/− mice (Fig. 5F). Bcl-2−/− osteoblasts exhibited an up-regulation of activated caspase-3 expression after serum starvation for 4 h, which was rescued by retrovirus-mediated Bcl-2 introduction (Fig. 5G). These results suggest that Bcl-2 is important for the survival of osteoblasts as well as their late stage differentiation.

Loss of a Single Bim Allele Restores Abnormal Bone Resorption, but Not Bone Formation, in Bcl-2−/− Mice—The mitochondrial apoptosis pathway is regulated in a balance of pro- and anti-apoptotic Bcl-2 family. Among pro-apoptotic Bcl-2 family members expressed in osteoclasts, we found that Bim plays an essential role in osteoclast apoptosis (5). The in vivo analysis of the role of Bcl-2 deficiency in the adult skeletal tissue is hampered by the ill health and early death of Bcl-2−/− mice, and Bouillet et al. (25) reported that the ill health and early death of Bcl-2−/− mice was rescued by hetero-deletion of Bim. We therefore generated Bcl-2−/− Bim−/− mice, in which a single Bim allele was inactivated, and compared them with their

FIGURE 6. Loss of a single Bim allele restored abnormal bone resorption but not bone formation in Bcl-2−/− mice, A, left, radiographic analysis of WT and Bcl-2−/− mice at 3 weeks of age, and Bcl-2+/− Bim−/− and Bcl-2−/− Bim−/− mice at 16 weeks of age. A, right, micro-computed tomography analysis of femurs in Bcl-2+/− Bim−/− and Bcl-2−/− Bim−/− mice at 16 weeks of age. Bar, 10 mm. B, quantification of micro-computed tomography data in Bcl-2+/− Bim−/− and Bcl-2−/− Bim−/− mice at 16 weeks of age. *, significantly different, p < 0.05. C, bone mineral density (BMD) analysis of femurs in Bcl-2+/− Bim−/− and Bcl-2−/− Bim−/− mice at 16 weeks of age. *, significantly different, p < 0.01. D, resorption pits generated by WT, Bcl-2+/−, and Bcl-2−/− Bim−/− osteoclasts. Bars = 100 μm. **, significantly different, p < 0.005; NS, no significant difference. E, survival of WT, Bcl-2−/−, and Bcl-2−/− Bim−/− osteoclasts after cytokine withdrawal. ***, significantly different, p < 0.005. F, alkaline phosphatase (ALP), von Kossa, and alizarin red S stainings of WT, Bcl-2−/−, and Bcl-2−/− Bim−/− osteoclasts. G, real time PCR analysis of runx2 and osterix expression in osteoblasts from Bcl-2+/− Bim−/− and Bcl-2−/− Bim−/− mice. * and ** significantly different; *p < 0.01; **, p < 0.005.
Bcl-2/−/Bim+/− littermates. Bcl-2/−/Bim+/− mice ameliorated growth retardation and polycystic kidney disease that were seen in Bcl-2/−/Bim+/− animals (Fig. 6A) (25). Microcomputed tomography and bone mineral density analyses revealed that Bcl-2/−/Bim+/− mice exhibited a decreased bone mass phenotype compared with Bcl-2+/−/Bim+/− mice at 16 weeks of age (Fig. 6, A–C). Although reduced bone resorbing activity and survival observed in Bcl-2−/− osteoclasts was restored in osteoclasts generated from Bcl-2−/−Bim+/− bone marrow cells (Fig. 6, D and E), the mineralization activity of Bcl-2−/−Bim+/− osteoblasts was still impaired (Fig. 6F). These results suggest that the inactivation of a single Bim allele restores the defective function caused by Bcl-2 deficiency in osteoclasts but is not sufficient to rescue the mineralization defect in Bcl-2-deficient osteoblasts.

Interestingly, although the expression of runx2 and osterix was equivalent between these two cell preparations on day 3 of culture, the increase in their expression observed in Bcl-2−/−Bim+/− cells was not apparent in Bcl-2−/−Bim+/− cells, and the levels of runx2 and osterix expression were significantly lower in Bcl-2+/−Bim+/− cells than Bcl-2−/−Bim+/− cells after 3 weeks of culture (Fig. 6G). This may be because the osteoblastic cells in the Bcl-2−/−Bim+/− preparation either lost their osteoblastic properties rapidly or the osteoblastic cells died in the course of culture.

**DISCUSSION**

We here demonstrated that the apoptotic molecule Bcl-2 plays a pivotal role in maintaining skeletal integrity by regulating both osteoclasts and osteoblasts. RANKL-dependent osteoclast differentiation was delayed in osteoclast precursors from Bcl-2−/− mice, which appeared to be due to the impaired induction of NFATc1 (38, 39). Only a limited number of studies have been published regarding the relationship between Bcl-2 and NFAT pathways. Interestingly, it was reported that Bcl-2 selectively impairs NFATc3 binding and nuclear translocation in Jurkat cells (24), which is contrary to our observation that NFATc1 is impaired by Bcl-2 deficiency. This discrepancy may be due to the cell-specific function of NFAT or the different role of NFATc1 and NFATc3. Further studies are required to elucidate the molecular interaction between Bcl-2 and NFATc1 activation in osteoclasts.

Nonreceptor tyrosine kinase c-Src plays an essential role for the activation of osteoclasts by regulating the cytoskeletal organization of the cells (36, 37, 40, 41). Bcl-2-deficient osteoclasts exhibited reduced c-Src activity and actin ring formation on dentine slices, which may lead to an impaired bone resorbing activity of the cells. The mechanism by which Bcl-2 deficiency affects c-Src activity remains unclear, and further studies are required to elucidate the molecular interaction between Bcl-2 and c-Src. Therefore, the reduced bone resorption observed in Bcl-2−/− mice may be caused by both impaired osteoclast differentiation through retarded NFATc1 induction and impaired bone resorbing activity of mature osteoclasts through reduced c-Src activity.

The reduced bone formation in Bcl-2−/− mice is at least partly caused by a cell autonomous defect of the osteoblasts, especially their terminal differentiation. It was reported that osteoblast-specific overexpression of Bcl-2 using the 2.3-kb fragment of the type I collagen promoter (Col2.3) increased the level of alkaline phosphatase, osteocalcin, Runx2, and osterix expression in osteoblasts, although the mineralization potential
was attenuated (42). This may be because Col2.3 induces Bcl-2 overexpression in immature osteoblasts, which may have affected mineralization potential of the cells.

The ill health and early death of Bcl-2–/– mice have precluded direct studies on the role of Bcl-2 in the long term bone metabolism that occurs in adult mice. One way around this problem would be to establish cell-specific knock-out mice, but this is difficult because no Bcl-2 floxed mice have been made available as yet. Another way would be to rescue the lethal phenotype of the mice by hetero-deletion of a proapoptotic molecule Bim, as reported previously by Bouillet et al. (25). Because loss of a single allele of Bcl-2 does not affect cell death in general (43), we generated Bcl-2–/–Bim+/– mice, and their skeletal phenotypes were compared with their Bcl-2–/–Bim+/– littermates. Although osteoclasts generated from Bcl-2–/–Bim+/– bone marrow cells displayed normal bone resorbing activity, the mineralization activity of Bcl-2–/–Bim+/– osteoblasts was still impaired. Bcl-2–/–Bim+/– mice exhibited a decreased bone mass compared with Bcl-2–/–Bim+/– mice, mainly due to impaired osteoblast function. These data indicate that the balance between the anti-apoptotic Bcl-2 and pro-apoptotic Bim plays a pivotal role in the regulation of apoptosis and the function of osteoclasts, although pro-apoptotic molecule(s) other than Bim presumably contribute to the osteoblast function.

While we were preparing this manuscript, Yamashita et al. (44) reported that osteoblasts from Bcl-2–/– mice were normal in terms of their proliferation, differentiation, and mineralization, the findings of which are obviously different from our own. However, their results also cannot account for previous studies from other groups that reported that Bcl-2–/– mouse bones exhibited a pseudo-woven appearance because of abnormal osteoblasts (29) and that the bone-targeted overexpression of Bcl-2 increased osteoblast differentiation and survival (42). They also found that Bcl2–/– osteoclasts exhibited a significantly larger size, which we failed to observe. The exact mechanism underlying this discrepancy is not entirely clear, but it is at least partly due to the different strains of mice we and they used (C57BL/6 and B6;129S2-Bcl2tm13Sk, respectively). They also reported that Bcl-2 is dispensable for the anabolic activity of parathyroid hormone. Therefore, it would be of interest to compare the effect of parathyroid hormone on the skeletal tissues of Bcl-2–/–Bim+/– mice and Bcl-2–/–Bim+/– littermates, which would yield important new information.

In summary, we have demonstrated that Bcl-2 promotes the differentiation, activation, and survival of both osteoblasts and osteoclasts. Moreover, Bcl-2 is important for osteoblast survival and function, although pro-apoptotic molecule(s) other than Bim may contribute to osteoblast function.

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