Immortalization of Osteoclast Precursors by Targeting *bcl*-X$_L$ and Simian Virus 40 Large T Antigen to the Osteoclast Lineage in Transgenic Mice

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Abstract

Cellular and molecular characterization of osteoclasts (OCL) has been extremely difficult since OCL are rare cells, and are difficult to isolate in large numbers. We used the tartrate-resistant acid phosphatase promoter to target the *bcl*-X$_L$ and/or Simian Virus 40 large T antigen (Tag) genes to cells in the OCL lineage in transgenic mice as a means of immortalizing OCL precursors. Immunocytochemical studies confirmed that we had targeted Bcl-X$_L$ and/or Tag to OCL, and transformed and mitotic OCL were readily apparent in bones from both Tag and *bcl*-X$_L$/Tag mice. OCL formation in primary bone marrow cultures from *bcl*-X$_L$, Tag, or *bcl*-X$_L$/Tag mice was twofold greater compared with that of nontransgenic littermates. Bone marrow cells from *bcl*-X$_L$/Tag mice, but not from singly transgenic *bcl*-X$_L$ or Tag mice, have survived in continuous culture for more than a year. These cells form high numbers of bone-resorbing OCL when cultured using standard conditions for inducing OCL formation, with ~ 50% of the mononuclear cells incorporated into OCL. The OCL that form express calcitonin receptors and contract in response to calcitonin. Studies examining the proliferative capacity and the resistance of OCL precursors from these transgenic mice to apoptosis demonstrated that the increased numbers of OCL precursors in marrow from *bcl*-X$_L$/Tag mice was due to their increased survival rather than an increased proliferative capacity compared with Tag, *bcl*-X$_L$, or normal mice. Histomorphometric studies of bones from *bcl*-X$_L$/Tag mice also confirmed that there were increased numbers of OCL precursors (TRAP + mononuclear cells) present in vivo. These data demonstrate that by targeting both *bcl*-X$_L$ and Tag to cells in the OCL lineage, we have immortalized OCL precursors that form bone-resorbing OCL with an efficiency that is 300–500 times greater than that of normal marrow. (***J. Clin. Invest.*** **1998. 102:88–97.)** **Key words: osteoclasts • *bcl*-X$_L$ • Simian Virus 40 large T antigen • precursors • transgenic • apoptosis**

Introduction

Osteoclasts (OCL)[1] are very rare cells in bone, and thus are difficult to isolate in sufficient numbers to characterize them fully at the cellular and molecular level. Immortalization of OCL precursors should greatly enhance our ability to study these parameters by facilitating production of large numbers of OCL in vitro. We previously targeted Simian Virus 40 large T antigen (Tag) to OCL in transgenic mice (TRAP-Tag mice) using the tartrate-resistant acid phosphatase (TRAP) promoter in an attempt to immortalize OCL precursors, but found that this strategy did not immortalize cells in the OCL lineage (1). Mitotic and morphologically transformed OCL developed in these transgenic mice, but OCL apoptosis was also increased, raising the possibility that Tag-induced cell death may have prevented immortalization. These results suggested that expression of an additional gene that could block apoptosis in OCL and their precursors might immortalize OCL precursors in these transgenic mice. Several apoptosis-inhibiting genes have been identified recently, and of these, members of the *bcl*-2 family are the best characterized (2). *Bcl*-2 prevents apoptosis induced by a number of agents, and transfection of the *bcl*-2 gene into hematopoietic precursors blocked apoptosis induced by growth factor withdrawal (3). *Bcl*-X$_L$ is a *bcl*-2–related gene expressed in a wide range of cell types (4). Like *bcl*-2, it has been shown to prevent apoptosis both in vivo and in vitro (5–8), and can function as a *bcl*-2–independent regulator of apoptosis in hematopoietic cells. Therefore, we targeted *bcl*-X$_L$ to cells in the OCL lineage, produced mice doubly transgenic for *bcl*-X$_L$ and Tag, and immortalized OCL precursors from the *bcl*-X$_L$/Tag mice. These cells form OCL with very high efficiency.

Methods

Construction of the mouse TRAP-bcl-X$_L$ hybrid transgene. We have previously described construction of the pBSmTRAP5′ plasmid, which contains 1294 bp of the 5′-flanking sequence, as well as the entire 5′-untranslated region of the murine *TRAP* gene (9). A plasmid containing the full-length murine *bcl*-X$_L$ cDNA with a 24-bp sequence encoding the FLAG epitope (10) inserted immediately after the AUG initiation codon, was kindly provided by Gabriel Nunez (University of Michigan, Ann Arbor, MI). A 985-bp fragment containing the *bcl*-X$_L$ cDNA was inserted into the unique EcoRI site of

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[1] *Abbreviations used in this paper:* GM, granulocyte macrophage; H & E, hematoxylin and eosin; mCTR, mouse calcitonin receptor; MNC, multinucleated cells; OCL, osteoclast; Tag, T antigen; TBS, Tris-buffered saline; TRAP, tartrate-resistant acid phosphatase.

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pBSpKCR3 (11), which contains part of the second exon, the second intron, and the third exon including the polyadenylation site of the rabbit β-globin gene. There are no AUG initiation codons within the β-globin sequences present, so translation of the Bcl-$\mathrm{X_L}$ protein starts at the normal *bcl*-$\mathrm{X_L}$ initiation codon. To generate the mTRAP-*bcl*-$\mathrm{X_L}$ hybrid transgene, the mTRAP promoter was cloned immediately upstream of the *bcl*-$\mathrm{X_L}$/globin construct. The transgene was excised by XhoI digestion from the resulting plasmid, TBX6 (Fig. 1), and was agarose gel–purified before microinjection.

Production and identification of transgenic mice. The mTRAP-*bcl*-$\mathrm{X_L}$ transgene was microinjected at a concentration of 3 μg/ml into the male pronucleus of fertilized one-cell mouse embryos by standard methods (12). The embryos were obtained from mating CB6F$_1$ (C57Bl/6 × BALB/c) males and females (Harlan Sprague Dawley Inc., Indianapolis, IN). The injected embryos were then reimplanted into the oviducts of pseudopregnant B6D2F$_1$ female mice. The presence of the transgene was identified in resulting offspring by Southern blot analysis of DNA (13), which was purified from a small piece of the tail taken at the time the animals were weaned (12). Transgenic mice of subsequent generations were identified by polymerase chain reaction analysis using *bcl*-$\mathrm{X_L}$–specific primers flanking a 416-bp region of the *bcl*-$\mathrm{X_L}$ cDNA. Probes for Southern blot analysis were generated by random oligonucleotide labeling (Pharmacia Biotech, Inc., Piscataway, NJ) using [α-^{32}P]dCTP (Dupont-NEN, Boston, MA).

Northern blot analysis. Total RNA was extracted from various tissues of transgenic and nontransgenic mice according to the procedure of Chomczynski and Sacchi (14). Northern blot analysis was carried out as described (15) using Nytran membranes and *bcl*-$\mathrm{X_L}$–specific probes generated by random oligonucleotide labeling, as above.

Assays of osteoclast formation. Murine bone marrow cells were cultured as described previously (16). In brief, the mice were killed by cervical dislocation, and the tibiae and femora were removed and dissected free from adhering soft tissues. The bone ends were cut off with a scalpel, and the marrow was flushed with α-MEM (GIBCO BRL, Gaithersburg, MD) containing 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL). Cells were centrifuged at 1,500 rpm for 10 min, and the pellet was resuspended in 10 ml of α-MEM containing 10% FCS (GIBCO BRL). Cells were allowed to attach to plastic for 2 h at 37°C in a 5% CO$_2$ incubator. Nonadherent cells were collected and centrifuged as previously described (16). Marrow cells (1×10^6/ml) were cultured in 24-well plates for 6 d in the presence of varying concentrations of either 1,25-(OH)$_2$D$_3$ (Dr. M. Uskokovic, Hoffmann-LaRoche, Nutley, NJ) or PTHrP (Bachem Bioscience Inc., King of Prussia, PA). Half of the media was changed every 3 d, and the 1,25-(OH)$_2$D$_3$ or PTHrP was replaced. At the end of the culture, the plates were fixed with 2% glutaraldehyde (Sigma Chemical Corp., St. Louis, MO) in PBS for 20 min, and were stained for TRAP using a histochemical kit (no. 387; Sigma Chemical Corp.). TRAP-positive multinucleated cells containing three or more nuclei were scored microscopically as osteoclast-like cells.

Isolation of immortalized osteoclast precursors from primary cultures of bone marrow obtained from transgenic mice. Mouse bone marrow cells (10^5 cells/ml) were suspended in 1.2% methylcellulose (Sigma Chemical Corp.) containing 30% FCS, 1% BSA (Sigma Chemical Corp.), 2.5×10^{-5} M L-mercaptoethanol, and 100 pg/ml of mGM-CSF (R & D Systems, Inc., Minneapolis, MN), and were cultured at 37°C in a humidified atmosphere of 5% CO$_2$-air in 35-mm petri dishes for 9 d (17) to induce CFU-GM–derived colonies that contain the earliest identifiable OCL precursor. This was done as a means of enriching OCL precursors. CFU-granulocyte macrophage (GM)–derived colonies were isolated individually, and the pooled cells were washed twice with α-MEM, and then plated in 96-well dishes at a cell density of 1,000–2,000 cells/well. These dishes had been plated 24 h previously with 2,500–5,000 MC3T3-E1 cells/well. We have shown in preliminary experiments that MC3T3-E1 cells support the growth of OCL precursors without inducing terminal differentiation of OCL precursors. After the cocultures reached confluence, they were treated with trypsin/EDTA (JRH Biosciences, Lenexa, KS) for 5 min at 37°C, and split at the ratio of 1:3. After 2 wk, cultures were transferred into 24-well plates, and, after reaching confluence, were transferred into 6-well plates. The cultures of *bcl*-$\mathrm{X_L}$/Tag cells cocultured on MC3T3-E1 cells have been maintained routinely for more than 18 mo.

Osteoclast formation assay: coculture with stromal cells. The OCL formation capacity of *bcl*-$\mathrm{X_L}$, Tag, or *bcl*-$\mathrm{X_L}$/Tag cells that had been cocultured with MC3T3-E1 cells as described above was determined using standard conditions that induce differentiation of OCL precursors to form multinucleated OCL that resorb bone (18). In brief, *bcl*-$\mathrm{X_L}$, Tag, or *bcl*-$\mathrm{X_L}$/Tag cells were cocultured with MC3T3-G2/PA6 stromal cells (1,000 MC3T3-G2/PA6 stromal cells/well in 96-well plates, or 5,000 PA6 cells/well in 48-well plates) that had been plated in the wells on the day before the addition of *bcl*-$\mathrm{X_L}$, Tag, or *bcl*-$\mathrm{X_L}$/Tag cells. Dexamethasone (10^{-7} M; Sigma Corp., St. Louis, MO) and 1,25-(OH)$_2$D$_3$ (10^{-9} M) were then added to the cultures, and the cultures continued for 7–14 d in α-MEM containing 10% FCS (18). One tenth of the coculture cells (MC3T3-E1 and marrow cells) from a 24-well plate or 1/20 of the cells from a 6-well plate were pipetted onto the PA6 cells/well. Half of the media was changed every third day. In selected cultures, cells were treated with interleukin-1, or varying concentrations of calcitonin added to cultures of *bcl*-$\mathrm{X_L}$/Tag cells treated with 1,25-(OH)$_2$D$_3$.

To assess the actual number of *bcl*-$\mathrm{X_L}$, Tag, or *bcl*-$\mathrm{X_L}$/Tag marrow cells plated in the assays, we used a cytotoxic antisera that was prepared against mouse fibroblasts (kindly provided by Dr. Yoneda, University of Texas Health Science Center at San Antonio), and that also lysed MC3T3-E1 feeder cells, but not marrow cells, to remove MC3T3-E1 cells before plating on the PA6 cells as follows: MC3T3-E1 cells were completely lysed by incubation with the cytotoxic antisera (1:100 dilution) and rabbit complement (1:50 dilution; Serotec, Oxford, England) for 1 h at 37°C, as determined by the complete absence of Mac-1–negative cells. Viable marrow cells were then harvested by treatment with trypsin/EDTA for 5 min. The marrow cells were washed with media, and the number of cells was counted. These cells were then cultured with PA6 cells as above to induce OCL formation. After 7 d of culture, the number of OCL-like multinucleated cells, identified by their expression of TRAP and the average number of nuclei in each multinucleated cell, was determined. Our cocultures generally contained ~ 5–10% marrow cells, and 90–95% feeder cells. Therefore, in the standard OCL formation assay, there were ~ 1,500–3,000 marrow cells/well.

Formation of resorption lacunae on dentine slices. PA6 stromal cells (10,000) were plated on a dentine slice/well in 24-well plates. The following day, 5,000 *bcl*-$\mathrm{X_L}$/Tag cells were added to each culture well, and the cells were cultured for 7–10 d in α-MEM containing 10% FCS, 2×10^{-9} M 1,25-(OH)$_2$D$_3$, and 10^{-7} M dexamethasone to induce OCL formation. Half of the media was changed every third day. The cells were fixed and stained for TRAP as described above. After counting TRAP-positive multinucleated cells (MNC), the cells were removed from the dentine slice by brushing, and resorption lacunae were stained with 1% toluidine blue (19). Resorption lacunae on dentine slices were scored by phase contrast microscopy using image analysis software (Bioquant, Nashville, TN) as described previously (17).

Effect of serum-free condition on the survival and osteoclast formation capacity of marrow cells from transgenic mice. Murine bone marrow cells obtained from transgenic and nontransgenic littermates were cultured in 24-well plates (4×10^6/ml) in α-MEM containing 0.1% BSA (CellPro, Inc., Pothell, WA) for 0–96 h at 37°C in a humidified atmosphere of 5% CO$_2$-air. 0, 24, 48, and 96 h after culture initiation, an aliquot of the cells was obtained, and its viability was assessed by trypan blue exclusion. Then, either 5×10^5 or 1×10^6 viable marrow cells per ml from each time point were cultured in 4-well plates (Nunc, Roskilde, Denmark) for 6 d in α-MEM–10% FCS with 2×10^{-9} M 1,25-(OH)$_2$D$_3$ to assess their capacity to form OCL (16). At the end of this second culture period, the cells were fixed and stained for TRAP activity, as described above.

Osteoclast precursor proliferation. Freshly isolated marrow cells (5×10^6/well) from *bcl*-X_L/Tag and Tag mice were incubated in 24-well plates in α-MEM containing 0.1% BSA for 24 h. 1 μCi ^{3}H-thymidine (specific activity 20 Ci/mmol ; Dupont NEN, Boston MA) was added. After an additional 48 h of culture, the cells were washed in standard media, and the number of viable cells was counted. 1×10^5 viable cells/well were plated in eight-chamber cultures slides (Nunc, Inc. Naperville IL), and 1,25-$(OH)_2D_3$ (2×10^{-9} M) was added. Cells were then cultured in α-MEM containing 10% FCS for 6 d to induce OCL formation. Cells were fixed with 2% glutaraldehyde in PBS for 20 min, and were stained for TRAP as described above. Slides were washed first with water and then with 5% trichloroacetic acid and processed for autoradiography (20).

Autoradiography and RT-PCR analysis for calcitonin receptor expression. Bcl-X_L/Tag cells or normal mouse marrow cells were cultured in the presence of 1,25-$(OH)_2D_3$ (10^{-9} M) and dexamethasone in 24-well plates as described above. After 7 d of culture, the cells were released with trypsin-EDTA and applied to Labtech chamber slides (Fisher Scientific Co., Pittsburgh, PA). ^{125}I-labeled salmon calcitonin (Amersham Corp., Arlington Heights, IL) was added to the slides in the presence or absence of 1 μM of unlabeled calcitonin (Peninsula Laboratories, Inc., Belmont, CA), and the slides were processed as described (17). The percentage of multinucleated cells expressing calcitonin receptors and the relative level of calcitonin receptor expression was compared with that of normal mouse marrow cultures.

To determine which isoform of calcitonin receptor was expressed by *bcl*-XL/Tag cells, equal amounts of RNA (2 μg) from cocultures of *bcl*-X_L/Tag cells with MC3T3-E1 cells, normal marrow cocultured with PA6 cells, or *bcl*-X_L/Tag cells cocultured with MC3T3-E1 cells and 1,25-$(OH)_2D_3$ (10^{-9} M) and dexamethasone (10^{-7} M) were reverse-transcribed into cDNA with a mouse calcitonin receptor (mCTR)–specific primer from the 3′UTR (5′-GTGGATCACAATGCTTGGGGTGGC-3′) using the Ready-To-Go You-Prime First-Strand Beads kit (Pharmacia Biotech Inc.) that contained cloned FPLCpure M-MuLV reverse transcriptase, RNase inhibitor, and nucleotides. For the PCR reaction, mCTR primers mT2F (5′-ATCATTATCATCATCCACC-3′) and mT3R (5′-CAGAGCATCCAGAAGTAG-3′) were used to generate mCTR PCR products that would include both the C1a (IN:111−) and C1b (IN:111+) isoforms. Equal amounts of each cDNA sample (by volume) were added to a total PCR reaction volume of 50 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1 μM each of the 5′ and 3′ primers, 2 mM dNTPs, and 2.5 U Taq DNA polymerase (Fisher Scientific Co., Pittsburgh, PA). PCR conditions were 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s for 40 cycles using Gene Amp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT). A negative control containing H_2O instead of cDNA was run with each PCR set. cDNA subclones containing both the mCTR C1a and C1b isoforms were included in the PCR reaction sets as positive controls.

Southern blot analysis of PCR products. PCR products were analyzed by electrophoresis in a 1.8% agarose gel containing TAE buffer, transferred onto nylon membrane (Micron Separation Inc., Westboro, MA) and cross-linked to the filter using Stratalinker™ (Stratagene, La Jolla, CA). The filter was prehybridized at 65°C for 2 h in a hybridization solution containing 6× SSPE, 5× Denhardt's solution, 0.5% SDS, 50 μg/ml denatured DNA, and 10% dextran sulfate. The hybridization was performed at 38°C overnight in hybridization solution containing 1.5×10^6 cpm/ml [α-^{32}P]dCTP labeled probe. The probe was an mCTR primer m2TF-v2 (5′-ATGGATCTGGTGCGGCGGGATC-3′) that does not overlap either m2TF or mT3R, end-labeled with [γ-^{32}P]dATP using the T4 kinase (Promega Corp., Madison, WI). The filter was then washed with 2× SSC and 0.5% SDS for 2 h, and autoradiography was performed using an intensifying screen.

Osteoclast apoptosis assay. Murine bone marrow cultures from control and transgenic mice were established as described previously (16). In brief, bone marrow cells were flushed out from the marrow cavity using a 1-ml syringe fitted with a 27-gauge needle, and containing α-MEM. The cells were pelleted by centrifugation, resuspended in α-MEM, and 0.5×10^6 cells were plated in 96-well plates. Osteoclast-like multinucleated cells were induced to form during the 7-d culture period by treating the cultures with 10^{-8} M 1,25-$(OH)_2D_3$. On day 7, control media or risedronate (10^{-7} M), an inducer of osteoclast apoptosis (21), was added to the cultures for 24 h. Cells were then fixed with 3.7% paraformaldehyde in PBS for 10 min, washed briefly with PBS, and stained for TRAP activity and with hematoxylin to visualize the nuclei. The percentage of osteoclasts with pyknotic and/or fragmented nuclei, the classic morphologic features of apoptosis, was scored in quadruplicate cultures with a microscope using a 10× objective as described previously (21).

Processing tissues for histology. Bones (fore and hind limbs, thoracic and lumbar vertebrae) from all animals were fixed in 10% phosphate-buffered formalin for 24–48 h, decalcified in 14% EDTA for 1–3 wk, processed through graded alcohols, and embedded in paraffin wax. Sections (3 μm thick) were cut at various levels from all bones, and were stained with hematoxylin, eosin, orange G, and phloxine. Histomorphometric analysis of cancellous bone volume (amount of bone matrix per cancellous space) and numbers of osteoclasts (including normal, morphologically transformed with enlarged hyperchromatic nuclei, mitotic, and apoptotic per mm^2 bone matrix in the cancellous space) were determined in sections of tibial metaphyses as described previously (1).

Bcl-X_L antigen immunostaining. Tissue sections were deparaffinized, rehydrated, and washed for 10 min in Tris-buffered saline (TBS), pH 7.3. Sections were given two 15-min treatments with 0.1% H_2O_2 in methanol. After rinsing, sections were blocked for 1 h with 50% normal rabbit serum in TBS, incubated with the anti-Bcl-X antibody (Transduction Laboratories, Lexington, KY) in dilutions of 1:10–1:200 with TBS, 1% BSA, and 0.02% Tween 20 for 1 h. This was followed by three 5-min washes in TBS with 0.02% Tween 20 (with stirring). The sections were incubated with biotin-conjugated Immunopure rabbit anti–mouse IgG [F(ab′)$_2$] (Pierce Chemical Co., Rockford, IL) at a 1:2,000 dilution in TBS, 1% BSA, and 0.02% Tween 20 for 45 min. After three 5-min washes in TBS and 0.02% Tween 20, the sections were incubated in peroxidase-conjugated streptavidin (DAKOPATTS, Copenhagen, Denmark) at a 1:1,500 dilution in TBS, 1% BSA, and 0.02% Tween 20. After three 5-min washes, the sections were treated with DAB (Sigma Chemical Co.), counterstained with methyl green and eosin, and viewed microscopically.

Results

Three transgenic founder mice were generated with the mTRAP-*bcl*-X_L transgene shown in Fig. 1, and lines of mice were established from each. Northern blot analysis of tissues from transgenic offspring demonstrated that expression of *bcl*-X_L was targeted to bone in each of the three lines of mice. In all mice examined, including both transgenic mice and nontransgenic controls, *bcl*-X_L mRNA was most abundant in the brain, thymus, and kidney. However, only in transgenic mice could *bcl*-X_L mRNA be detected in long bone and calvaria (Fig. 2). A low level of expression was also detected in the brown fat of transgenic mice, consistent with the pattern of tissue-specific expression we previously observed with the murine TRAP promoter (9). The highest level of *bcl*-X_L expression in the bone was observed in the mTRAP-*bcl*-X_L line 2, and therefore, most of the subsequent analyses and interbreeding to mTRAP-Tag mice to generate *bcl*-X_L/Tag doubly transgenic mice was performed with mice of this line.

Immunohistochemical analysis confirmed that we had targeted expression of *bcl*-X_L to osteoclasts. Bcl-X_L was not detectable in osteoclasts from nontransgenic littermates (Fig. 3 *A*), but was detected in osteoclasts from both *bcl*-X_L (Fig. 3 *B*) and

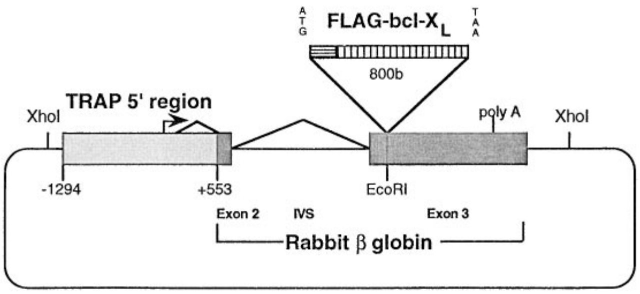

Figure 1. Map of the TRAP-*bcl*-X$_L$ construct. The mTRAP-*bcl*-X$_L$ hybrid transgene was excised by XhoI digestion from this plasmid construct for the production of transgenic mice.

Table I. Histomorphometry of Transgenic Mouse Bones

Group	TRAP+ mono. cells/marrow	No. of OCL in tibiae/mm^2	Transformed OCL	Mitotic OCL	Apoptotic OCL
	%		%	%	%
Control	0.73±0.13	163±52	0	0	0
bcl-X$_L$	0.24±0.04	220±157	0	0	0
Tag	9.40±5.77	172±49	57±7	2±1	7±2
bcl-X$_L$/Tag	35.88±5.46*	228±104	62±11	3±1	6±3

Histomorphometric analysis of decalcified mouse bone was performed as described in Methods. The number of TRAP+ mononuclear cells was significantly increased in *bcl*-X$_L$ /Tag mice when compared with Tag mice ($P < 0.016$).

bcl-X$_L$/Tag doubly transgenic mice (Fig. 3 *C*). Surprisingly, the level of Bcl-X$_L$ in osteoclasts of *bcl*-X$_L$ mice appeared to be lower than in *bcl*-X$_L$/Tag mice (as assessed by the strength of the signal at similar dilutions of the anti-Bcl-X antibody) for reasons that are unclear.

Bone sections from *bcl*-X$_L$ transgenic mice showed normal histology (Fig. 3 *D*) and normal osteoclasts (Fig. 3 *E*) when examined at 3 wk, 3 mo, and 6 mo of age, and they never developed osteopetrosis, as determined radiologically and histologically. In contrast, some of the mice transgenic for either Tag or *bcl*-X$_L$/Tag had developed mild osteopetrosis when killed at the age of 2–4 mo. In addition, transformed osteoclasts were readily apparent in both Tag and *bcl*-X$_L$/Tag mice (Fig. 3 *F*). In *bcl*-X$_L$/Tag mice, 62% of osteoclasts appeared to be transformed, and 3% were mitotic (Table I). In mice transgenic for Tag alone, these numbers were 57% and 2%, respectively. In both lines, the numbers of apoptotic osteoclasts were similarly increased (Tag: 7%, *bcl*-X$_L$/Tag: 6%) compared with either control littermates (0%) or *bcl*-X$_L$ mice (0%). Osteoclast numbers/mm^2 bone area in tibial metaphyseal cancellous bone were not significantly different in *bcl*-X$_L$, *bcl*-X$_L$/Tag, or Tag mice compared with nontransgenic littermates. However, higher numbers of osteoclasts/mm^2 in bone area were observed in the femurs of transgenic mice, with 391±55 in Tag mice and 528±110 in *bcl*-X$_L$/Tag mice vs. 232±60 in control mice (Table I). In addition, the number of TRAP-positive mononuclear cells was significantly increased in *bcl*-X$_L$/Tag mice in comparison with Tag mice (Fig. 3 *G* and Table II).

We next examined the capacity of marrow obtained from the various classes of transgenic mice to form OCL in response to 1,25-(OH)$_2$D$_3$ (10^{-10} to 10^{-7} M) and PTHrP (0.1 ng/ml to 1 g/ml) in cultures, as a means of assessing the sensitivity of OCL precursors from these transgenic mice to these osteotropic factors and the relative number of OCL precursors present in marrow from these mice. Osteoclast formation in response to varying concentrations of 1,25-(OH)$_2$D$_3$ or PTHrP showed similar dose-response patterns compared with nontransgenic controls (Fig. 4, *A* and *B*). However, the numbers of osteoclasts formed in the primary marrow cultures from transgenic mice were significantly increased compared with their normal littermates. For example, marrow cells from mice transgenic for either *bcl*-X$_L$, Tag, or *bcl*-X$_L$/Tag formed two times more osteoclasts per 10^6 marrow cells plated in response to 10^{-9} M 1,25-(OH)$_2$D$_3$ than did control marrow cultures (Fig. 4 *A*), demonstrating that OCL precursors were increased in marrow samples from the transgenic mice. Osteoclast formation was increased to a similar extent in all 3 *bcl*-X$_L$ mouse lines (data not shown).

Marrow cells derived from these transgenic mice also showed distinct differences in their capacity to be passaged. Normal cells survived less than a month, and 0.15% of cells formed osteoclasts at that time. Marrow cells obtained from either Tag or *bcl*-X$_L$ transgenic mice survived for 2–4 mo, and formed few osteoclasts at that time. In contrast, marrow cells

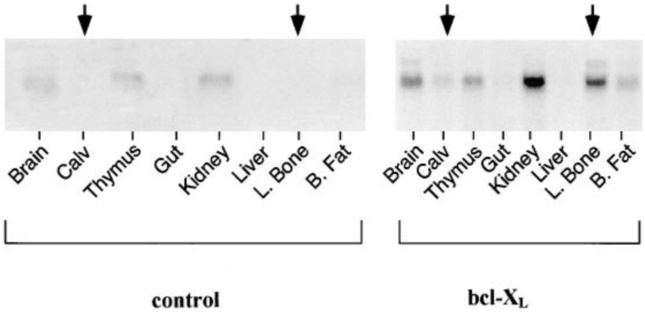

Figure 2. Northern blot analysis of tissues from *bcl*-X$_L$ transgenic mice. Bcl-X$_L$ was detectable in brain, thymus, and kidney in normal mice. Increased levels of expression of *bcl*-X$_L$ mRNA were found in calvaria and long bones from transgenic animals compared with control littermates (*arrows*), where the signal was undetectable.

Table II. Survival of Primary Marrow Cells from Transgenic Mice in Continuous Cultures

Source of marrow	Survival in vitro	Cells incorporated into OCL
		%
Normal	< 1 mo	0.15
bcl-X$_L$	~ 2 mo	~ 0
Tag	~ 4 mo	0.36
bcl-X$_L$/Tag	> 18 mo	30–50

Marrow cells from control and transgenic mice were cultured with MC3T3-E1 cells as described in Methods. Aliquots of the cultures were assessed at the time indicated for their capacity to form OCL. The percentage of cells incorporated into OCL was determined by counting the average number of nuclei per 100 OCL, multiplying this number by the number of OCL formed, and dividing the product by the number of cells originally plated.

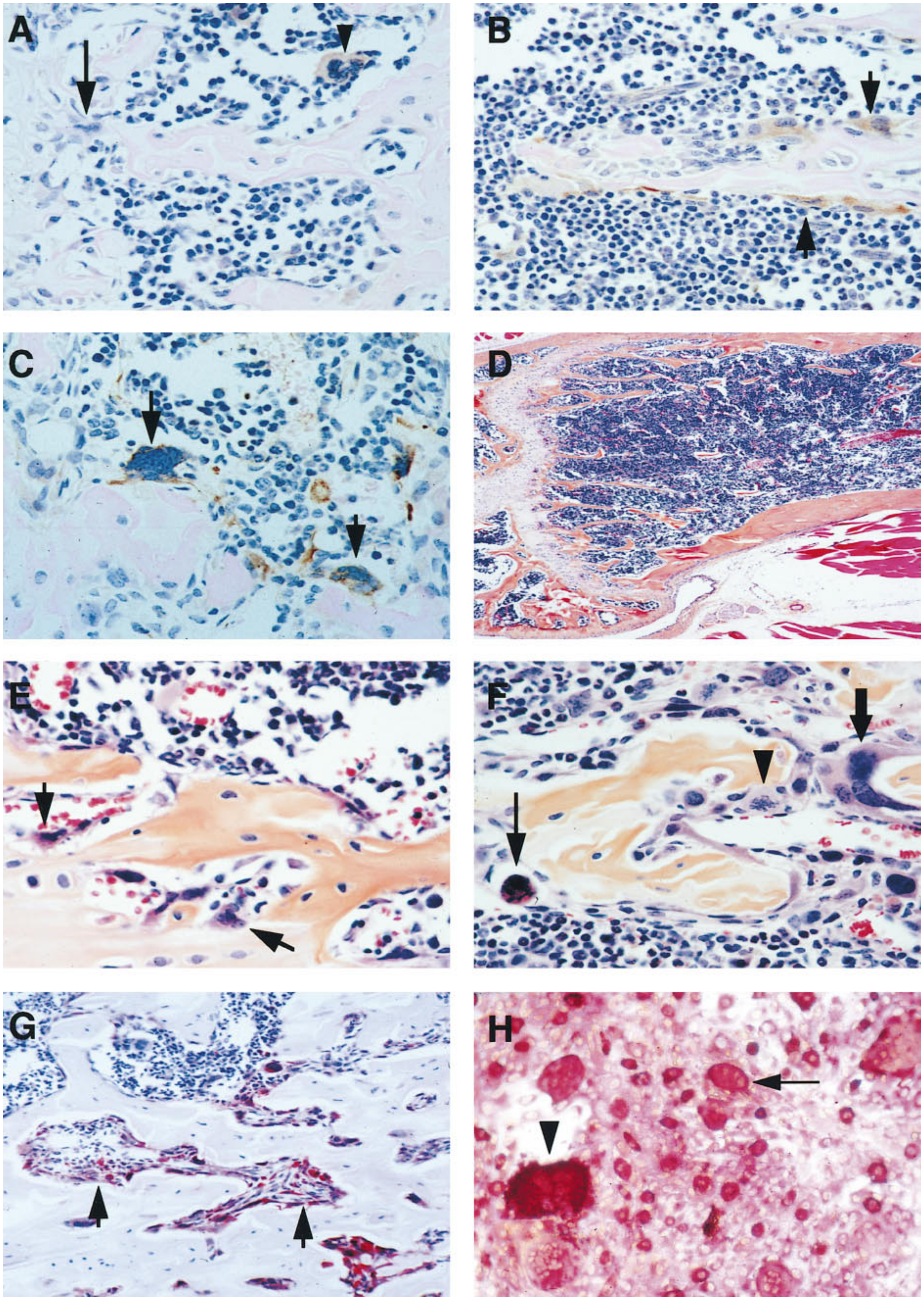

Figure 3. (*A*–*C*) Immunostaining for Bcl-X_L in decalcified sections of proximal tibiae. (*A*) A moderately strong signal for *Bcl*-X_L is seen in the cytoplasm of a megakaryocyte (*arrowhead*) in the bone marrow of this normal littermate of *Bcl*-X_L transgenic mice, but no signal is seen in the cytoplasm of osteoclasts (*arrow*) on the bone surface in the primary spongiosa. Primary antibody dilution, 1/50; hematoxylin and eosin (H & E) counterstain; original magnification 100×. (*B*) A strong signal for *Bcl*-X_L is seen in the cytoplasm of osteoclasts (*arrows*) along the bone surface in a *bcl*-X_L transgenic mouse. Primary antibody dilution, 1/25; H & E counterstain; original magnification 100×. (*C*) A stronger signal for *Bcl*-X_L

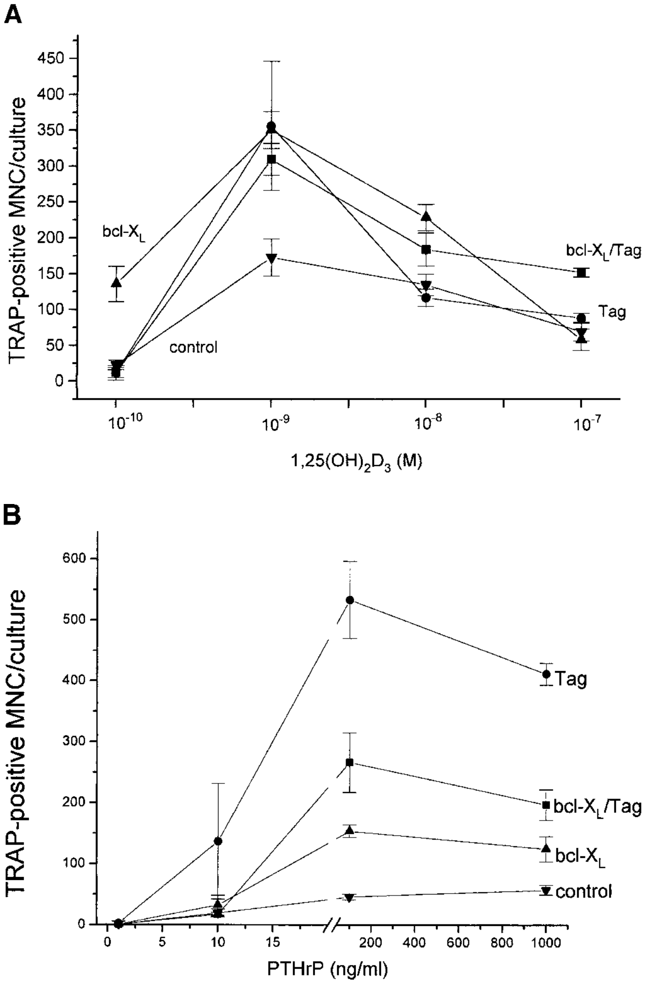

Figure 4. Effects of 1,25-$(OH)_2D_3$ (*A*) and PTHrP (*B*) on osteoclast-like cell formation in the cultures of marrow obtained from transgenic mice. Marrow cells were isolated and cultured for osteoclast formation as described in Methods. The results are of a representative experiment done in quadruplicate. A similar pattern of results was seen in three independent experiments.

Table III. Effects of IL-1, 1,25-$(OH)_2D_3$, Calcitonin, and Dexamethasone on MNC Formation by bcl-X_L/Tag Cells

Treatment	MNC/2,000 Bcl/Tag/ cells plated
Control	1.3±0.3
1,25-$(OH)_2D_3$ (10^{-9} M)	25.3±0.5[§]
1,25-$(OH)_2D_3$ + calcitonin (50 ng/ml)	13.8±1.0[‡]
Dexamethasone (10^{-6} M)	6.8±0.8*
1,25-$(OH)_2D_3$ + dexamethasone	201.5±11.2[§]
IL-1β (10 ng/ml)	8.5±0.6[‡]

Bcl-X_L/Tag cells (2,000 cells/well) were cultured with PA6 cells in the presence of varying concentrations of IL-1 (10 ng/ml), salmon calcitonin (50 ng/ml), dexamethasone (10^{-6} M), or 1,25-$(OH)_2D_3$ (10^{-9} M) for 7 d. The cultures were fixed, and the number of TRAP-positive MNC was scored. MNC contained 6±1 nuclei per cell. Results represent the mean±SEM for four determinations from a typical experiment. *$P < 0.05$ compared to control. [‡]$P < 0.05$ compared to control. [§]$P < 0.001$ compared to control.

derived from *bcl*-X_L/Tag doubly transgenic mice have now been maintained in continuous culture for more than 18 mo, and on repeated assays at least 30–50% of these marrow cells are incorporated into osteoclasts that resorb bone (Table II, Fig. 3 *H*, Fig. 5), reflecting the increased survival of OCL precursors compared with nonosteoclastic marrow cells in these cultures with passage. This enhanced survival of OCL precursors resulted in enrichment of OCL precursors as the cells were passaged. Treatment of cultures of passaged *bcl*-X_L/Tag cells with 1,25-$(OH)_2D_3$ demonstrated that they formed large numbers of OCL, and that calcitonin markedly inhibited OCL formation by these cells (Table III). Furthermore, IL-1 modestly enhanced formation of OCL by *bcl*-X_L/Tag cells (Table III).

RT-PCR and Southern blot analyses of the PCR products demonstrated that *bcl*-X_L/Tag cells did not express calcitonin receptors unless they were stimulated with 1,25-$(OH)_2D_3$. Both *bcl*-X_L/Tag cells and normal mouse marrow cells cultured with 1,25-$(OH)_2D_3$ expressed the C1a isoform of calcitonin receptor. None of these cultures expressed the C1b isoform of calcitonin receptor (Fig. 6). Autoradiographic studies demonstrated that > 95% of MNC formed by *bcl*-X_L/Tag cells expressed calcitonin receptor at levels similar to MNC formed in marrow cultures from normal mice (Fig. 7, *A–C*).

When primary bone marrow cells obtained from the various classes of transgenic mice or nontransgenic littermates were preincubated in α-MEM without serum for varying periods of time, the viability of the total marrow cells present was decreased over time in a similar manner (data not shown).

Figure 3 legend (Continued)

is seen in the cytoplasm of osteoclasts (*arrows*) in the *bcl*-X_L/Tag doubly transgenic mouse than in the cytoplasm of osteoclasts in *bcl*-X_L transgenic mice despite a doubling of the primary antibody dilution (1/50). Note also the larger size of the osteoclasts in these mice (*F*). H & E counterstain; original magnification 100×. (*D* and *E*) Proximal humerus of a 6-wk-old bcl-X_L transgenic mouse. (*D*) The appearances of the epiphysis, metaphysis, and cortical bone are indistinguishable from those of normal littermates. H & E; original magnification 6.5×. (*E*) Osteoclasts (*arrows*) at the epiphyseal plate appear normal, and have several nuclei with regular shape and size. H & E; original magnification 100×. (*F*) Osteoclasts in a bcl-X_L/Tag doubly transgenic mouse. Morphologically transformed (i.e., having enlarged nuclei with dense irregular chromatin and multiple large nucleoli; *thick arrow*), mitotic (*arrowhead*), and apoptotic (*thin arrow*) osteoclasts similar to those reported previously in Tag transgenic mice (1) are present on the bone surface of this 6-wk-old mouse. H & E; original magnification 100×. (*G*) TRAP-stained section of proximal humerus of a bcl-X_L/Tag doubly transgenic mouse. TRAP-positive osteoclasts are seen adjacent to bone surfaces near the epiphyseal plate of an 8-wk-old mouse. Sheets of TRAP-positive mononuclear cells (*arrows*) have replaced normal hematopoietic cells in parts of the section. Hematoxylin counterstain; original magnification 50×. (*H*) TRAP staining of cultured bcl-X_L/Tag cells. *Bcl*-X_L cells were cultured in the presence of PA6 stromal cells, 1,25-$(OH)_2D_3$ and dexamethasone for 1 wk. Numerous TRAP-positive multinucleated cells (*arrow*), some with intense staining (*arrowhead*), and TRAP-positive mononucleated cells are present. Original magnification 200×.

Figure 5. Formation of resorption lacunae by MNC formed by *bcl*-X_L/Tag cells cocultured with 1,25-$(OH)_2D_3$ and dexamethasone on PA6 cells. Magnification 50×.

However, osteoclast precursor survival showed distinct differences after prolonged exposure to serum-free conditions. The number of OCL formed by nontransgenic marrow mononuclear cells preincubated in serum-free conditions decreased steadily, and was 2.4% of the initial value after 96 h of serum-free preculture (Fig. 8). In contrast, marrow cells from *bcl*-X_L mice after 48 h of serum-free preculture retained their capacity to form OCL, but then began progressively to lose this capacity. OCL formation in cultures of *bcl*-X_L marrow that were preincubated in serum-free conditions was modestly better than results with nontransgenic marrow cultures. OCL formation in cultures of Tag marrow cells preincubated for up to 48 h in serum-free conditions initially increased, but after 96 h of serum-free preculture, was decreased by 32% compared with the initial value. In contrast, the relative proportion of osteoclast precursors in the viable marrow cells remaining from *bcl*-X_L/Tag mice after 96 h of preincubation increased sixfold compared with the initial value (Fig. 8).

To determine if the increased OCL formation capacity of *bcl*-X_L/Tag marrow cells in serum-free cultures was due to resistance to apoptosis vs. increased proliferation of the marrow cells, marrow cells from *bcl*-X_L/Tag mice were labeled with [^{3}H]thymidine, preincubated for 48 h in serum-free media, and then assayed for their capacity to form OCL. Approximately 2% of the nuclei in the OCL formed in *bcl*-X_L/Tag cultures were labeled, as compared with 4% for Tag mice, indicating that an increase in proliferation did not account for the increased numbers of OCL formed in *bcl*-X_L/Tag cultures.

To determine if the survival advantage seen in OCL precursors from *bcl*-X_L/Tag mice was also conferred to multinucleated OCL formed from those precursors, the percentage of mature OCL undergoing apoptosis in marrow cultures from these transgenic mice was then determined. Based on the morphology and nuclear fragmentation patterns in OCL formed in the cultures, no differences were detected in the percentage of apoptotic OCL under basal conditions between control and transgenic mice of the various classes (control, 31±9%; *bcl*-X_L, 29±7%; Tag, 32±5%; *bcl*-X_L/Tag, 31±4%). Adding 10^{-5} M risedronate, a potent inducer of OCL apoptosis (21), induced a similar percent of apoptotic OCL in marrow cultures from control and all classes of transgenic mice (control, 60±5%; *bcl*-X_L, 50±8%; Tag, 60±7%; *bcl*-X_L/Tag: 54±4%), further indicating that no survival advantage was conferred to mature OCL.

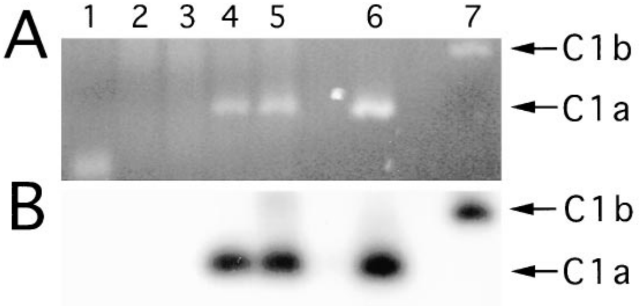

Figure 6. Expression of calcitonin receptors by *bcl*-X_L/Tag cells. (*A*) RT-PCR; (*B*) Southern blot. Negative water control (lane *1*); RNA samples from MC3T3-G2/PA6 stromal cells alone (lane *2*); untreated *bcl*-X_L/Tag cells (lane *3*); *bcl*-X_L/Tag cells treated with 1,25-$(OH)_2D_3$ and dexamethasone (lane *4*); and normal mouse marrow cocultured with stromal cells and 1,25-$(OH)_2D_3$ (lane *5*). Both the treated *bcl*-X_L/Tag cells and normal mouse marrow expressed predominantly the C1a isoform of the murine calcitonin receptor. Positive controls, C1a (lane *6*) and C1b (lane *7*) isoforms of the murine calcitonin receptor.

Discussion

Targeting *bcl*-X_L and Tag to cells in the OCL lineage allowed us to immortalize OCL precursors that can form OCL at high efficiency. Tissue-specific targeting of SV40 Tag expression in transgenic mice has been used to transform many cell types, permitting the establishment of a variety of novel cell lines (22–25). Before our current studies, immortalized OCL precursors that form OCL at high efficiency and resorb bone had not been described. We previously targeted Tag expression to OCL of transgenic mice using the murine TRAP promoter (1). While mTRAP-Tag transgenic mice exhibited mitotic and transformed OCL, we were unsuccessful in immortalizing OCL precursors from the bone marrow of these mice (1). Because we observed a significant number of apoptotic OCL in the bones of these mice, we postulated that Tag expression had induced apoptosis concomitantly with the transformation of OCL. High levels of apoptosis have also been observed in other Tag-expressing cell types in transgenic mice (26–28), despite presumptive inactivation of p53 by Tag. We therefore thought that introducing an apoptosis-inhibiting gene might block Tag-induced apoptosis, and permit immortalization of OCL precursors. To this end, we generated mTRAP-*bcl*-X_L transgenic mice, and interbred these mice to the mTRAP-Tag mice to create *bcl*-X_L/Tag doubly transgenic mice. Immunohistochemical and histomorphometrical analyses confirmed that we had targeted *bcl*-X_L to OCL, and that the *bcl*-X_L/Tag and Tag mice had mitotic and transformed OCL.

Culture studies of marrow from the various transgenic cell lines demonstrated that targeting *bcl*-X_L and/or Tag to cells in the OCL lineage increased the number of OCL precursors. Marrow cells from TRAP-Tag, TRAP-*bcl*-X_L, and TRAP-*bcl*-X_L/Tag transgenic mice formed two times more osteoclasts in the presence of 10^{-9} M 1,25-$(OH)_2D_3$ compared with control mouse marrow cells, even though the sensitivity of marrow cells from TRAP-Tag, TRAP-*bcl*-X_L or TRAP-Tag/*bcl*-X_L mice to either 1,25-$(OH)_2D_3$ or PTHrP did not differ from nontransgenic littermate controls.

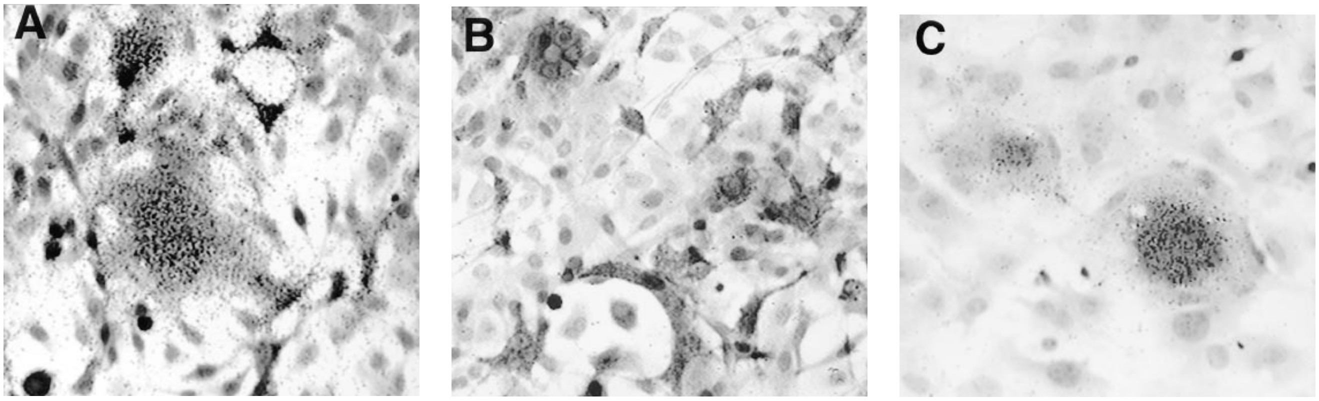

Figure 7. Autoradiography of MNC formed by *bcl*-X_L/Tag cells in the presence of 1,25-$(OH)_2D_3$ demonstrated that they express calcitonin receptors (*A*), and that binding of [^{125}I]salmon calcitonin to the MNC was competed by excess unlabeled calcitonin (*B*). Similar levels of expression for calcitonin receptors were detected in multinucleated cells formed from normal mouse marrow cultures (*C*).

However, expression of *bcl*-X_L and Tag together, rather than either alone, enhanced survival of OCL precursors. Culture of transgenic marrow cells from these different transgenic mouse strains in serum-free conditions, to deprive the cells of growth factors, demonstrated that OCL precursors from *bcl*-X_L/Tag mice persisted longer in vitro than OCL precursors from *bcl*-X_L and Tag mice. This survival advantage was not conferred to the total marrow cell population, but only to the OCL precursor subpopulation, since total marrow cell numbers from transgenic mice and control mice decreased in serum-free media at the same rate. OCL precursors are a very small subpopulation of total marrow cells (~ 0.15%), and differences in viability of this subpopulation would not be readily apparent by simply measuring total cell viability. However, when OCL precursors were assayed by their capacity to form OCL, we found that OCL precursors in *bcl*-X_L/Tag marrow cells were progressively enriched after 96 h of serum-free preincubation. In contrast, significantly fewer OCL precursors survived in serum-free cultures of marrow cells from *bcl*-X_L, Tag, or control mice after 96 h.

Targeting the antiapoptotic *bcl*-X_L gene to OCL by itself did not have a major effect on the survival of osteoclast precursor cells when cocultured with osteoblastic cells. Instead of 1 mo (survival of control marrow cells), OCL precursors from *bcl*-X_L marrow survived for ~ 2 mo in continuous culture. Tag alone was clearly superior in its capacity to enhance survival of OCL precursors compared with *bcl*-X_L, since these cells survived for 4 mo. However, when both Tag and *bcl*-X_L were targeted to cells in the OCL lineage, the resulting OCL precursors had a greatly increased survival capacity. Cells from the doubly transgenic mice have now been continuously cultured for more than 18 mo, and they continue to form OCL that resorb bone. This enhanced survival capacity of OCL precursors from *bcl*-X_L/Tag mice compared with other marrow cell types resulted in progressive enrichment of OCL precursors as the marrow cells were passaged.

Histomorphometry demonstrated that *bcl*-X_L/Tag mice had significantly more TRAP-positive mononuclear cells compared with nontransgenic littermates, Tag mice, or *bcl*-X_L mice, consistent with our in vitro findings of increased OCL precursor numbers and survival in *bcl*-X_L/Tag mice. Since TRAP is an in vivo marker enzyme for cells in the OCL lineage, these data suggest that OCL precursors were increased in *bcl*-X_L/Tag mice in vivo. OCL numbers in vivo in transgenic mouse bones and control mouse bone were about the same, suggesting that OCL in *bcl*-X_L and *bcl*-X_L/Tag mice do not live longer than OCL in nontransgenic littermates or in Tag mice, and that the survival advantage of *bcl*-X_L/Tag genes occurs predominantly at the OCL precursor stage of differentiation. These data were consistent with our in vitro OCL apoptosis assay. In all our three transgenic mouse lines and in nontransgenic littermates, the percentage of OCL undergoing apoptosis in marrow cultures was ~ 30%, and was increased to 50–

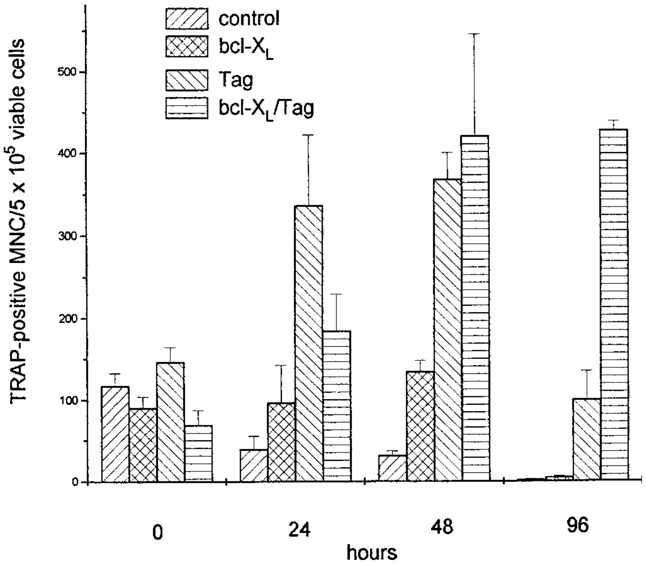

Figure 8. OCL formation capacity of OCL precursors obtained from transgenic mice in serum-free culture for up to 96 h. Marrow cells from control and transgenic mice were precultured in serum-free media for up to 96 h. At 0, 24, 48, and 96 h, aliquots of the cells were tested for their viability by Trypan blue exclusion, and then 5 × 10^5 viable cells were assayed for their capacity to form OCL as described in Methods. Results represent the mean±SEM for quadruplicate determinations. A similar pattern of results was seen in three independent experiments.

60% by adding 10^{-5} M risedronate in a similar manner in all these groups.

The OCL formation capacity of *bcl*-X_L/Tag cells is approximately 300–500 times higher than that of normal mouse marrow, with 30–50% of *bcl*-X_L/Tag cells being incorporated into OCL when cocultured with PA6 cells and 10^{-7} M dexamethasone and 10^{-9} M 1,25-$(OH)_2D_3$, and appears superior to previously reported OCL precursor cell lines developed from murine sources. The MNC, which were generated in these cultures, formed resorption lacunae on dentine, and also expressed the C1a isoform of the calcitonin receptor. The C1a isoform of the calcitonin receptor is the predominant isoform of the receptor expressed in murine osteoclasts (29). Additionally, the MNC that formed expressed calcitonin receptors at similar levels as normal marrow cells. Further, calcitonin inhibited OCL formation by *bcl*-X_L/Tag cells to a similar degree as normal marrow cells. Takahashi and coworkers (16) reported that calcitonin inhibited OCL by normal marrow cultures by ~ 50%. Hence, the MNC express the characteristics of OCL. However, preliminary experiments suggest that *bcl*-X_L/Tag cells respond differently to some osteotropic factors than do normal marrow cells. For example, IL-1 is not a potent stimulator of OCL formation by *bcl*-X_L/Tag cells, and prostaglandin E2 does not induce OCL formation by *bcl*-X_L/Tag cells.

Chambers and coworkers (30) also developed OCL precursor cell lines from mice transgenic for temperature-sensitive Tag driven by interferon-inducible MHC complex H-$2K^b$ promoter. The initial OCL formation capacity of these cell lines was 2.5% (Chambers, personal communications). However, these M-CSF and IFN-dependent OCL precursor cell lines were not committed to the OCL lineage, and eventually lost their OCL characteristics. Similarly, two murine hematopoietic cell lines—FDCPA4 (31) and FDCP-C2-GM (32)—form low numbers of resorbing OCL when cocultured with either stromal cells or fetal bone rudiments in the presence of 1,25-$(OH)_2D_3$. The murine macrophage cell line BDM-1 also has been reported to have a 10% subpopulation of F4/80-negative cells that effectively formed OCL. The OCL formation capacity of this cell line was reported to be 5–9% (33).

To conclude, our data show that concomitant expression of *bcl*-X_L and Tag in OCL precursors enhances their survival, permitting us to produce immortalized OCL precursors that form OCL with high efficiency. Although *bcl*-X_L/Tag cells do not respond to all osteotropic factors in a similar manner as normal marrow cells, the high levels of OCL formation induced by 1,25-$(OH)_2D_3$ and dexamethasone, and inhibition of OCL formation by calcitonin, suggest that *bcl*-X_L/Tag cells should be useful to characterize further the molecular events associated with OCL development.

Acknowledgments

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