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Characteristics of Clodronate-Induced Apoptosis in Osteoclasts and Macrophages

KATRI S. SELANDER, JUKKA MÖNKKÖNEN, EEVA-KAISA KARHUKORPI, PIRKKO HÄRKÖNEN, RITVA HANNUNIEMI, and H. KALERVO VÄÄNÄNEN

Department of Anatomy and Biocenter Oulu, University of Oulu, Oulu, Finland (K.S.S., E.-K.K., H.K.V.), Department of Pharmaceutics, University of Kuopio, Kuopio, Finland (J.M.), Department of Anatomy and Medicity, University of Turku, Turku, Finland (P.H.), and Leiras Biomedical Research Center, Leiras (H.K.V.), Finland

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SUMMARY

Bisphosphonates (BPs), such as clodronate and pamidronate, are inhibitors of bone resorption and are used on a widespread basis in the treatment of hyper-resorptive bone diseases. At the cellular level, BPs inhibit osteoclasts, but the precise molecular mechanisms are unclear. BPs have also been shown to affect the survival of macrophages, cells ontogenetically related to osteoclasts. We show that both clodronate and pamidronate induce apoptosis in isolated osteoclasts. Clodronate, when administered in liposomes, also induced apoptosis in rat peritoneal macrophages *in vitro* and in liver macrophages of mice *in vivo* but not in murine macrophage-like RAW-264 cells. The subcellular localization and staining intensity of Bcl-2, an anti-apoptotic protein known to protect several cell types against drug-induced apoptosis, were similar in RAW-264 and peritoneal macrophage cells, as revealed by immunofluorescence. The clodronate-induced apoptotic pathway was further characterized in isolated osteoclasts cultured on glass coverslips through the use of clodronate-containing liposomes and several inhibitors of the apoptotic cascade. None of the agents tested could totally prevent clodronate-induced osteoclast death. Partial protection was, however, obtained by the addition of staurosporine or homocysteine. The results suggest that primarily cytoplasmic, protein kinase C-activated mechanisms are involved in the execution of clodronate-induced apoptosis of osteoclasts.

BPs, such as clodronate and pamidronate, are pyrophosphate analogs that bind with high affinity to bone hydroxyapatite (1). They are potent inhibitors of osteoclast-mediated bone resorption and clinically used on a widespread basis in the treatment of metabolic bone diseases associated with accelerated bone resorption, such as Paget's disease, hypercalcemia of malignancy, and osteoporosis (2). The precise molecular mechanism behind the action of these compounds is not known, and it probably varies among BP molecules (3–7). The effects of clodronate and pamidronate, however, have been linked to osteoclast death (3, 7). The results of several studies suggest that the specificity of BPs to osteoclasts is due to the high affinity of these compounds to bone hydroxyapatite and thereby to the delivery of the drug into osteoclasts via resorption (3, 5, 7). Although highly hydrophilic, clodronate and pamidronate are endocytosed by macrophages when encapsulated in liposomes, which has been shown to result in the elimination of these cells *in vivo* and *in vitro* (8–11).

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In studying the mechanism of action of clodronate and pamidronate, we set out to determine whether the mode of death induced by these drugs is apoptosis, which is an evolutionarily well-conserved pathway of death and an active process resulting in self-destruction of the cell (for a review, see Ref. 12). Apoptosis is necessary for normal development and maintenance of tissue homeostasis (12). It is the end result of the use of radiation therapy as well as of many cancer drugs in their target cells (13–15). There also is accumulating evidence that cellular damage induced by a wide range of physiological and toxicological agents may induce common signals that initiate the apoptotic cascade (16, 17). Cells undergoing apoptosis exhibit typical morphological features, including cytoplasmic shrinkage and nuclear fragmentation. Ultrastructurally, apoptotic cells show membrane blebbing, chromatin condensation, and margination at the periphery of nuclei, whereas mitochondria and other cytoplasmic organelles remain intact (12). Biochemical features of apoptosis in many cells also include internucleosomal DNA digestion (18, 19). Finally, apoptotic bodies are eliminated by phagocytes or neighboring cells without initiation of an in-

ABBREVIATIONS: BP, bisphosphonate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TEM, transmission electron microscopy; TUNEL, TdT mediated dUTP-biotin end labeling; TRAP, tartrate-resistant acid phosphatase; PMA, phorbol-12-myristate 13-acetate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATA, aurintric carboxylic acid; AI, apoptosis index; TdT, terminal deoxynucleotidyl transferase.

flammatory response (12). Despite these well-characterized morphological and biochemical features of apoptosis, relatively little is known about the precise molecular events by which this form of cell death is executed.

The effects of clodronate and pamidronate were studied by using the well-established pit-formation assay with bone precoated with these agents as a substrate for osteoclasts (3, 5, 7). We further characterized clodronate-induced changes in osteoclasts by delivering the highly hydrophilic drug to the cells in liposomes. The liposomal delivery of the drug was chosen because it has been shown earlier that BPs that are free in solution (i.e., without bone in the cultures) do not readily affect osteoclasts (3). Macrophages are ontogenetically related to osteoclasts and under certain conditions share functional homology with these primary bone-resorbing cells (20, 21). Because BPs have also been shown to affect macrophages, we compared the response to clodronate in osteoclasts with those in isolated peritoneal macrophages (p.m. cells) and in murine macrophage-like RAW-264 cells (8–11, 20, 21). For *in vivo* studies, the well-characterized "macrophage elimination technique," originally introduced by Van Rooijen and Van Nieuwmegen, was used (8). With this technique, the intravenous administration of clodronate-containing liposomes results in rapid destruction of spleen and liver macrophages (8, 9). Our aim was to characterize whether this phenomenon is also the result of apoptosis.

Materials and Methods

Tissue culture materials and BPs. Clodronate and pamidronate were donated by Leiras Biomedical Research Center, Turku, Finland. Kit 387-A (to stain TRAP), Hoechst 33258, and all tissue culture materials were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The CellTiter Aqueus 96 Kit for cell viability assays was from Promega (Madison, WI), Lab-Tek Chamber Slides were from Nunc (Naperville, IL), and the ApopTaq Kit from Oncor (Gaithersburg, MD).

Unilamellar distearoylphosphatidylglycerol liposomes were prepared by reversed-phase evaporation from phospholipid/cholesterol (67:33) and from a 200 mM stock solution of clodronate, as described previously in detail (10).The drug and the phospholipid contents of the obtained liposomes were analyzed spectrophotometrically and by phosphorus analysis, respectively, as described previously (11, 22). In all liposome experiments, the phosphorus content of the nonloaded liposomes was adjusted to match that of the corresponding concentration in the clodronate liposomes.

Slices of devitalized bovine cortical bone, used as substrate for osteoclastic bone degradation, were precoated with BPs as described previously (7). In brief, bone slices (0.5 cm^2) were incubated for 30 min in 1 ml of 10 mM clodronate or 10 mM pamidronate (diluted in deionized H_2O, pH adjusted to 7.4 with NaOH, and filter sterilized) at room temperature, followed by a rinse in d-H_2O for 5 min.

Osteoclast cultures. The experiments were performed while following the small animal protocols approved by the Faculty of Medicine, University of Oulu, Oulu, Finland. The procedure for osteoclast isolation and culture on clodronate- or pamidronate-pretreated bone slices has been described in detail (3, 7). Briefly, mechanically harvested osteoclasts from the long bones of 1–2-day-old rat pups were allowed to attach to clodronate- or pamidronate-covered or control slices of cortical bovine bone for 30 min. A longer time of attachment (60 min) was allowed for osteoclasts to be cultured on glass coverslips. The cells on the bone slices or on the coverslips were then transferred to wells containing 0.5 ml (coverslips) or 1 ml (bone slices) of plain medium (Dulbecco's modified Eagle's medium buffered with 20 mM HEPES and containing 0.84 g/liter sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum). All cultures used in the current study were mixed cell populations that contain osteoclasts as well as other cell types from the bone marrow environment. To the wells containing coverslips, 0.5 ml of medium with or without liposomes containing 2×10^{-5} to 10^{-7} M clodronate or a corresponding concentration of nonloaded liposomes was added. In further studies, 10^{-5} M clodronate-containing liposomes were added to the bone-derived cells on glass coverslips simultaneous with protein kinase C inhibitor staurosporine (1 nM); the intracellular chelator of Ca^{2+}, BAPTA (25 μM); protein kinase C agonist PMA (10 nM); nuclease inhibitors $ZnSO_4$ (100 nM) or ATA (10 μM); tyrosine kinase inhibitors herbimycin (100 nM) or genistein (100 nM); the microtubule-stabilizing agent taxol (100 nM paclitaxol); or 1 mM homocysteine. These tested agents and concentrations have been shown to interfere with various apoptotic pathways in other cells (23–26). In the current study, all cell culture experiments were carried out at 37° in 95% air/5% CO_2. The cultures were terminated after 4, 6, 12, or 24 hr by fixing the cells with 3% PFA in PBS for 5 min at room temperature, followed by a rinse in PBS (twice for 5 min). The cells were then stained for TRAP, which is an enzyme commonly accepted as a marker for osteoclasts (27), for 10 min at 37° according to the manufacturer's instructions. After a brief rinse in d-H_2O, the TRAP-stained specimens were subjected to Hoechst 33258 staining (1 mg/ml stock diluted 1:800 in PBS) for 5 min at room temperature to counterstain nuclei. Specimens were finally rinsed for 3 min in d-H_2O. In some specimens, F-actin was stained with rhodamine-labeled phalloidin (R-PHD, Molecular Probes, Eugene, OR) at 5 units/ml before the TRAP and Hoechst 33258 stainings, as described previously in detail (7). Samples were then mounted in PBS-glycerol and viewed with conventional and UV light under appropriate filters in a Leitz Ariztoplan microscope. The numbers of morphologically apoptotic and intact osteoclasts remaining attached to the culture substratum (bone slices or coverslips) were enumerated microscopically using the 25× objective. The AI of osteoclasts was calculated as the percentage of apoptotic osteoclasts [i.e., osteoclasts with fragmented nuclei and shrunken, highly vacuolized cytoplasm (12, 23)] of the total number of TRAP-positive, multinucleated cells.

TEM. TEM was carried out on cells derived from bone cell suspensions (described above). Aliquots of 100 μl were allowed to attach to the wells of chamber slides for 1 hr, followed by the addition of 400 μl of medium containing 10^{-5} mol/liter concentrations of clodronate- or nonloaded liposomes. After being in culture for 6 hr, the cells were immediately fixed in 2% glutaraldehyde after gentle removal of the medium, washed in PBS, osmicated, and dehydrated in a series of graded alcohols before being suspended in Epon 812 resin and polymerized at 60° overnight. Ultrathin sections of 100 nM were collected on copper grids, stained with uranyl acetate and lead citrate, and observed under a Philips 410 LS transmission electron microscope.

Macrophage cultures. Peritoneal macrophages were collected from peritonea of 1–2-week-old rats after the animals were killed with CO_2. Briefly, 5 ml of a 0.34 M concentration of ice-cold saccharose was injected into the abdominal cavity; the peritoneum was massaged; and the peritoneal fluid containing the cell suspension was collected in a sterile centrifuge tube. The cells were spun down (5 min at 400 × *g* at 4°) and resuspended in culture medium (the same as for bone cells). Aliquots of 100 μl were allowed to attach to glass coverslips for 1 hr, followed by the addition of 0.9 ml of the same medium. Murine macrophage-like RAW-264 cells were cultured on glass coverslips (100 μl/coverslip) in 1 ml of culture medium (Dulbecco's modified Eagle's medium buffered with 0.84 g/liter sodium bicarbonate and supplemented with 2 mM glutamine, 100 IU/liter penicillin, 100 μg/liter streptomycin, and 10% heat-inactivated fetal calf serum). To the wells of both p.m. and RAW-264 cell cultures, soluble clodronate (10^{-5} to 10^{-7} M), clodronate-containing (10^{-5} to 10^{-7} M) liposomes, or nonloaded liposomes were added. After being in culture for 24 hr, both the p.m. and RAW-264 cells were fixed with 3% PFA in PBS and stained for acid phosphatase (with

Sigma Kit 387-A) and with Hoechst 33258, as described above. The numbers of morphologically apoptotic macrophages and those with intact nuclei that were attached to the coverslips were enumerated microscopically from five random fields (~150 cells/coverslip) using the 25× objective and Hoechst filter. The AI values for both p.m. and RAW-264 cells were counted as percentages of apoptotic cells of the total number of cells that were viewed.

Immunofluorescent detection of Bcl-2. The possible role of Bcl-2 in clodronate-induced cell death was studied with immunofluorescence because overexpression of this protein can alter the cellular response to drug-induced death (17, 28). After 24 hr of culture, RAW-264 or p.m. cells were fixed with 2% PFA in PBS for 30 min on ice, rinsed twice in PBS (5 min each), permeabilized with 0.1% Triton X-100 in PBS containing 0.1% BSA, and rinsed in PBS three times for 5 min. The cells were blocked with 3% BSA in PBS for 30 min at room temperature, and the primary antibody (mouse monoclonal antibody to human Bcl-2, diluted 1:40 in 1% BSA/PBS; Dako, Glostrup, Denmark) was applied to the cells in an overnight incubation at 4°. The specimens were then rinsed three times in PBS for 5 min each. Bcl-2 was detected with rhodamine-conjugated rabbit anti-mouse antibody (diluted 1:100 in 1% BSA/PBS; Dako) for 1 hr at room temperature. After a final rinse in d-H_2O for 5 min, the nuclei were stained with Hoechst 33258 as described above.

***In vivo* studies.** Adult mice weighing 45–50 g were injected intravenously with 0.2 ml of 10 mM liquid clodronate (diluted in d-H_2O, pH adjusted to 7.4 with NaOH, and filter sterilized), 10.6 mM liposome-encapsulated clodronate, nonloaded liposomes, or vehicle (8, 9). Livers were removed at 3 hr after the injection and routinely processed into paraffin sections.

TUNEL assay. For the detection of internucleosomal DNA nicks in cells undergoing apoptosis, either on glass coverslips or in paraffin sections of livers, ApopTaq Kits were used according to the manufacturer's recommendations. Three types of controls were used to ensure the specificity of labeling of apoptotic nuclei: 1) cells or tissue sections were pretreated with DNase (20 μg/ml) for 30 min at 37° before the TUNEL reaction to obtain positive controls or 2) TdT enzyme or 3) fluorescein-conjugated anti-digoxigenin monoclonal antibody was omitted from the reaction to obtain negative controls (19). After the TUNEL reaction and for visualization of all nuclei, propidium iodide was added to the coverslips before being mounted in PBS/glycerol.

Statistical analyses. Values are reported as mean ± standard error, and they represent the combined results of two or three independent experiments. In a typical control experiment, ~100-150 osteoclasts were detected per control bone slice or coverslip. Differences between groups were assessed by Student's two-tailed t test, and a value of $p < 0.05$ was considered statistically significant.

Results

Osteoclast cultures on bone. Staining the cells on both bone slices and glass coverslips (for TRAP and with Hoechst 33258) allowed us to specifically view the nuclear integrity of osteoclasts (Fig. 1). Simultaneous F-actin detection allowed us to also visualize the cytoskeleton of osteoclasts. In resorbing and intact osteoclasts, actin was typically seen in ringlike structures (7). In apoptotic osteoclasts, both in controls and BP-treated groups on bone slices or on glass coverslips, actin cytoskeleton was disorganized or almost undetectable (Fig. 2). The numbers of intact and morphologically apoptotic osteoclasts attached to the bone slices were counted microscopically. There was no difference in the osteoclast AI between the BP groups and the controls after 4 hr of culture (the AI values of osteoclasts after 4 hr of culture were 2.2 ± 0.5, 2.1 ± 0.6, and 3.1 ± 0.5 in control, pamidronate, and clodronate groups, respectively; mean ± standard error). After 6 hr of culture and later, the percentage of morphologically apoptotic osteoclasts was significantly higher for clodronate- and pamidronate-covered bone slices, whereas on control bone slices, the AI for osteoclasts remained at the same level throughout the culture (AI for osteoclasts: 2.3 ± 0.4, 4.5 ± 0.6, and 6.1 ± 0.7; 3.2 ± 0.4, 6.2 ± 0.5, and 22.1 ± 1; and 2.3 ± 0.3, 5.3 ± 0.7, and 13.2 ± 1.9 for control, pamidronate, and clodronate groups at 6, 12, and 24 hr, respectively; mean ± standard error; clodronate and pamidronate versus controls, $p < 0.05$ at 6, 12, and 24 hr). Of the two BPs

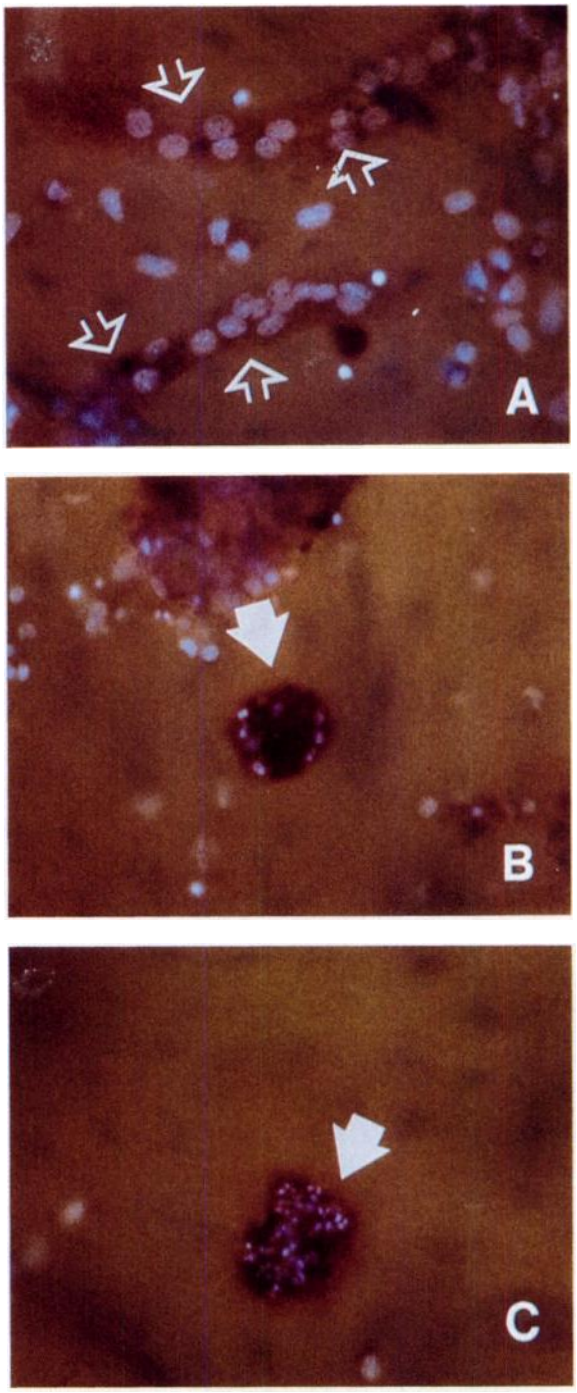


Fig. 1. TRAP- and Hoechst 33258-stained osteoclasts on (a) control and (b) pamidronate- and clodronate- (c) covered bone slices after 24 hr of culture. *Open arrows*, intact osteoclasts. *Filled arrows*, apoptotic osteoclasts. Magnification, 340×.

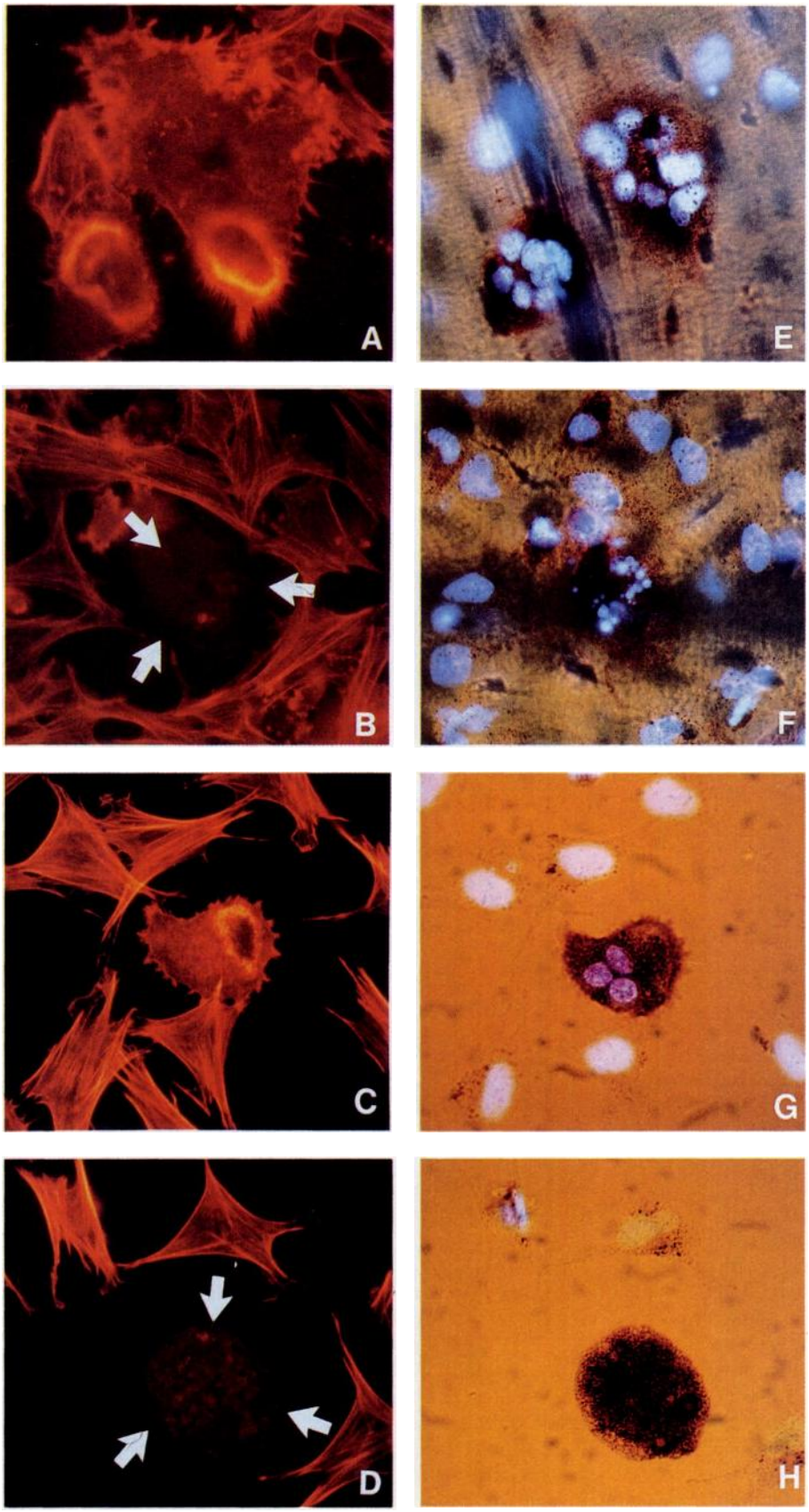
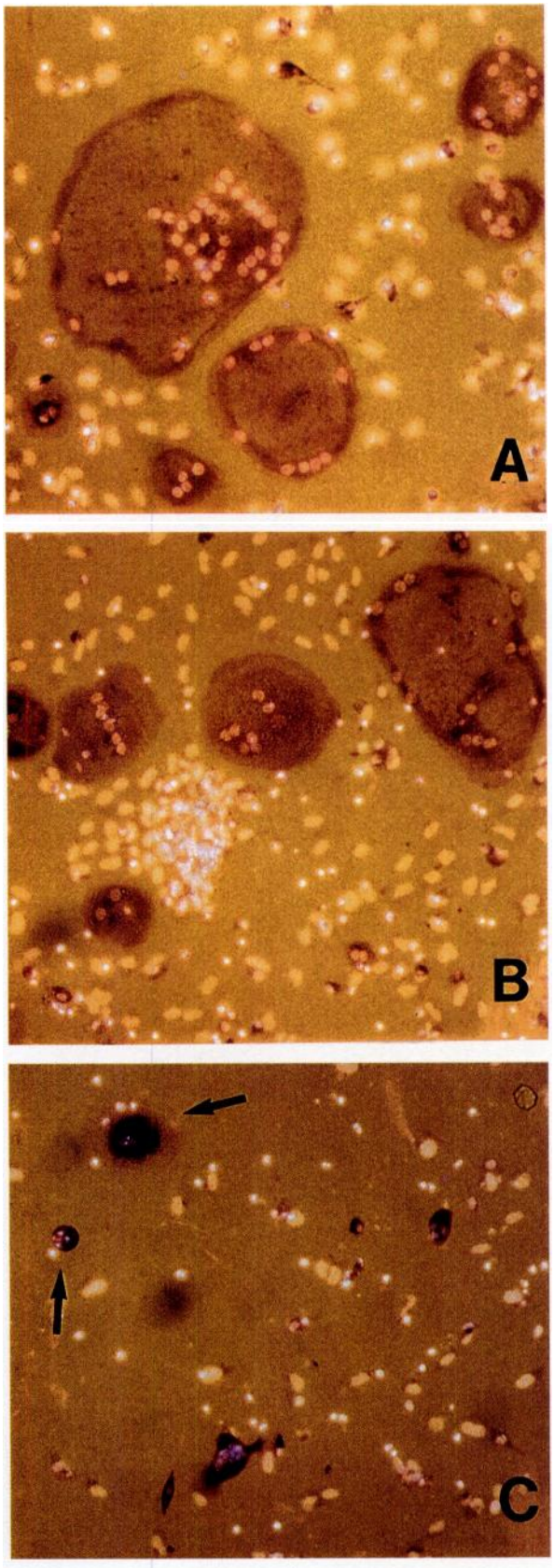


Fig. 2. Rhodamine/phalloidin-stained osteoclasts on (A and B) bone slices and (C and D) glass coverslips after 24 hr of culture. E–H, TRAP- and Hoechst 33258-stained corresponding cells. Note the changes in apoptotic cytoskeleton, including (*arrows*, B and D) loss of definition and shrunken size. A, C, E, and G, Controls. B and F, Pamidronate-covered bone slice. D and H, Osteoclasts cultured with 10^{-5} M clodronate-liposomes. Magnification, 528×.



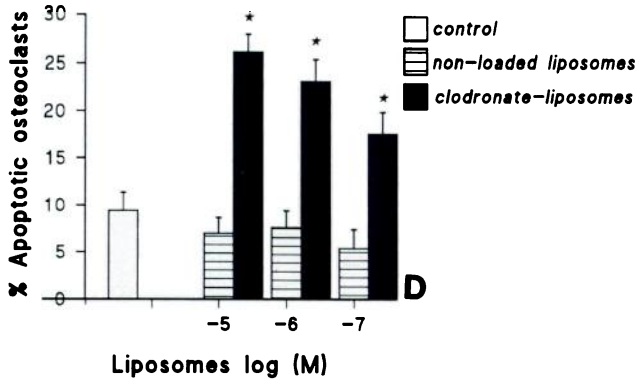


Fig. 3. TRAP- and Hoechst 33258-stained osteoclasts from the (a) control group or from cultures containing (b) nonloaded or (c) 10^{-5} M clodronate-containing liposomes. *Arrows*, apoptotic osteoclasts. Magnification, 133×. d, Percentage of apoptotic osteoclasts grown on glass coverslips with or without nonloaded or 10^{-5} to 10^{-7} M clodronate-containing liposomes. Cells were cultured for 24 hr and stained for TRAP and with Hoechst 33258, and the number of intact and apoptotic osteoclasts was counted microscopically. Data are mean ± standard error from five coverslips that were viewed in each group. *, $p < 0.05$, clodronate liposome group versus controls.

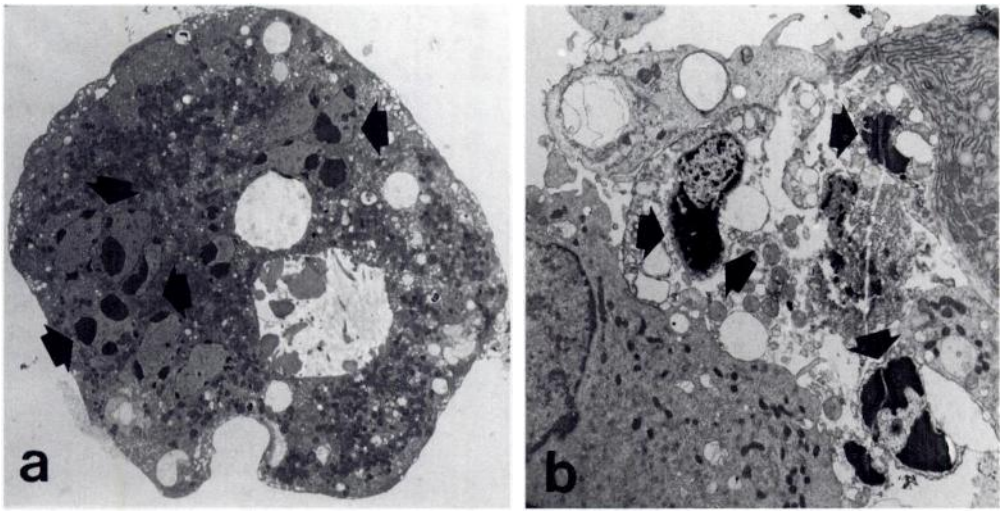


Fig. 4. Ultrastructure of apoptotic osteoclasts cultured on glass coverslips from (a) a control group and from (b) cultures containing 10^{-5} M clodronate liposomes, exhibiting a more advanced stage of degradation. *Arrows*, cytoplasmic vacuolization and condensation and margination of chromatin at the nuclear periphery. Magnification: a, 900×; b, 2400×.

examined, bone-bound clodronate was found to be a stronger inducer of osteoclast apoptosis.

Osteoclast cultures on glass coverslips. After culture for 24 hr, the cells cultured on glass coverslips were stained for TRAP and with Hoechst 33258, and the AI of osteoclasts determined counted microscopically. A significant, dose-dependent increase in the AI of osteoclasts was detected in the presence of 10^{-5} to 10^{-7} M clodronate-containing liposomes, whereas nonloaded liposomes did not affect the AI of osteoclasts relative to controls (Fig. 3). The cells from these cultures were also viewed by TEM after 6 hr of culture, and the typical ultrastructural findings of apoptosis (i.e., condensation of chromatin, fragmentation of the nucleus, and highly vacuolized cytoplasm with mainly intact organelles) were detected in the apoptotic osteoclasts (Fig. 4). On the basis of TEM observations, we did not detect any specific morphological features that could separate clodronate-induced apoptosis of osteoclasts from that seen in control experiments. Cells on glass coverslips were also subjected to the TUNEL method, which revealed internucleosomal DNA nicks in the nuclei of apoptotic osteoclasts (Fig. 5). Thus, morphologically abnormal osteoclasts fulfill all the criteria of programmed cell death (12, 19, 23); therefore, in the following experiments, when the AI of osteoclasts was quantified, it was done on the basis of morphological determinations.

The apoptotic pathway induced by clodronate. Several inhibitors that are known to inhibit the apoptotic cascade in other cells (23–26) were added to the cultures simultaneously with 10^{-5} M clodronate-containing liposomes, the concentration that induced apoptosis of ~30-50% osteoclasts in these cultures. The cells were cultured for 24 hr and stained for TRAP and with Hoechst 33258, and the AI of osteoclasts was calculated microscopically. The addition of 10 μM ATA, 25 μM BAPTA, 10 nM PMA, 100 nM genistein, 100 nM herbimycin, 100 nM taxol, or 100 nM $ZnSO_4$ could not prevent the clodronate-induced death of osteoclasts. A slight but significant decrease in the number of apoptotic osteoclasts was detected when protein kinase C was inhibited by the simultaneous addition of 1 nM staurosporine with 10^{-5} M clodronate-containing liposomes. Partial protection against clodronate-induced apoptosis was also obtained with the simultaneous addition of 1 mM homocysteine, which, through metabolism to 3-deazaadenosylhomocysteine, is probably able to interfere with DNA methylation reactions but may also affect cAMP levels and, indirectly, cytoskeletal function (25, 29) (Fig. 6).

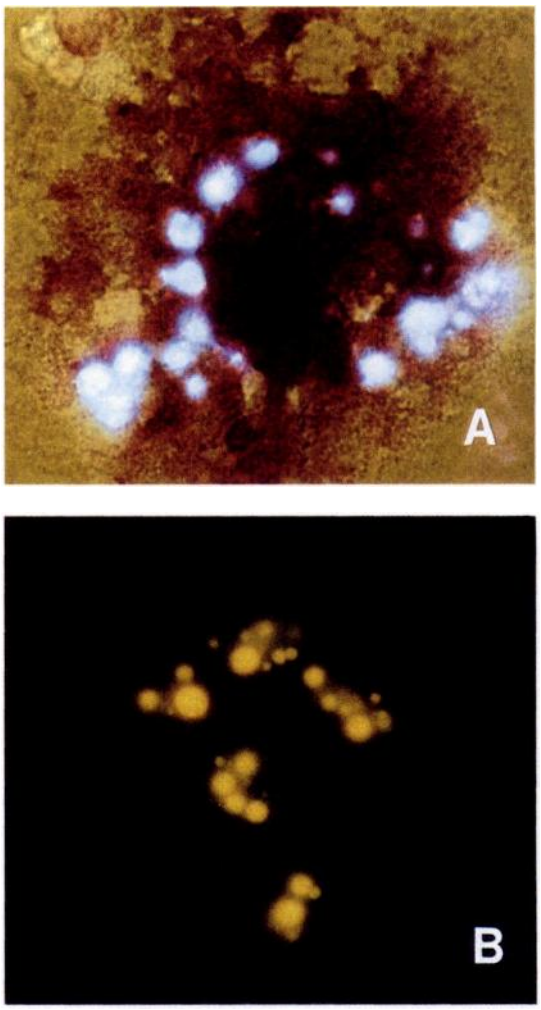


Fig. 5. b, *In situ* detection of internucleosomal DNA breaks in the nuclei of an apoptotic, multinucleated osteoclast on a glass coverslip using the fluorescent TUNEL method. a, For comparison, an apoptotic osteoclast is shown after staining for TRAP and with Hoechst 33258. Magnification, 1060×.

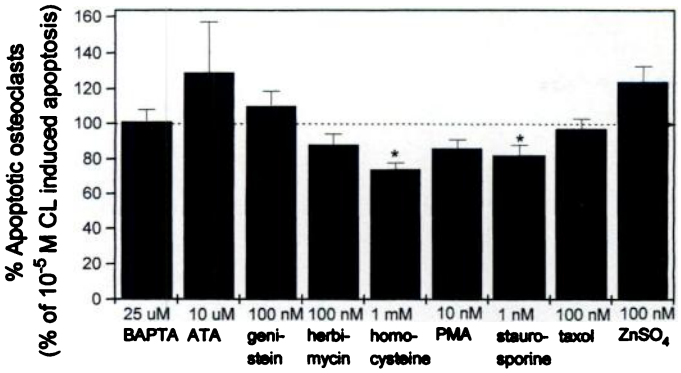


Fig. 6. Signal-transduction studies of clodronate-induced apoptosis in osteoclasts. Bone cell suspensions were cultured on glass coverslips with 10^{-5} M clodronate-containing liposomes (*CL*) and 25 μM BAPTA, 10 μM ATA, 100 nM genistein, 100 nM herbimycin, 1 mM homocysteine, 10 nM PMA, 1 nM staurosporine, 100 nM taxol, or 100 nM $ZnSO_4$. After 24 hr of culture, cells were stained for TRAP and with Hoechst 33258, and the percentage of apoptotic osteoclasts was evaluated microscopically. Data are mean ± standard error and presented relative to 10^{-5} M clodronate-containing liposome-induced osteoclast apoptosis (*dotted line*). Seven to 11 coverslips were viewed in each group. *, $p < 0.05$ versus 10^{-5} M clodronate-containing liposome-induced osteoclast apoptosis.

The effects of clodronate on macrophages. Isolated rat peritoneal macrophages (p.m. cells) or murine macrophage-like RAW-264 cells were cultured in the presence of 10^{-5} to 10^{-7} M clodronate-containing or nonloaded liposomes or with liquid clodronate for 24 hr. The cells were then stained for acid phosphatase and with Hoechst 33258, and the AI of the p.m. and RAW-264 cells was determined microscopically (Fig. 7). In p.m. cells, clodronate-containing liposomes induced a significant and dose-dependent increase in the AI of these cells, whereas liquid clodronate or nonloaded liposomes did not affect the AI values of p.m. cells. The AI values of the macrophage-like RAW-264 cells remained unaltered with all of the agents tested relative to controls (quantitative data not shown). We next questioned whether the delayed onset of apoptosis in RAW-264 cells in response to clodronate is related to altered expression or subcellular localization of Bcl-2. Overexpression of this protein has been shown to confer resistance to drug-induced apoptosis in several cell types (17, 28). Immunofluorescent staining intensity and subcellular localization of Bcl-2 were, however, similar in both p.m. and RAW-264 cells within the various treatment groups. In both cell types, the most-intense punctate-like staining of Bcl-2 was localized to the nuclei, whereas no Bcl-2 staining was detected in apoptotic nuclei (Fig. 8).

Liver sections of mice treated intravenously with various clodronate formulations were subjected to the TUNEL method and viewed microscopically. A dramatically increased apoptotic signal was observed in liver sections from clodronate liposome-treated mice, and most of the apoptotic

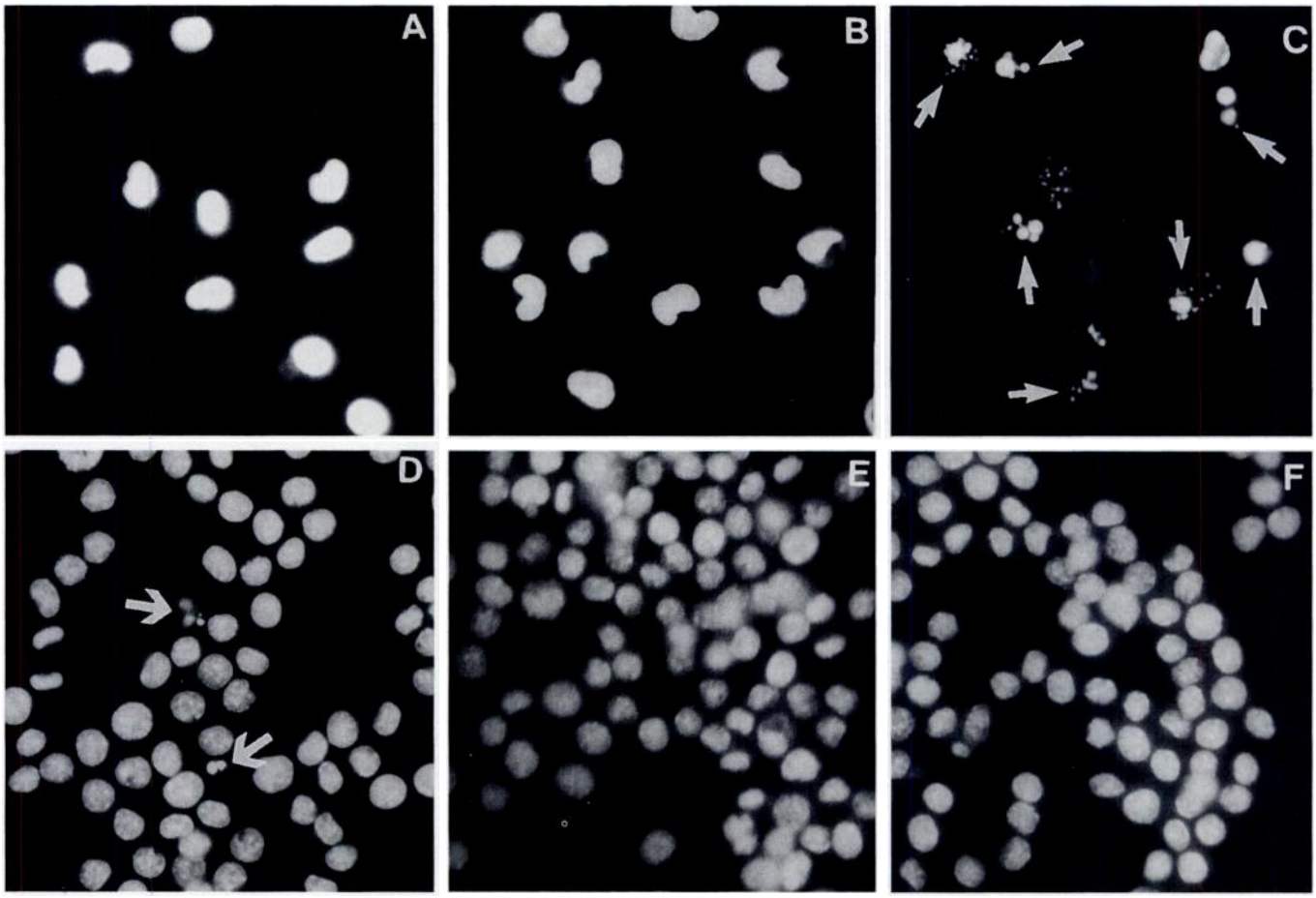


Fig. 7. Hoechst 33258-stained nuclei of (A–C) peritoneal macrophages (p.m. cells) and (D–F) RAW-264 cells from (A and D) controls or (B and E) cultures containing nonloaded or (C and F) 10^{-5} M clodronate-containing liposomes after 24 hr of culture. *Arrows*, apoptotic macrophages. Magnification, 528×.

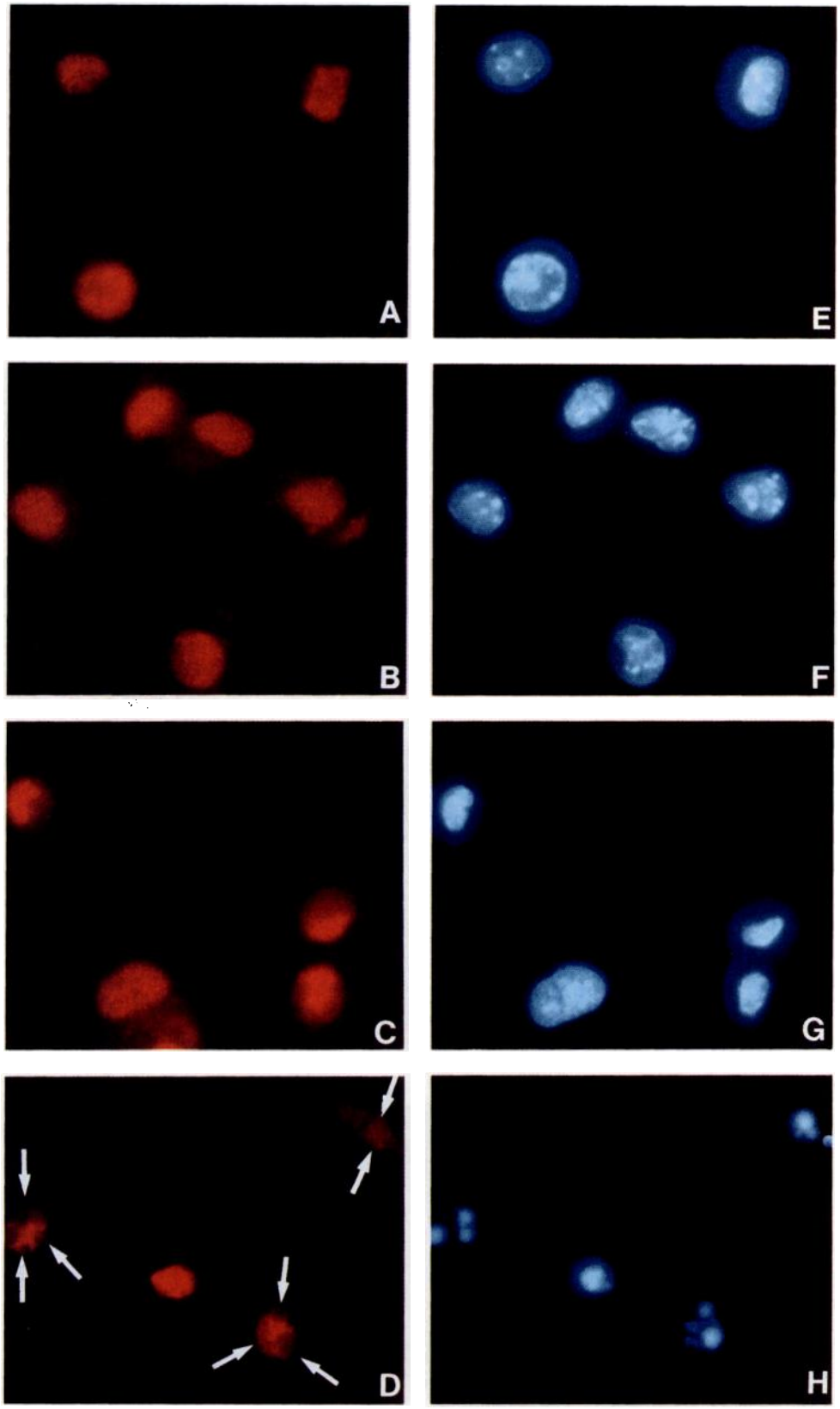


Fig. 8. Immunofluorescent visualization of Bcl-2 in (A and B) RAW-264 and (C and D) p.m. cells. E–H, Hoechst 33258-stained nuclei from corresponding cells. The staining intensity and subcellular localization of Bcl-2 were similar in intact cells cultured with (B and D) 10^{-5} M clodronate-containing or (A and C) nonloaded liposomes. *Arrows*, from apoptotic nuclei, the staining for Bcl-2 eventually disappeared. Magnification, 1060×.

nuclei were located at the walls of hepatic sinusoids. A slightly increased signal was also detected in liver sections from mice treated with liquid clodronate. In liver sections of mice that were treated with nonloaded liposomes or vehicle, essentially no apoptotic nuclei were observed (Figs. 9 and 10).

Discussion

Resorption of either clodronate- or pamidronate-covered bone resulted in a clear increase in the number of morphologically apoptotic osteoclasts as a function of time. Of the

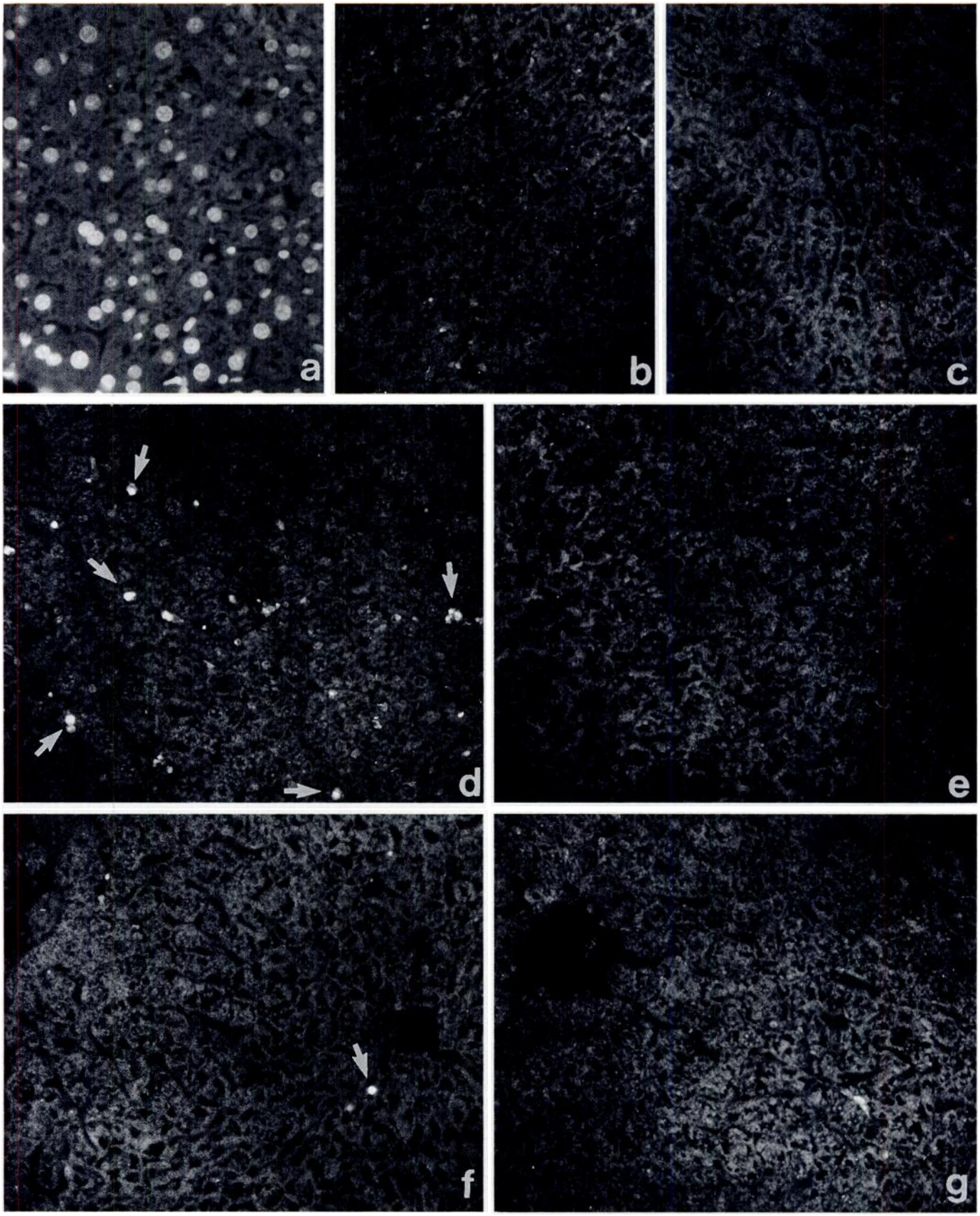


Fig. 9. *In situ* detection of DNA-breaks in liver sections from mice with the use of the TUNEL method. a, After DNase I pretreatment of the sections, nearly all nuclei were labeled. Negative controls included omission of the TdT enzyme (b) and the anti-digoxigenin antibody detecting the labeling site of the DNA nicks (c). b and c, No positive reactions. *Arrows*, an enhanced apoptotic signal was detected in (d) the clodronate-liposome group compared with (e) the nonloaded liposome, (f) liquid clodronate, or (g) vehicle groups. Magnification, 270×.

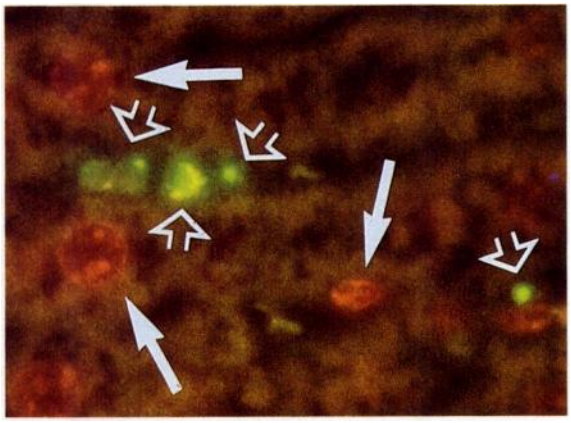


Fig. 10. *Open arrows*, TUNEL-labeled, apoptotic nuclei located at the wall of hepatic sinusoids; *filled arrows*, nuclei of intact liver cells. Magnification, 1060×.

two BPs studied, clodronate was found to be a stronger inducer of osteoclast apoptosis, which agrees well with earlier findings showing that clodronate exceeds pamidronate in osteoclast cytotoxicity *in vitro* (3, 5, 7). Our results also fit well with the idea that osteoclasts obtain BPs by resorbing bone matrix (3, 5, 7, 20). The apoptotic mode of osteoclast death by clodronate was confirmed by the typical morphology, internucleosomal DNA nicks in the nuclei of apoptotic osteoclasts, and by the typical ultrastructural features of apoptosis (12, 19, 23).

Intravenous administration of clodronate liposomes into adult mice resulted in an enhanced apoptotic signal from the nuclei of cells located at the walls of hepatic sinusoids, which coincides with the well known elimination of Kupffer cells from the liver in response to this treatment (8, 9). Clodronate also induced apoptosis in isolated peritoneal macrophages but did not increase the apoptosis rate of murine macrophage-like RAW-264 cells when the drug was administered in a phagocytosable form in liposomes. However, the results of earlier studies have shown that RAW-264 cells stop proliferating in response to engulfment of clodronate-containing liposomes (10, 11). Therefore, this treatment eventually results in apoptosis (12), although it was not directly seen here. We concluded from these findings that the primary target or targets for clodronate in RAW-264 cells are present but that initiation or execution of the apoptotic pathway in response to clodronate might be defective. We therefore compared the cellular distributions of Bcl-2, an oncoprotein whose overexpression can confer resistance to drug-induced apoptosis, in RAW-264 and p.m. cells (17, 28). The staining intensity and subcellular localization were similar in all treatment groups of RAW-264 and p.m. cells. In intact macrophages, the most intense punctate-like staining was localized in the nuclei, but it was not found in apoptotic nuclei (28, 30). Our findings thus suggest that the delayed onset of clodronate-induced apoptosis in RAW-264 cells is not caused by overexpression of Bcl-2 but rather by other, Bcl-2-independent mechanisms. The role of p53, a tumor suppressor protein, whose function is essential for chemotherapeutic agents to induce apoptosis in their target cells, remains to be characterized in clodronate-induced apoptosis in RAW-264 cells (31). It is also possible that Bcl-X, a Bcl-2-related protein that is known to prevent apoptosis in several hematopoietic cell lines, retards the onset of clodronate-induced apoptosis in RAW-264 cells (16, 32).

To study the signal-transduction pathways of clodronate-induced apoptosis in osteoclasts, we used liposomal delivery of clodronate, instead of offering the drug to osteoclasts in the bone-bound form. In this way, we could overcome resorption-inhibitory effects and, thus, inhibition of drug delivery that some of the tested compounds may have in the pit formation assay (33, 34). Culture of bone-derived mixed cell population with 10^{-5} to 10^{-7} M clodronate-containing liposomes induced a significant, dose-dependent increase in the number of apoptotic osteoclasts, whereas nonloaded liposomes had no effect. Several inhibitors were used to analyze the mediation of clodronate-induced apoptosis of osteoclasts. The inhibition of tyrosine kinases through the use of genistein and herbimycin did not have an effect on clodronate-induced apoptosis of osteoclasts, suggesting that tyrosine kinase-mediated pathways are not involved. Our observation of the decreasing effect of staurosporine, a potent inhibitor of protein kinase C, on clodronate-induced apoptosis suggests that protein kinase C-mediated intracellular phosphorylations are involved in mediation of BP-initiated apoptosis. In murine thymocytes, inhibition of protein kinase C has been reported to similarly prevent glucocorticoid-induced apoptosis (35). Protein kinase C agonist PMA could not protect osteoclasts against clodronate-induced apoptosis either. There are conflicting reports on staurosporine activation and PMA inhibition of apoptosis in some other contexts (36–38), but our findings support the idea that in osteoclasts protein kinase C-mediated events are required to execute, not to oppose, BP-initiated apoptosis. It is of interest that Kameda *et al.* (1995) also concluded that protein kinase C-mediated pathways are the major mechanisms underlying the execution of spontaneous/physiological apoptosis in osteoclasts.

Homocysteine was another agent that partially prevented clodronate-induced apoptosis in osteoclasts. By facilitating the formation of *S*-adenosyl-homocysteine, homocysteine can affect *trans*-methylation reactions (29). This may eventually lead to increased susceptibility of hypomethylated DNA to degradation by nucleases. However, for clodronate, this did not seem to occur. Another possible effect is interference with the function of microfilaments either directly or by elevating the levels of cAMP, as shown in homocysteine-caused partial blockage of the 3-deazaadenosine-induced apoptosis in HL-60 cells (25, 29). It is conceivable that homocysteine also decreased clodronate-induced apoptosis by affecting cytoskeletal function. The well-known disorganization of the microfilament network by clodronate (4, 7) may have an important role in clodronate-induced apoptosis. The same mechanism could be involved in the protective effect of staurosporine, which may inhibit protein kinase C-dependent cytoskeletal alterations.

Some of the known apoptosis-inducing drugs cause cell death by disrupting the microtubular architecture (26, 39). To test whether clodronate affects microtubules, we used taxol, a microtubule-stabilizing agent that has been shown to prevent colchicine-induced apoptosis (26). It did not inhibit but rather, at higher concentrations (data not shown), potentiated the clodronate-induced death of osteoclasts. Regardless of this fact and especially when damage primarily affecting microtubules is sufficient to induce apoptosis (26, 39), the interactions between different bisphosphates and the GTP-

binding ras-proteins (40), which have a role in the maintenance of the cytoskeletal polarization, are of special interest and remain to be clarified.

The clodronate-induced apoptosis was associated with typical nuclear changes that are consistent with condensation and degradation of DNA. Some BPs have been shown to be metabolized to nucleotide analogs in the *Dictyostelium* slime mold (41). This could lead to DNA damage and thus increase the sensitivity of DNA to nucleases. The inhibition of the Ca^{2+}/Mg^{2+}-dependent nucleases with ATA or $ZnSO_4$ did not, however, rescue osteoclasts from clodronate-induced apoptotic changes. Also, treatment with the Ca^{2+} chelator BAPTA, which obviously affects the function of Ca^{2+}/Mg^{2+}-dependent nucleases, did not decrease clodronate-associated cell death. This is consistent with earlier observations in other cells and the suggestion that DNA degradation may be a characteristic feature of but not a necessary step for the execution of apoptosis (42). It is also important to remember the marked redundancy of apoptotic pathways (43). Thus, it is possible that although one pathway is blocked by an inhibitor, another pathway may be available. Our results do not exclude the possibility that other mechanisms, such as the ICE-like family of cysteine proteases, play an important role in clodronate-induced apoptosis (44).

Cytotoxicity is not, however, the sole mode of action of BPs; in particular, molecules of the new BP generation do not result in osteoclast death. Alendronate treatment, for example, results in impaired formation of osteoclast ruffled borders (5), and tiludronate has been suggested to act as an inhibitor of osteoclast H^+-ATPase, located at the ruffled border (6, 45). Based on the results of earlier studies, it is clear that BPs affect osteoclasts directly, via resorption (3, 5, 7). It is, however, also possible that indirect mechanisms, via the mediation of osteoblasts or other cells present in bone, contribute to the BP-induced apoptosis of osteoclasts (46). However, this is unlikely because apoptotic cell death could also be induced in cultured peritoneal macrophages.

Taken together, our results show that clodronate induces apoptotic dell death in cultured osteoclasts. This mode of action could account for its ability to inhibit bone resorption *in vivo*. The primary molecular target of clodronate in osteoclasts and the detailed apoptotic pathway remain to be clarified, although the current results suggest that protein kinase C-dependent cellular reactions are probably involved.

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Send reprint requests to: Dr. Katri Selander, Department of Anatomy, University of Oulu, Kajaanintie 52 A, 90220 Oulu, Finland.