Journal of Cellular Biochemistry 90:42–58 (2003)

Cell Death in Cultured Human Saos2 Osteoblasts Exposed to Low-Density Lipoprotein

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Abstract Osteoporosis (OP) and atherosclerotic-cardiovascular diseases (and possibly dementia) constitute emerging age-related co-morbidity states that might share risk factors. Blood-born lipids, like LDL involved in atherosclerosis and apolipoprotein-E4 (ApoE4) involved in dementia, may also be implicated in development of OP. We examined osteoblast cell lines as a culture model for OP by exposure to lipoproteins. ApoE expression in Saos2 and U2OS osteoblasts was confirmed by PCR. ApoE4 did decrease cell counts relatively to ApoE3, especially in Saos2 cells in which it was less selective for cells with higher alkaline phosphatase (ALP, an osteoblast marker) activity than ApoE3. This associates with ApoE4, being a risk factor for both dementia and OP. Saos2, but not U2OS, showed a decrease in cell counts after 48 h exposure to native LDL (NLDL). Both cell lines had decreased cell counts already after 24 h when exposed to oxidized-LDL (OxLDL) for which Saos2 also showed a higher sensitivity than U2OS. Exposure of Saos2 to both, OxLDL at low concentration (5 μg/ml) and NLDL revealed a shrunken size cell fraction of 17–23% on the fluorescence-activated cell sorter (FACS) analysis. Such shrunken cell fraction was not seen when Saos2 cells were exposed to 50 μg/ml of OxLDL or to OxLDL combined with 10 nM dexamethasone (DEX, a stimulator of osteoprogenitor differentiation). DEX treatment has lysed the cells earlier than 24 h post exposure and has selected more resistant cells that did not show apoptotic shrinkage in the FACS analysis done after 24 h. We interpret this as a failure to detect the apoptotic cell fraction due to their lysis prior to the FACS analysis. Western blots performed at different time points (10 min, 30 min, 4 h, 24 h, and 48 h) under OxLDL + DEX revealed a fall in the positive regulator of pp60Src-kinase phosphotyrosine (pY)418 relative to the DEX controls during the first 4 h. This is consistent with DEX osteogenic induction, known to be negatively regulated by c-Src, although the pY418/pY529 ratios (negative/positive kinase regulation) fell only at the 10 min time point. Contrarily the pY418/pY529 ratio increased, relative to untreated controls, under 5 μg/ml and 50 μg/ml of NLDL at the 4 h time point and under 50 μg/ml NLDL only at the 10 min time point, being consistent with the ability of a higher dose of LDL to antagonize osteoblast differentiation. This could be even more acceptable if the NLDL would have become minimally oxidized during its long purification procedure. Under NLDL, the Bcl-2/Bax ratio was pro-apoptotic at 10 min, 30 min, and 4 h only under 50 μg/ml, whereas under OxLDL + DEX it was pro-apoptotic only after 4 h suggesting that additional pathways contribute to cell death. These results indicate that lipid effects on human osteoblast lines in culture may be used as a model to identify molecular targets shared between OP and atherosclerosis for intervention in this co-morbidity. J. Cell. Biochem. 90: 42–58, 2003.

Key words: co-morbidity; osteoporosis; atherosclerosis; oxidized-LDL; apolipoprotein; c-Src-kinase; Bcl-2; Bax; apoptosis; alkaline phosphatase

Osteoporosis (OP) is becoming a major health burden in the industrialized countries and so are cardiovascular disease entities like atherosclerosis. These disease entities have been, and still are, dealt with separately in terms of the biochemical mechanism in their pathology. Upon observation on postmenopausal health status it becomes obvious that cardiovascular diseases do progress in parallel with accelerated bone loss. In fact indirect evidence on a possible linkage between these two diseases have been accumulated during the last decade by

Grant sponsor: Hadassah Women Organization for Women's Health; Grant sponsor: Public Committee for Designation of Estate Funds.

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Received 3 March 2003; Accepted 22 May 2003
DOI 10.1002/jcb.10603

epidemiological studies [Moon et al., 1992; Vogt et al., 1997; Kado et al., 2000] and has been shown recently to exist in animal models [Drake et al., 2001; Parhami et al., 2001]. There is also a study showing that apolipoprotein-E4 (ApoE4) aside from being a risk factor for Alzheimer disease (sporadic type), it is associated with increased risk for hip fractures in women [Cauley et al., 1999]. OP and atherosclerosis (and perhaps other cardiovascular morbidities) are now establishing themselves as an emerging co-morbidity of ageing with the possible sharing of biochemical processes. Within this co-morbidity one can observe two striking reciprocal phenomena, on one hand the diminished bone mineral density and on the other, the gain in mineralized vascular wall matrix. It would be presumptuous and even baseless to suggest that mineral flows directly from ostopenic bone into the calcifying arterial plaques. Alternatively, the assumption that bone and vascular tissues respond to the same signals in an opposite manner is more reasonable. By the use of cell culture models it has been shown that minimally oxidized-LDL (OxLDL) inhibits osteoblast differentiation markers and cell-mediated mineralization, while in contrast it causes vascular cell-mediated mineralization [Parhami et al., 2001]. This reciprocal response indicates that LDL may be implicated in OP. A link between OP and atherosclerosis is also supported by an experiment of nature, namely a loss-of-function mutation in the LDLR related protein 5 (LRP 5) gene, found in two families, that has shown association with increased bone density [Boyden et al., 2002; Hofbauer and Schoppet, 2002; Hofbauer et al., 2002]. The mechanism for high bone density association with malfunctioning LDLR is not known, although osteoblasts do express LDLR related proteins [Dong et al., 1998] and also ApoE [Bachner et al., 1999]. Lack of LDLR function may affect bone density via decreased osteoclasts activity, like, e.g., a mutated LDLR related protein (*LRP5*) gene [Van Hul et al., 2002], as yet an unexplored subject. It is however known that osteoblasts and pre-adipocytes develop from common precursors and share differentiation markers like bone alkaline phosphatase (ALP) and osteocalcin. The two cell lineages can shuttle between their differentiation states [Nuttall et al., 1998], for which serum fatty acids serve as a trigger [Diascro et al., 1998], possibly by modulation of PPARγ2 (peroxisome proliferator activated receptor) [Lecka-Czernik et al., 2002]. In animals an atherogenic diet has induced bone loss [Parhami et al., 1999] suggesting that the oxidized fatty acids that are OxLDL-derived have a direct modulating effect on osteoblast PPARγ2 [Lecka-Czernik et al., 2002]. Alternatively, a direct interaction of OxLDL with osteoblasts in culture and in vivo has induced oxidative stress and could inhibit differentiation of osteoprogenitors into osteoblasts [Parhami et al., 1999], and perhaps induce cell death in differentiated osteoblasts. To directly interact with osteoblasts or with their precursors in vivo, LDL has to reach these cells physically, similarly to the scenario in vascular walls where LDL penetrates into the intima and looses the plasma-contained antioxidant protection. There are two hypothetical ways that such scenario could take place in relation to osteoblasts, one is a time and space window in which LDL might cross the capillary sinosoidal structure that nourishes the cutting cones generated by actively resorbing osteoclasts. Here LDL could become minimally oxidized, interact with young osteoblasts, and impair their function or even cause osteoblast apoptosis, while sparing the osteoclast bone-resorbing function. A second possibility is that some of the osteoblast precursors are present well within vascular walls [Doherty et al., 1998] and is therefore exposed to OxLDL before migrating into cutting cones. Whatever the way is for LDL to interact with osteoblasts in vivo, it is important to understand the signals that LDL induces in osteoblasts especially if this indeed represents part of the pathological mechanism in OP. Because of the reciprocal mineralization phenomenon seen in the above mentioned co-morbidity of OP and atherosclerosis, we sought to examine a protein with reciprocal responses or activities in cell types with opposite functions. As a player of such role pp60Src was chosen, since bone-resorbing osteoclast activity depends on its expression [Boyce et al., 1992] whereas bone-forming osteoblast differentiation depends on its fading away [Marzia et al., 2000]. This approach to shared signaling targets may (in the future) be carried further to occurrences of reciprocal pp60Src involvement in vascular wall cells during generation of atheromatosis, vis-à-vis its activity in osteoblast. In the present work, we describe the response of human osteoblastic cell lines to native LDL (NLDL) and OxLDL, we

show the ability of LDL to kill osteoblasts by lysis and under certain conditions they may show at least two features of apoptosis. We show the kinetics of pp60Src-kinase regulatory phosphotyrosine states under LDL exposure. We have also followed the kinetics of Bax/Bcl-2 ratio since it is implicated in apoptosis. The results set a baseline in Saos2 osteoblasts for further studies to identify shared molecular targets for preventive or therapeutic intervention in the above co-morbidity of ageing.

MATERIALS AND METHODS

Reagents

Anti Bcl-2, and anti Bax were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphorylated Src (pY529 and pY418) was purchased from BioSource Int (Camarillo, CA). For Src protein detection, we used mAb 327 [Lipsich et al., 1983]. Peroxidase-conjugated second antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), fetal calf serum (FCS) was purchased from Life Technologies Laboratories (Grand Island, NY). Tissue cultures reagents, tissue culture media, antibiotics, and trypsin–EDTA were purchased from Biological Industries (Beit Haemek, Israel). ApoE3 and ApoE4 PanVera.

Cell Culture

Saos2 and U2OS osteoblastic cell lines were cultured in maintenance medium DMEM supplemented with 10% FCS antibiotics and 10 mM glutamine. For experiments of cell-growth, kinetics cells were seeded in 96-well microtiter plates, 5×10^3 cells/well in separate plates for each time point. Cells were then cultured for 72 h in maintenance medium, subsequently either ApoE or LDL was added in fresh medium at different concentrations as specified in the text. The LDL was added with 2% final concentration of lipid-free human serum that replaced the FCS. Cells were counted at 24 and 48 h time points, following lipoprotein or LDL addition using the methylene blue (MB) staining method. ALP activity was measured in parallel plates.

Quantitative Cell Staining

Cells were stained using the MB method [Klein et al., 1997b]. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with distilled water, and air-dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed exhaustively with water, and air-dried. The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. Optical density (OD) of the eluted MB was measured at 620 nm by an optical densitometer.

ALP Activity Assay

ALP activity was measured in situ in microtiter plates. The medium was removed and cells were washed twice in situ with 0.2 ml TBS (50 mM Tris, 150 mM NaCl pH 7.6). ALP substrate, pNPP (*p*-nitrophenyl phosphate) in TBS, 1.33 mg/ml was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min and OD of the hydrolyzed pNPP was measured in a optical densitometer at 405 nm. ALP specific activity was expressed as nMol/90 min/50,000 cells and converted into percentage of untreated cultures from the first time point referred to as 100%.

Electrophoresis and Western Blot Development

Cells were seeded 2×10^4/well in 6-well plates, 72 h later LDL containing medium with or without 10 nM dexamethasone (DEX) was added the FCS was replaced with human serum (2%) from which LDL and other lipid fraction had been removed. LDL or OxLDL was added to a final concentration of 0.0, 5, and 50 μg/ml. Plates were harvested for electrophoresis on indicated time point by cell lysis. Wells, placed on ice, were washed twice with 6 ml of cold PBS removing washing solution to dryness. Ice cold electrophoresis sample buffer [with 7% 2-mercaptoethanol and 3% sodium dodecylsulfate (SDS)] 50 or 100 μl was added to each well and incubated on ice for 5 min. The lysates were quickly scraped off the plastic with a rubber policeman and transferred to cold tubes, boiled for 5 min, after removal of 10 μl for protein determination they were cooled on ice and stored at −70°C until further use. Samples of 75 or 50 μl of lysed cultures were adjusted to equal volumes with plain sample buffer and fractionated by electrophoresis on 7–15% SDS–polyacrylamid gradient gels. Gels were then electroblotted onto nitrocellulose filters which

were blocked with 3% bovine serum albumin (BSA) and 0.2% Tween 20 in PBS. For phospho-c-Src detection, filters were first incubated with anti-phosphorylated protein antibody and after washing they were incubated with appropriate second antibody conjugated to peroxidase. The filters were exposed to ECL buffer to generate chemilluminense by the activated peroxidase-conjugates, detected by photo-radiography.

After radiography, the anti-phosphotyrosine antibodies were stripped off the filters and the above procedure was repeated with the appropriate antibody that recognizes either a second phosphorylated tyrosine or an unphosphorylated epitope in the same protein or other proteins (Bcl-2 or Bax). Stripping was performed by incubation of the filters in 62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS at 50°C for 30 min.

Protein Determination

Duplicates of 5 μl electrophoresis sample were socked into 3 MM filter papers cut in pieces of 1 cm^2, dried, and stained with 2 mg/ml Coomassie blue for 20 min, destined with 20% methanol, 7% acetic acid until clearing of the unstained background, the stained protein spots staining dye was eluted with 3% SDS, protein quantities determined by Coomassie blue OD at 620 nm compared with a similarly treated and serially diluted BSA standard.

Lipoprotein Isolation

LDL was isolated by stepwise ultracentrifugation by Havel's method as in Havel and Bragdon [1955]. Oxidation of LDL was carried out after removal of EDTA by incubation of 1 mg LDL protein/ml with 5 M Cu^{2+} for 24 h. Both kinds of LDL samples were quantified by protein measurement.

Fluorescence-Activated Cell Sorter (FACS) Analysis

FACS analysis of cell cycle stages determined by size was used [as done before, Ben-Bassat et al., 1999] to exhibit the apoptotic fraction of shrunken cells. Saos2 cells were seeded and treated in 6-well plates as in the Western blot experiments, on the 24 h time point cells were trypsinized and washed twice with PBS, suspended in 0.1ml PBS to which Triton X-100 was slowly added to a 0.7% final concentration. After 10 min, 0.03 ml of propidium iodide (PI) was added (0.5 mg/ml in 0.1% sodium citrate) to stain cellular DNA for fluorescence emission. The FACS was calibrated with PI-free cells and properly gated to present cell population sizes on the X-axis (light scatter) and cell number (PI fluorescence) on the Y-axis. The percent of shrunken cells fraction relative to total cell population was derived as the percent of apoptotic cells.

RT-PCR on ApoE mRNA

The two-step procedure is based on initial RT of RNA to cDNA followed by amplification of cDNA by PCR, as described below. The cDNA was prepared from 10^6 non-stimulated osteosarcoma cells using total RNA by mixing with 200 ng of a random oligonucleotide primer and heated to 65°C for 4 min and slowly cooled to 25°C to anneal the primer. The reaction mixture (total volume 50 μl) consisted of 50 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 4 mM dNTP, 4 mM dithiothreitol, and 40 U of avian myeloblastosis virus RT. The reactions were carried out at 42°C for 2 h. PCR reactions for ApoE were prepared in a final volume of 20 μl, and contained 5 μl of cDNA reaction mixture, 23 mM dNTP, 18.87 mM $(NH_4)_2SO_4$, 76 mM Tris (pH 8.8), 7.67 mM $MgCl_2$, 11.36 mM dithiothreitol, 193 μg/ml BSA, 11.36% dimethyl sulfoxide (DMSO), 10 Ci of [-^{32}P]dCTP (1 Ci = 37 GB), and 250 ng each of oligonucleotide primers, A (5′-GAGAAGCTTGCGGCGCAGGCCCGGCTGGGCGCG-3′) and B (5′-TGAAGCTTCGCTCGGCGCCCTCGCGGGCCCGGG-3′). The reaction mixture was heated to 98°C for 15 min and Taq polymerase (Roche Boehringer Mannheim, Germany), 1 μl per reaction, was added at 88°C. cDNA was amplified using a PCR thermal cycler (Minicycler, MJ Research, Waltham, MA). The reaction mixture was heated to 95°C for 5 min and this was followed by 25 cycles consisting of 1.5 min for denaturation at 94°C, 1 min for annealing at 60°C, and 1 min for extension at 72°C, with a step cycle for 7 min at 72°C for 1 cycle. The amplified products, digested with *Hha*I [Hixson and Vernier, 1990] were subsequently analyzed on a 2% agarose gel. The household gene L19 ribosomal protein mRNA was reverse transcribed and its 194 bp PCR product was used as a transcription comparative reference between both osteoblast cell lines. L19 PCR primer sequences used were: 5′-CTGAAGGTGAAGGGGAATGTG-3′,

and 5′-GGATAAAGTCTTGATGATCTC-3′, as forward and reverse primers, respectively.

RESULTS

ApoE and LDL Receptor Expression in Osteoblasts

Figure 1 shows RT-PCR amplification of ApoE isoforms mRNA in non-stimulated Saos2 and U2os human osteoblast cell lines. ApoE mRNA was detected in both cell lines, ApoE showed a higher constitutive expression in Saos2 cells. This has raised the question as to how would ApoE affect the longevity and ALP activity in the same osteoblast cell lines.

Osteoblast Response to ApoE4 and ApoE3

Figure 2 shows the proliferative and ALP activity (differentiating osteoblast marker) responses of osteoblast cell lines to ApoE4 and to ApoE3 in the absence and presence of DEX. Saos2 osteoblasts showed a slight decrease (-27.6% of controls, $P < 0.001$) in cell count on day 1 under ApoE4 (Fig. 2a), accompanied by a minute increased ALP activity (Fig. 2b, only $+9\%, P < 0.05$), while U2os osteoblasts showed a minute but significant decrease (-8.5%, $P < 0.05$) in cell counts under ApoE4, however, there was a slight increase in ALP activity (26% Fig. 2b, $P < 0.001$) and a net increase (95%, $P < 0.0001$) on day 2. The negative cell count response of U2os cells to ApoE3 is reciprocal to that of Saos2 and to that obtained by ApoE4. ApoE3 decreased cell counts in U2os (-30%, Fig. 2a, $P < 0.0001$) accompanied by a slight increase ($+21\%$, Fig. 2b, $P < 0.0001$) in ALP activity on day 1 and an almost net increase ($+118\%$, $P < 0.0001$) on day 2. Thus these cell lines respond differentially by ALP activity to both ApoE isoforms especially on day 2 where U2os but not Saos2 cells showed a substantial increment. DEX has decreased cell counts under both ApoE isoforms in both cell lines (Fig. 2c), generally DEX has amplified responses to ApoE. There was, however, only one DEX-induced differential response between the two cell lines, it tended to oppose the ApoE3-induced proliferation arrest in U2os reciprocally to its effect on Saos2 cells (compare Fig. 2c with Fig. 2a in U2os versus Fig. 2c with Fig. 2a for Saos2). It can be concluded that the decreased osteoblast counts in response to ApoE4 (a risk factor in dementia) is more prominent in Saos2 than in U2os cells.

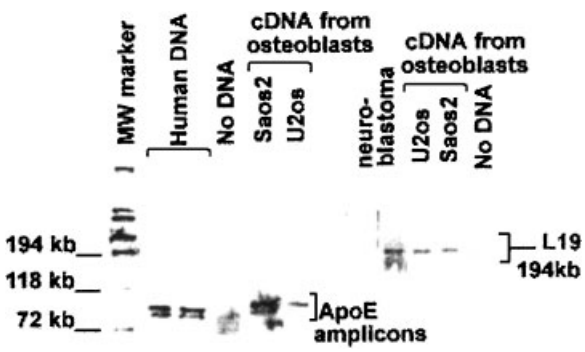

Fig. 1. RT-PCR on RNA extracts of non-stimulated osteoblast cell lines to detect apolipoprotein-E (ApoE) transcripts. RNA extracts from non-stimulated Saos2 and U2OS cell lines were subjected to RT, fragments of the resulting cDNA underwent amplification with PCR primers specific for ApoE. PCR products digested with *Hha*I were fractionated by agarose gel electrophoresis, in parallel with ApoE amplicons PCR product of human genomic DNA. The restriction fragment sizes were of 89 and 82 bp. Loading controls on the right are 194 bp, PCR products of household L19 ribosomal protein from the Saos2 and U2os cDNA, and a human neuroblastoma.

Osteoblast Cell Lines Response to NLDL and OxLDL

In addition to ApoE expression in these osteoblast lines, others have shown expression of LDL receptor (LDLR) in osteoblasts [Dong et al., 1998]. LDL has been implicated in vascular pathology that resembles mineralization of bone matrix [Parhami et al., 1997] and LDLR mutation has been shown to be associated with increased bone density [Boyden et al., 2002]. We therefore examined the response of cell proliferation and ALP activity in these osteoblastic cell lines.

Figures 3 and 4 show the response of Saos2 and U2os cells to LDL, respectively after 24 and 48 h. NLDL did not decrease cell counts in neither of these cell lines on the first day of exposure (Figs. 3a and 4a), however, after 48 h of exposure there was a decrease in cell count in Saos2 (Fig. 3a, Day 2, especially under 50 μg/ml, $P < 0.05$) but not in U2os cells (Fig. 4a, Day 2) as expressed by total cell protein. OxLDL did not decrease cell counts in both cell lines after 24 h (Figs. 3c and 4c, Day 1) but after 48 h (Day 2) it has decreased it in Saos2 ($P < 0.05$ for 5 and 50 μg/ml OxLDL) more than in U2os cells (not significant for 5 and 50 μg/ml), indicating that Saos2 cells are more sensitive to OxLDL than U2os cells. In general, both cell lines showed a decrease in specific ALP activity in the control cultures by day 2 (untreated bars), ALP activity

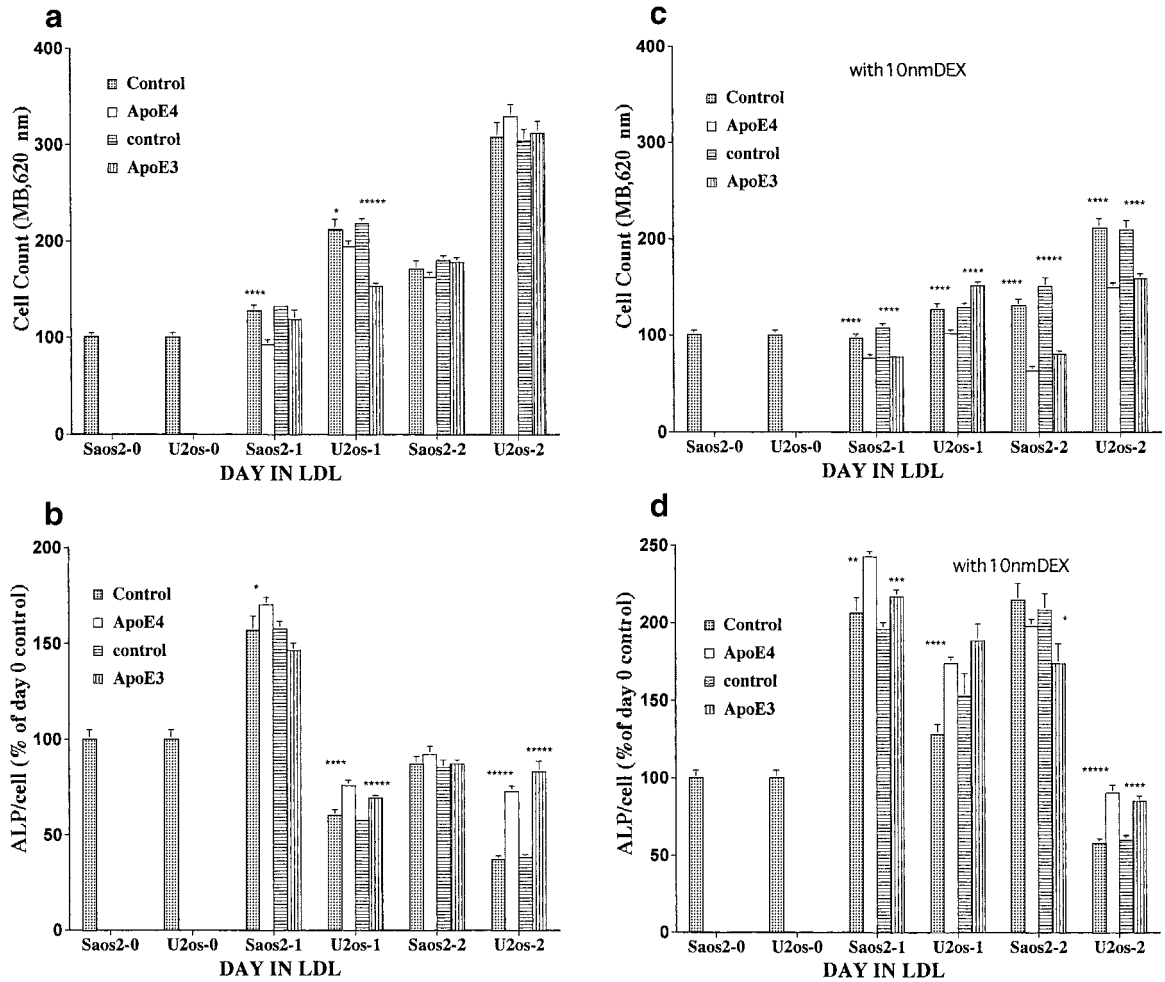

Fig. 2. Effects of ApoE4 and ApoE3 on Saos2 and U2os cell proliferation and alkaline phosphatase (ALP) activity with and without dexamethasone (DEX). Saos2 and U2os cells seeded 3,000 cells/well were exposed to 2 μg/ml ApoE on day 0, without DEX (**a**, **b**) and with DEX (**c**, **d**). Twenty-four and 48 h later, cells were counted by methylene blue (MB) staining (a, c) and in parallel other cultures underwent ALP activity assay (b, d). Specific ALP was calculated nMol hydrolyzed substrate per 90 min per 50,000 cells. All the results are expressed as percentage of day 0 untreated cultures, mean ± SEM, n = 20. Levels of significance in the differences between treated and untreated cultures is derived by the Student's *t*-test (two-tailed) and presented by asterisks: $1^* = P < 0.05$, $2^* = P < 0.02$, $3^* = P < 0.002$, $4^* = P < 0.001$, $5^* = P < 0.0001$.

in LDL and OxLDL-treated Saos2 cells has further decreased dose dependently (Fig. 3b) as opposed to U2os cells (Fig. 4b). Figures 5 and 6 show the response of Saos2 and U2os cells, respectively, to LDL but this time in the presence of DEX. DEX, a stimulator of osteoblast differentiation, has amplified the decrease in cell count of Saos2 in response to LDL (Fig. 5a) and OxLDL (Fig. 5c). DEX has caused U2os to respond to LDL by a relative decrease in cell count (compare Fig. 6a with Fig. 4a) and has amplified their response to OxLDL (compare Fig. 7c with Fig. 5c). DEX has also amplified the decreased ALP activity in Saos2 cells on day 1, but after 48 h it has rather increased ALP activity. Note that this is because DEX itself decreases Saos2 absolute cell count and is capable of selecting the cell population with higher ALP activity. DEX also amplified the relative ALP activity in U2os cells of what appears to be its opposite effect on day 1 in Saos2 cells (compare Fig. 6b,d, Day 1 with Fig. 5b,d, Day 1). The higher sensitivity of Saos2 to LDL and/or DEX relatively to that of U2os could be due to the difference in the level of ApoE isoform expression (Fig. 1), or because Saos2 doesn't express p53 oncogene and the RB tumor suppressor. Therefore, we preferred Saos2 as a sensor of LDL effects on osteoblast signaling that enables, e.g., exclusion of p53

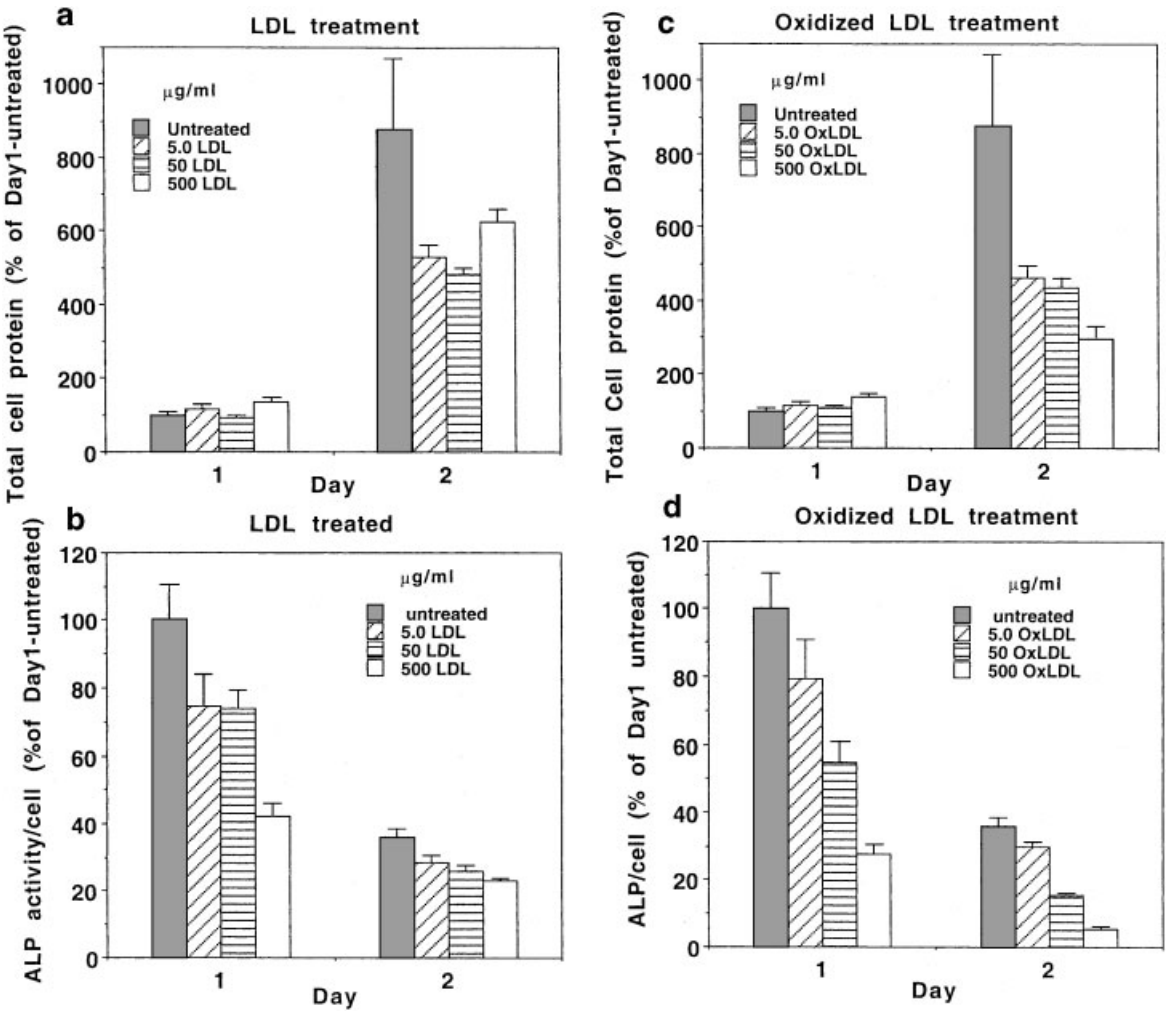

Fig. 3. Effect of native LDL (NLDL) and oxidized-LDL (OxLDL) on Saos2 osteoblast cell proliferation and ALP activity. Saos2 cells seeded 3,000 cell/well, after 48 h the medium was changed with one that contained indicated concentrations of LDL (**a**, **b**) or OxLDL (**c**, **d**), the fetal calf serum (FCS) was replaced with human lipoprotein free serum. After 24 and 48 h of exposure, the cultures were subjected to cell counts by MB total protein assay (a, c) and parallel cultures (b, d) were assayed for ALP activity as in Figure 2. Results are presented as mean ± SEM, n = 20.

involvement in LDL-induced apoptosis or in cell cycle arrest.

Src Phosphorylation in Saos2 Osteoblasts Exposed to LDL

We have looked at the two extreme effects, NLDL versus OxLDL + DEX on pp60Src regulatory phosphorylation states in Saos2 osteoblasts. Cell cultures exposed to LDL have been harvested on indicated time points and prepared for SDS–PAGE fractionation and the electroblotted filters were reacted with two different anti-phosphotyrosine antibodies. The first reaction was with anti-pY529 antibody that recognizes the negative regulator and later after stripping it off the filters they were reacted with anti-pY418 that recognizes the positive regulator. Figure 7 shows the pp60Src phosphorylation differences in OxLDL + DEX (Fig. 7a.1,b.1) and NLDL treated osteoblasts (Fig. 7a.3,b.3) as detected by band density scanning. For each LDL concentration a separate bar-graph shows the ratio between positive/negative phosphorylated regulator tyrosine residue, 5 and 50 µg/ml LDL in Figure 7a.2,b.2, respectively. The superimposed linear curves represent the control pY418/pY529 ratios of bands resulting from DEX alone and from untreated cultures. The figure shows that at the 4 h time point the DEX alone control has

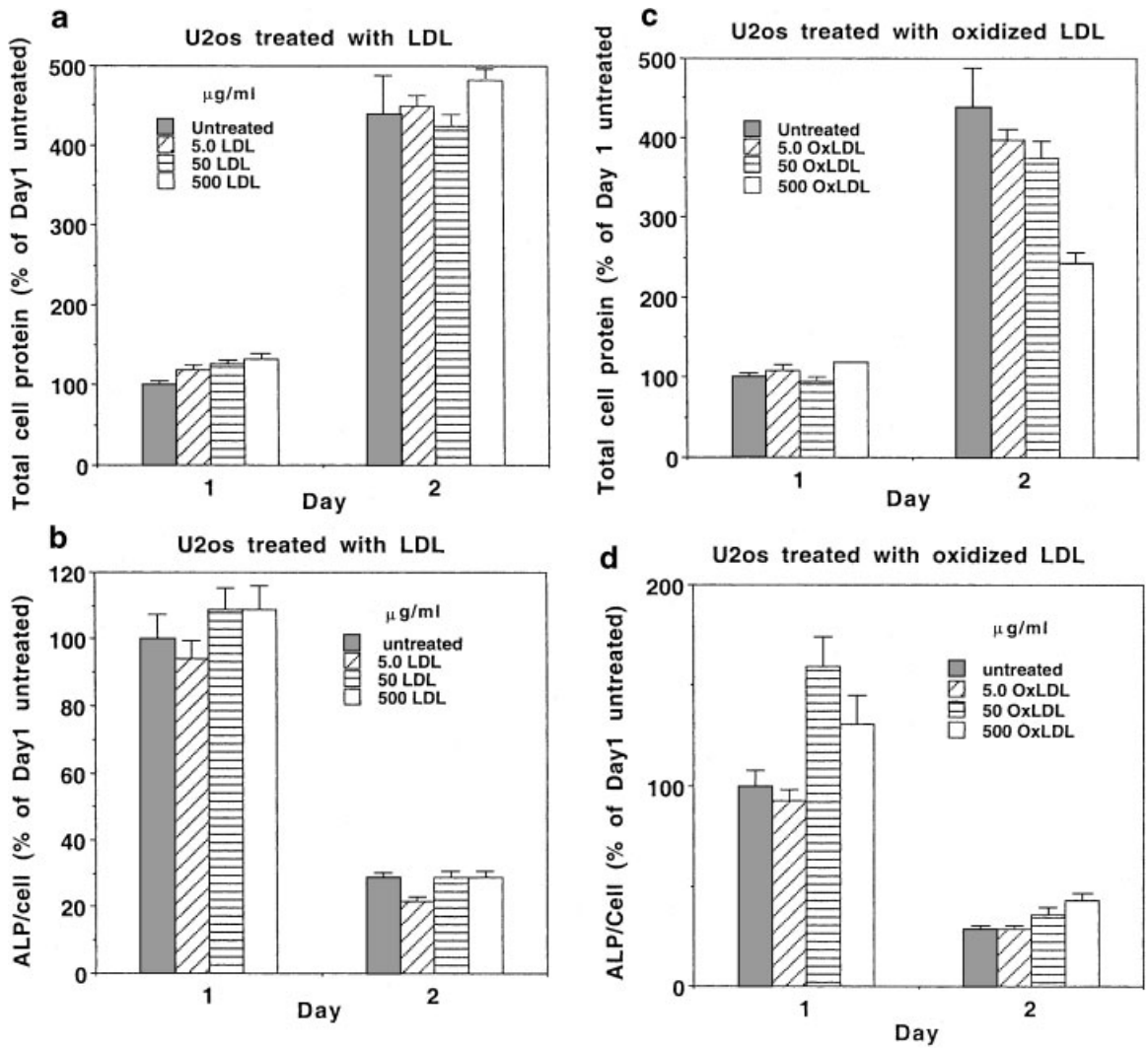

Fig. 4. Effects of NLDL and OxLDL on U2OS osteoblast cell proliferation and ALP activity. U2OS cells seeded 3,000 cell/well, after 48 h the medium was changed with one that contained indicated concentrations of LDL (**a**, **b**) or OxLDL (**c**, **d**), the FCS was replaced with human lipoprotein free serum. After 24 and 48 h of exposure, the cultures were subjected to cell counts by MB total protein assay (a, c) and parallel cultures (b, d) were assayed for ALP activity as in Figure 3. Results are presented as mean ± SEM, n = 20.

increased the activity ratio of c-Src-kinase by at least three folds relative to the untreated controls (Fig. 7a.2,b.2). At the 24 h time point, the kinase activity ratio is again at the negative activity level below unity and is consistent with the cell cycle progress and with an approximate completion of a cell cycle turn. This undulant advance of pp60Src-kinase activity ratios reflects the decrease in phosphorylated tyrosine-529 under exposure to 5 and 50 μg/ml of both LDL forms at 10 min, 30 min, and 4 h parallel to an increase in Src phosphorylation at tyrosine-418. The 10 min time point under 50 μg/ml both LDL forms and the 4 h time point under both LDL concentrations show a deviation of the kinase phosphorylation ratios from their relevant controls. Therefore, these time points are suspected to bear a functional meaning in regard to c-Src effect on cell proliferation, differentiation, and survival under LDL-induced signals.

FACS Analysis of Saos2 Cells Exposed to LDL

We have looked for the fraction of shrinking cells under the effect of NLDL and OxLDL in the presence and absence of DEX. Saos2 cells were

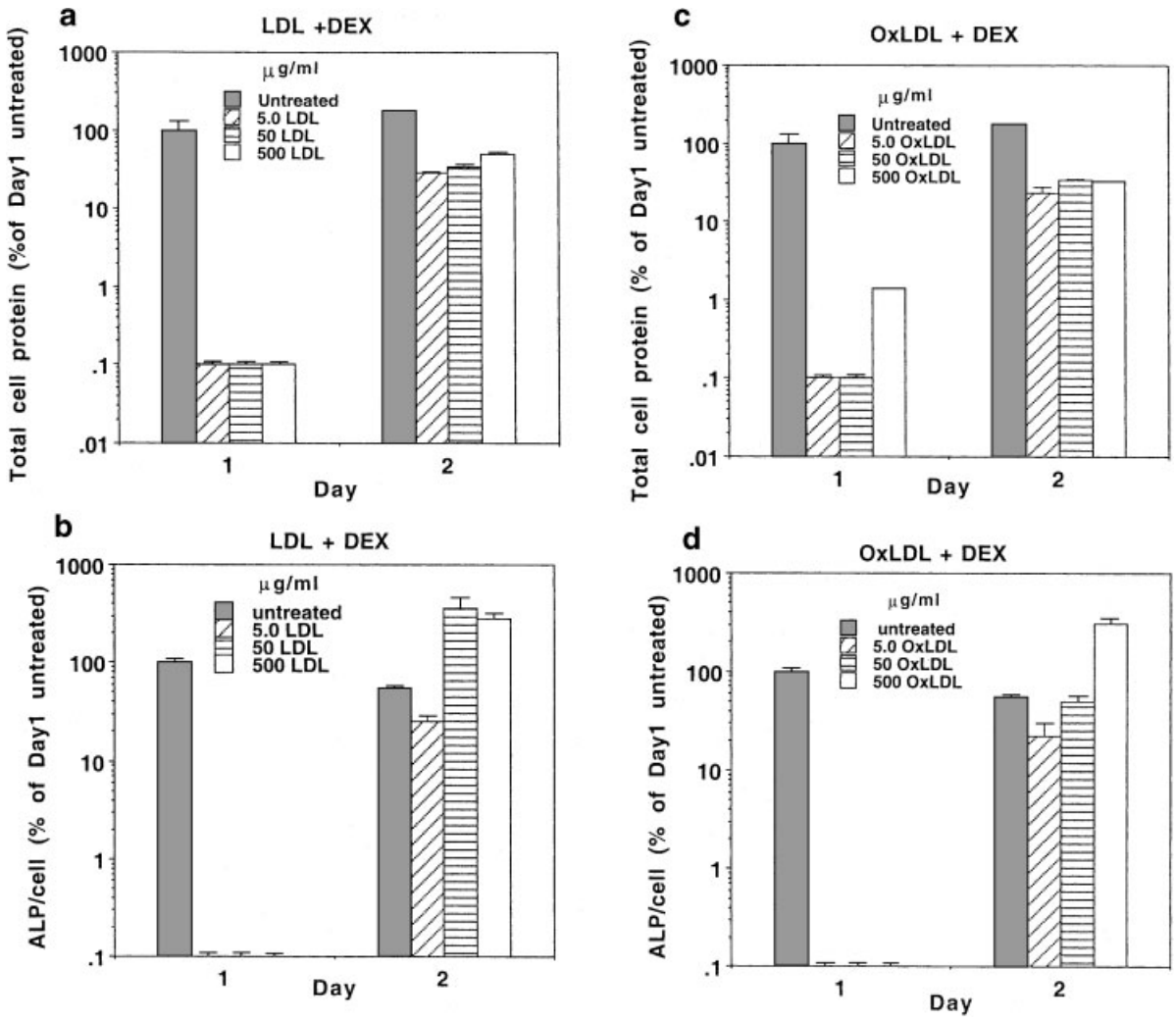

Fig. 5. Effect of NLDL and OxLDL in the presence of DEX on Saos2 osteoblast cell proliferation and ALP activity. Saos2 cells seeded 3,000 cell/well, after 48 h the medium was changed with one that contained indicated concentrations of LDL + DEX (**a**, **b**) or OxLDL + DEX (**c**, **d**), the FCS was replaced with human lipoprotein free serum. After 24 and 48 h of exposure, the cultures were subjected to cell counts by MB total protein assay (a, c) and parallel cultures (b, d) were assayed for ALP activity as in Figure 3. Results are presented as mean ± SEM, n = 20.

exposed to various LDL containing media for 24 h, removed by trypsinization fixed and stained with PI for analysis on a fluorescent cell sorter. Figure 8 shows the distribution of cells according to cell size in cultures treated with NLDL and OxLDL and without DEX. The cells with sizes smaller than the G_0/G_1 population, at the left side of the graphs, are indicated and their proportion as percentage of the total population is shown in each graph, this cell fraction is considered apoptotic. It follows that the cultures treated with OxLDL revealed less apoptotic cells than the ones treated with NLDL, the culture treated with 50 μg/ml of OxLDL revealed 5% apoptotic cell similar to the untreated control. None of the cultures treated with the same LDL doses combined with DEX revealed apoptotic cells proportions higher than controls (not shown), probably because of the harsh effect of this treatment that has rapidly lysed cell populations destined to show apoptotic features.

Bcl-2 and Bax Proteins in Saos2 Cells Under NLDL

The kinetics of Bcl-2 and Bax changes were examined on Western blots of proteins extracted from Saos2 cells that have been exposed to

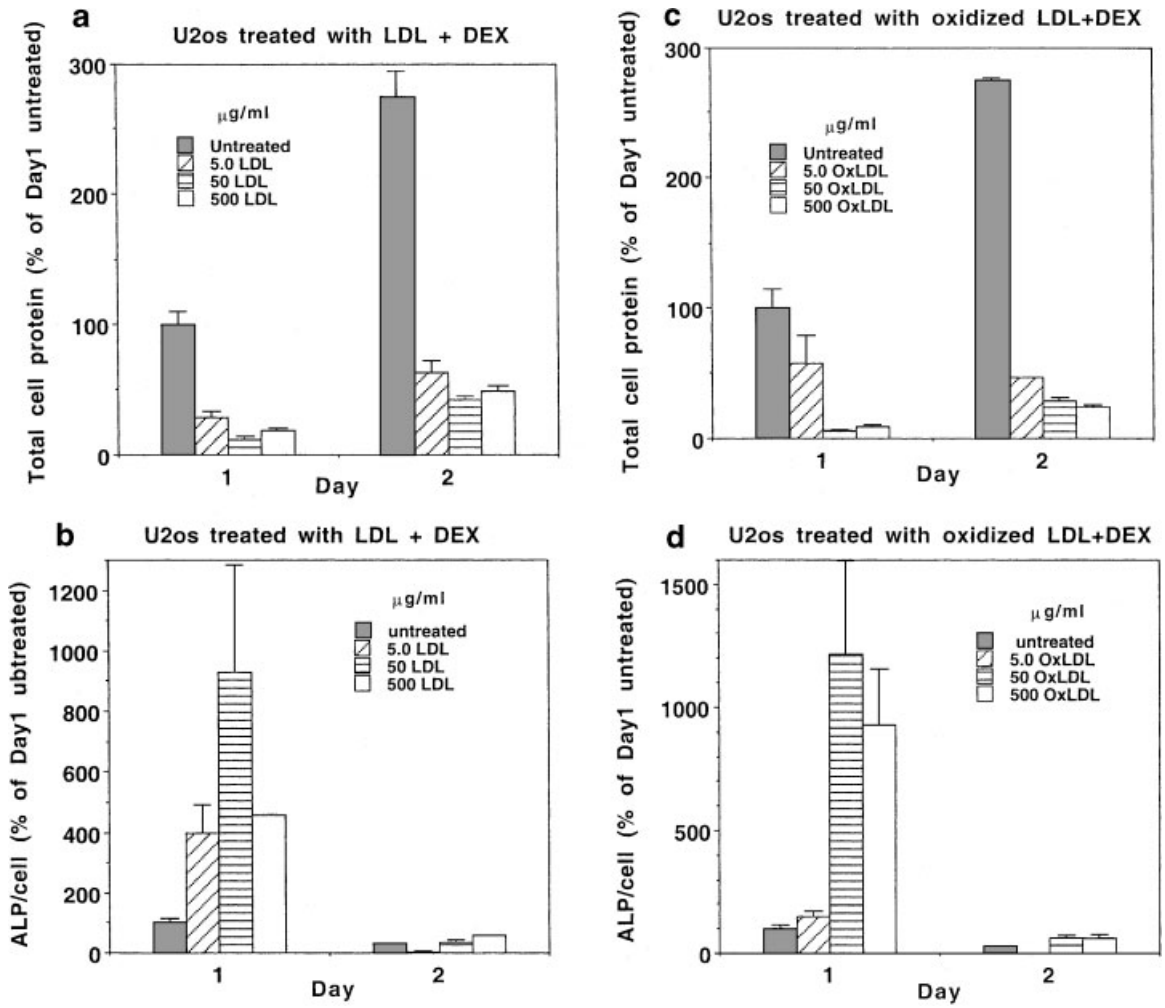

Fig. 6. Effect of NLDL and OxLDL in the presence of DEX on U2OS osteoblast cell proliferation and ALP activity. U2OS cells seeded 3,000 cell/well, after 48 h the medium was changed with one that contained indicated concentrations of LDL + DEX (**a**, **b**) or OxLDL + DEX (**c**, **d**), the FCS was replaced with human lipoprotein free serum. After 24 and 48 h of exposure, the cultures were subjected to cell counts by MB total protein assay (a, c) and parallel cultures (b, d) were assayed for ALP activity as in Figures 2 and 3. Results are presented as mean ± SEM, n = 20.

NLDL at different time intervals. Figure 9 shows the Bcl-2 and Bax proteins response under two extreme conditions one is under NLDL alone and the other is under OxLDL combined with DEX. The control cultures are represented as Bax/Bcl-2 ratios by continuous and discontinuous lines (no treatment and DEX alone, respectively, in Fig. 9a.2,b.2). The figure shows that at the 4 h time point, DEX has induced a surge in Bax/Bcl-2 ratio twice as high as in the untreated cultures, however, it declined at the 24 h time point, while the ratios in the untreated cultures continued to rise at the 24 and 48 h time points. This is consistent with the ability of DEX to kill half the cell population at an early stage and select the cells with high ALP activity as seen above. At 24 h under DEX alone (Fig. 9a.2,b.2) and DEX + 5 μg/ml OxLDL (Fig. 9a.2) the cells show Bax/Bcl-2 ratios that are similar to one another and far below the level seen at 4 h under DEX alone. This is consistent with Bax/Bcl-2 ratios in a selected cell population that is about to start a new cell cycle; it is also consistent with our failure to show dead cells of shrunken volume, among the DEX + OxLDL treated populations, in the FACS analysis after 24 h. The absence of shrunken cell occurred perhaps due to early lysis by additional mechanisms induced by the combination of DEX + OxLDL as opposed to NLDL alone. The only instance in which the Bax/Bcl-2 ratio is consistent with apoptosis that

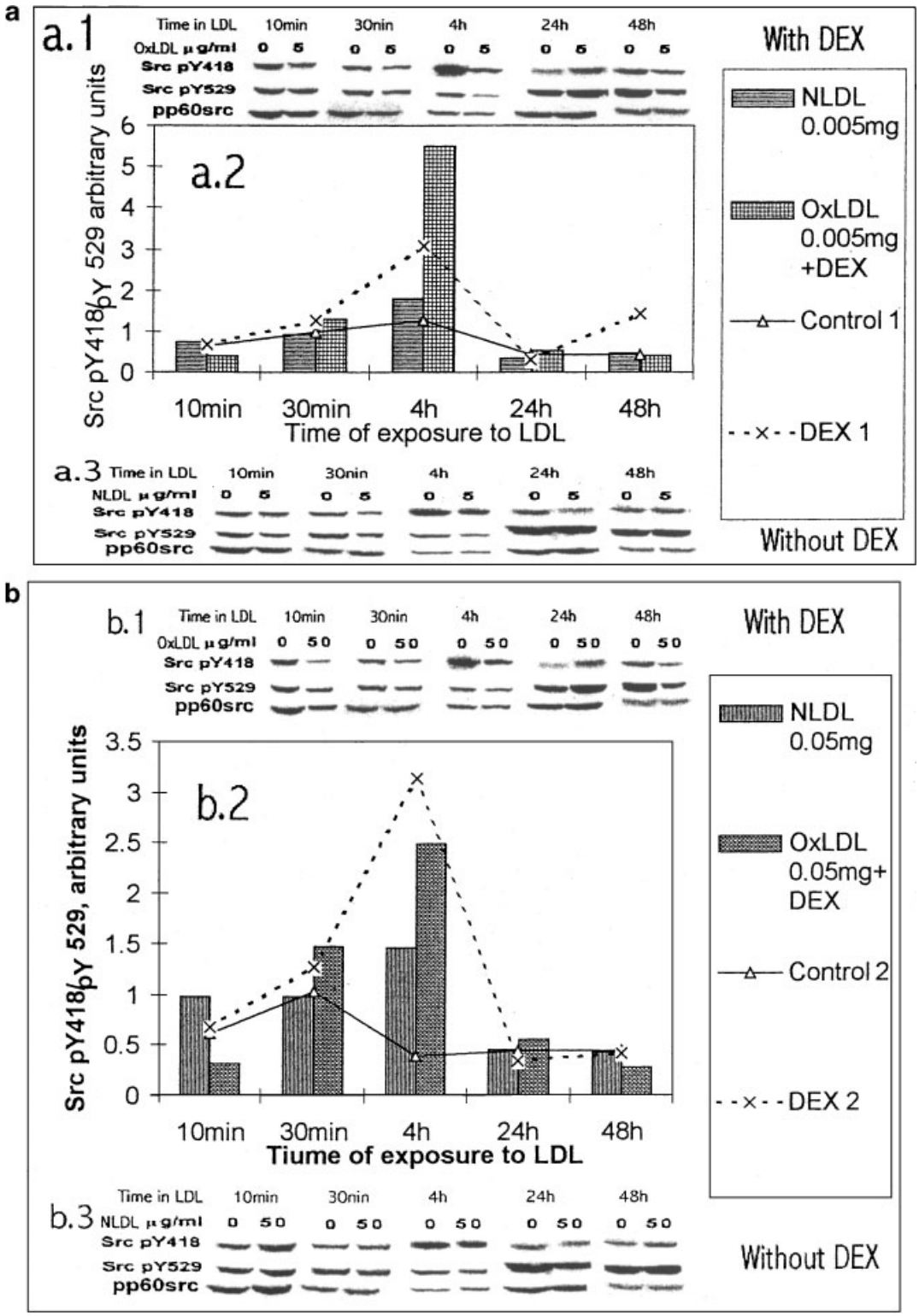
a
a.1
Time in LDL
10min
30min
4h
24h
48h
OxLDL µg/ml
0 5
Src pY418
Src pY529
pp60src
With DEX
NLDL 0.005mg
OxLDL 0.005mg +DEX
Control 1
DEX 1
a.2
Src pY418/pY 529 arbitrary units
Time of exposure to LDL
a.3
NLDL µg/ml
Without DEX
b
b.1
OxLDL µg/ml
0 50
NLDL 0.05mg
OxLDL 0.05mg+ DEX
Control 2
DEX 2
b.2
Src pY418/pY 529, arbitrary units
Tiume of exposure to LDL
b.3
NLDL µg/ml

Fig. 7.

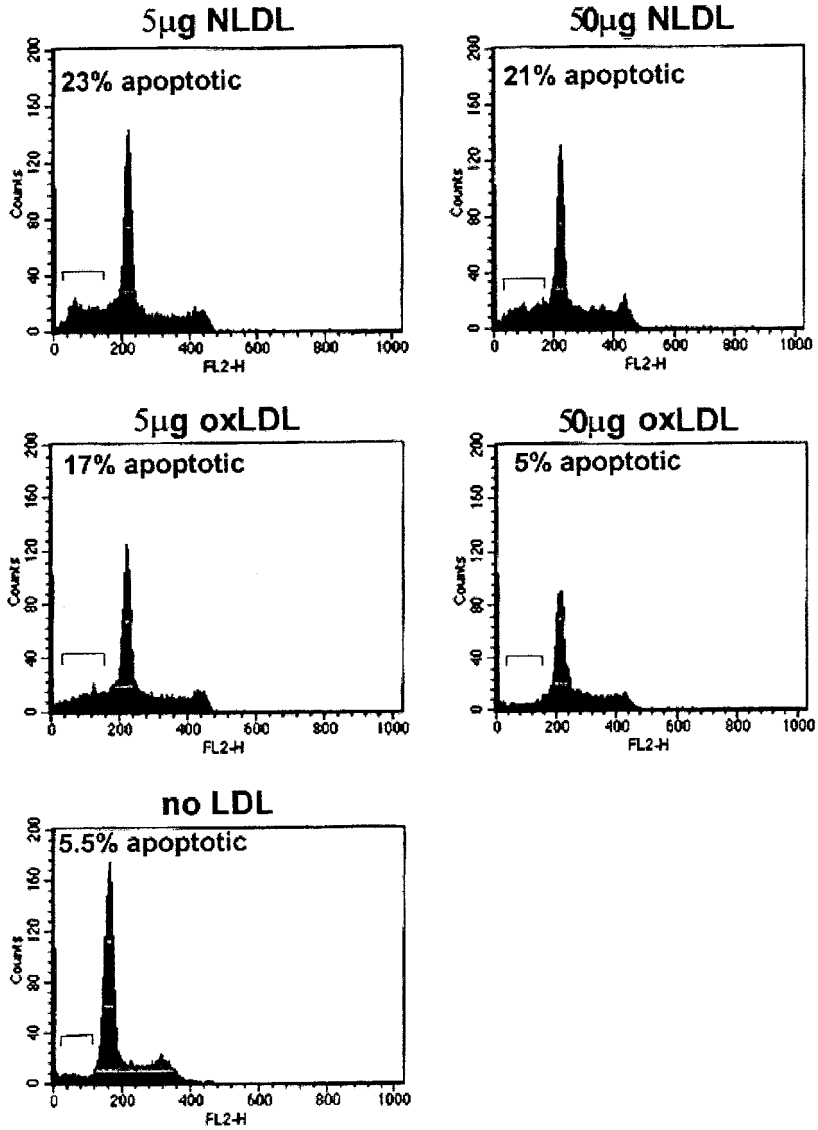

Fig. 8. Fluorescence-activated cell sorter (FACS) analysis of Saos2 for the percentage of apoptotic cell fraction under the effect of NLDL and OxLDL. Saos2 cells, exposed to the same conditions described in Figure 7 legend, were prepared for FACS analysis as described in ''Materials and Methods'' after 24 h. Each graph represents a culture of a different (indicated) treatment. The X-axis shows the relative cell size distribution along the cell cycle and the Y-axis shows the number of cells. The smallest normal fraction of cells represents the G_0/G_1 stage shown as a sharp pick, on the left side of this pick indicated by a staple pin is the shrunken cell fraction considered apoptotic cell fraction. The percentage of apoptotic cell relative to the entire population is indicated for each culture treatment.

Fig. 7. Changes in the ratios between phosphotyrosine (pY) residues, positive and negative regulators of pp60Src-kinase activity in Saos2 cells, under LDL. Saos2 cells in 6-well plates at subconfluence were exposed to indicated concentration of LDL, with and without DEX (10 nM DEX) and 2% of lipid-free human serum. At indicated time intervals, cell were lysed and 75 mg protein samples were run on SDS–PAGE 7–15% gradient. Western blots were reacted separately with two different mAbs specific for pp60Src-kinase regulation residues. Bands representing pY418 (positive) and pY529 (negative) regulation of c-Src were scanned and their density values derived, using NIHimage software adapted to PC computers (Scion Corp., Frederick, MD). The ratios between pY418 and pY529 are presented as columns, NLDL and OxLDL. The DEX 1 & 2 controls (discontinuous lines) and the untreated 1 & 2 controls (discontinuous lines) are superimposed on the LDL treated columns for clarity. Phosphoprotein bands are also shown, **upper panels** (**a**) represent treatment with 5 μg/ml LDL (OxLDL + DEX: a1 and NLDL: a3), **lower panels** (**b**) represent treatment with 50 μg/ml LDL (OxLDL + DEX: b1 and NLDL: b3). Control bands of the total pp60Src protein are presented below the corresponding phosphoprotein bands.

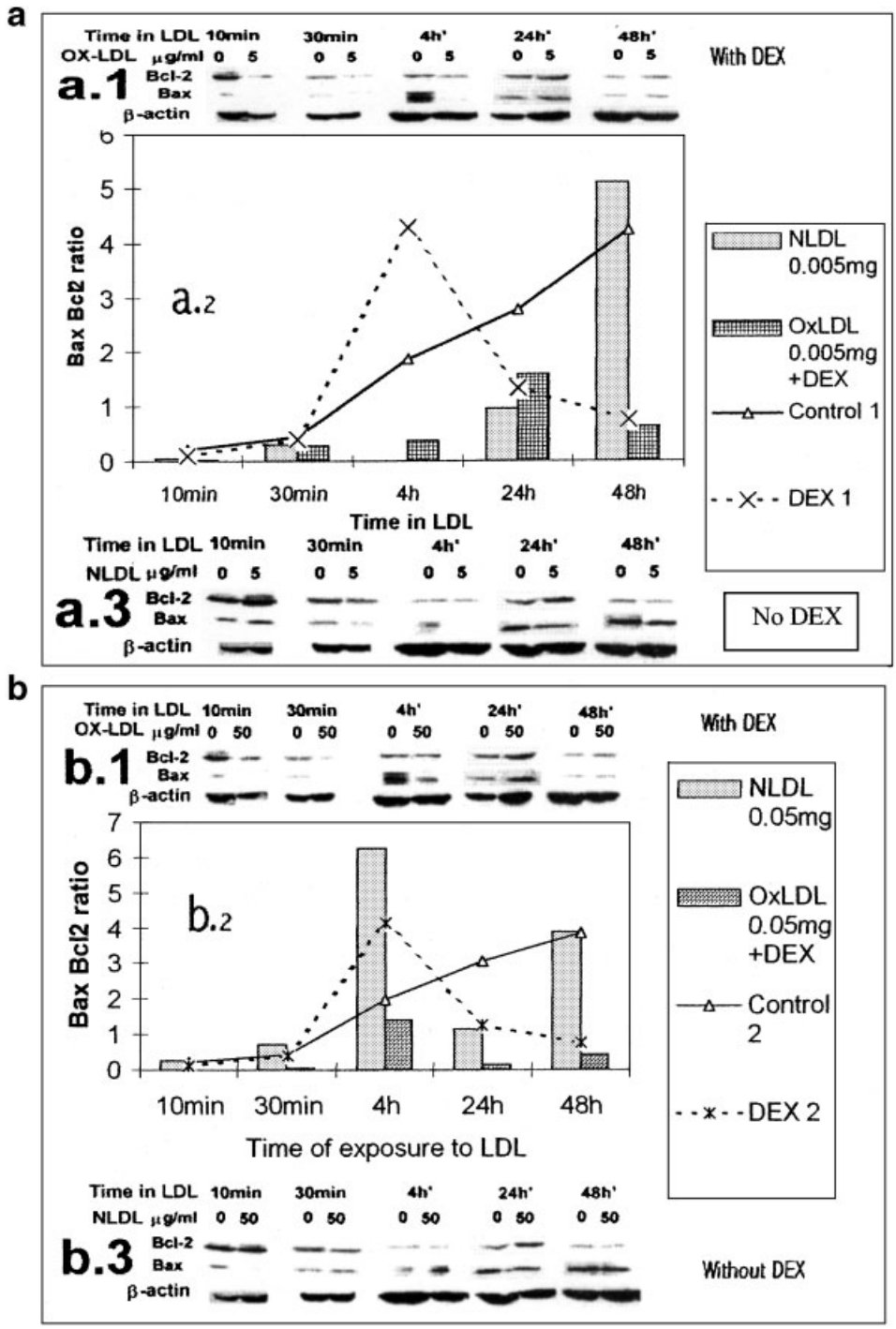

Fig. 9. Changes in the ratios between pro-apoptotic Bax and anti-apoptotic Bcl-2, proteins in Saos2 cells, under LDL. The experimental conditions are identical to those shown in Figure 7, with the exception that the detection antibodies recognized Bax and Bcl-2 pro- and anti-apoptotic proteins, and the internal control is β-actin.

corroborate with the FACS analysis is the surge at the 4 h time point under 50 μg/ml of NLDL alone (Fig. 9b.2,9b.3) that is more than twice the control level. The discrepancy between the FACS analysis results and the Bax/Bcl-2 ratios under NLDL at 24 h time points, relative to the controls, suggests that BAX may have more then one function in osteoblasts and that

LDL-induced cell death may occur by a mechanism in which Bax activity is not the essential component.

DISCUSSION

The non-stimulated osteoblast cell lines showed transcriptional expression of ApoE, however, it is not clear whether ApoE mRNA is translated into secreted proteins in these cell lines. Expression of ApoE in osteoblastic cell lines is not surprising at least Saos2 expresses BMP-2 that has been shown to trans-activate *ApoE* genes in murine embryonic tissues including progenitors of bone cells and adipocytes [Bachner et al., 1999]. It is also not clear as to whether Saos2 and U2OS osteoblasts express ApoB/E receptors, although when exposed to ApoE4 (that is neurotoxic [Tolar et al., 1999] and is implicated as risk factor in sporadic type Alzheimer disease) a decrease in cell count occurred in Saos2 cells (Fig. 2a). ApoE4 might be a risk factor in the development of OP [Cauley et al., 1999], perhaps in combination with other risk factors. This makes osteoblast cell lines a model for studying cellular mechanisms of ApoE signaling in osteoblasts, a line of research that we haven't expanded. Another putative lipid-related risk factor for OP is the cholesterol carrier complex LDL that is implicated in causing vascular wall pathology and cell death [Colles et al., 2001], especially when it is oxidized. As a blood-born factor, LDL was suggested to act reciprocally on mineralization in atherosclerotic arteries versus loss of mineralized matrix in bone [Parhami et al., 1997], therefore it is reasonable to expose osteoblast cell lines to NLDL and OxLDL in order to examine the proliferative response in osteoblast cultures. Both cell lines showed sensitivity to LDL but Saos2 were more sensitive to OxLDL and NLDL than U2OS cells. The difference in sensitivity could be due to several differences between the two cell lines, e.g., Saos2 do not express p53 and the retinoblastoma (*Rb*) genes [Yamabe et al., 1998] that are important in the control of the cell cycle [Hofbauer and Denhardt, 1991; Leake, 1996]. Therefore, the effects seen in Saos2 under the various treatments are independent of p53 and Rb proteins. NLDL has decreased the cell counts in Saos2, for which we cannot role out the possibility that the non-oxidized LDL became minimally oxidized spontaneously, similar to LDL in vivo that its separation from the original plasma causes loss of natural plasma-contained protection against oxidants. This may also explain the cases in which OxLDL had a lesser effect than its NLDL (minimally oxidized) counterpart. In some experiments, the OxLDL could have been damaged to a degree of lesser effectiveness in stress-induction relative to the NLDL that might have become inadvertently minimally oxidized during its purification. The ability of LDL to decrease ALP activity in Saos2 cells indicates a negative effect on osteoblasts and together with the decrease in cell counts may constitute a model for lipid-induced bone pathology, e.g., OP.

The use of DEX caused Saos2 cell death in less than 24 h of at least half of the cell population selecting survived cells with higher ALP activity, this may explain the lack of apoptotic cells in the FACS analysis (not shown) at 24 h. DEX is conventionally used in studying cell-mediated mineralization and osteoblastic differentiation in culture [Leboy et al., 1991; Kamalia et al., 1992], its positive stimulation of osteoprogenitor differentiation takes place in early stages but becomes negative later during mineralization [our unpublished results and Cheng et al., 1994]. We have examined the kinetics of pp60Src phosphorylation under LDL as a marker of osteogenic differentiation in osteoblasts. In previous studies, we have shown that pp60Src expression has diminished during osteoprogenitor differentiation, [Klein et al., 1998] and others [Marzia et al., 2000] have shown that osteoblast differentiation depends on the decreased expression of pp60Src. This phenomenon may be important in anti-osteoporotic drug targeting since bone resorption by osteoclasts depends on increased expression of pp60Src [Horne et al., 1992; Roodman, 1999] in reciprocity to its function in osteoblasts. Osteoclasts and macrophages are differentiation relatives both derived from monocytes, therefore it is possible that vascular wall foam cells also undergo increase in pp60Src expression similarly to marrow macrophages [Abu-Amer et al., 1998]. Monocytic progenitors of macrophages show increased c-Src activity during migration [Kintscher et al., 2001] and c-Src activity is also increased in proliferating vascular smooth muscle cells under the effect of minimally OxLDL [Watanabe et al., 2001]. The ability of LDL to increase c-Src activity in cultured osteoblasts might also be the case

in vivo, thus inhibition of c-Src could serve two disease entities that evolve in parallel, OP and atherosclerosis. We have compared two extreme conditions, one in which Saos2 cells were exposed only to NLDL for which Figure 8 showed apoptosis and in the other extreme OxLDL was used together with DEX which is an inducer of osteoblast differentiation and had a fast lytic effect on part of the cell population that resulted in selection of ALP-active cells. The later underwent dose dependent decrease in Src-kinase activity in the presence of DEX (pY418 fell sharply at 10 min with a slower fall in the inactive pY529) that is consistent with osteoblast differentiation pattern of c-Src. In contrast, the cultures treated with NLDL (perhaps being minimally oxidized) showed a reciprocal pattern at 10 min, i.e., a relative increase in the active Src-kinase that was prominent especially at the 4 h time point under 5 μg/ml. This increase of the pY418/pY529 ratio above the untreated controls is consistent with an anti-differentiation pattern in osteoblasts. It should be noted that the c-Src-kinase activity ratios are changing during the cell cycle perhaps related to cell cycle progression they are, however, influenced by extrinsic signaling (4 and 24 h time points). The effect of DEX alone is seen at the 4 h time point, however, improved synchronization and more time intervals are needed for better characterization of the interdependence between c-Src-kinase and cell cycle progression under these conditions.

The ratios between the pro- and anti-apoptotic proteins Bax/Bcl-2 are more variable than those of the pY418/pY529 at each time point and this variability is extended to the 24 and 48 h time points perhaps depending also on culture density. Based on the FACS results, we expected an increase in Bax/Bcl-2 ratios by both NLDL doses relative to the untreated controls, however, this occurred only at the 4 h time point under 50 μg/ml (starting at 30 min, Fig. 9b.2). Under 5 μg/ml of LDL the increase in Bax/Bcl-2 ratio characteristic of apoptosis occurred only at the 48 h time point (Fig. 9a.2), and doesn't explain the apoptotic cells seen at 24 h in the FACS analyses (Fig. 8). The discrepancy between the results of the FACS analysis and the lack of increased Bax/Bcl-2 ratios at 5 μg/ml NLDL indicates induction by LDL of apoptotic factors other than Bax protein. Other apoptotic pathways will have to be examined in the future, e.g., one of the MAPK pathways that involve either Src phosphorylation that may respond to LDL-induced stress [Abe et al., 1997; Watanabe et al., 2001; Suzaki et al., 2002], or p38 activation. OxLDL may induce apoptosis by stress in which ceramide release is involved [Deigner et al., 2001]. Changes in intracellular distribution of ceramide may shorten Bcl-2 half-life via dephosphorylation by PP2 at the mitochondria level [Ruvolo et al., 1999] and thereby tilt the Bax/Bcl-2 ratio in favor of apoptosis. As yet an unsorted issue is on one hand the involvement of Bax and Bcl-2 in the mitochondrial participation in apoptosis and on the other hand the involvement of the mitochondria in the initiation of bone matrix mineralization [Klein et al., 1993b]. It has been suggested that the cell-mediated mineralization is associated with uncoupling that enables thermogenesis [Klein et al., 1996a]. This process might start with temporary coupling of oxidative-phosphorylation, reflected by a surge in membrane potential, that is, subsequently discharged [Klein et al., 1996b] and being followed by mineralization. One effect of the DEX on the coupling period is its ability to increase the membrane potential [Klein et al., 1993a], this effect of DEX is associated (as seen in Fig. 9, 4 h time point) with a surge in Bax/Bcl-2 ratio. Bax interaction with Bcl-2 at the mitochondrial membrane may regulate the pore-forming domain of the Bcl-2 family molecules [Antonsson et al., 1997; Reed, 1997]. Cyclophilins make part of mitochondrial membrane permeability transition pores (MPTP) that may release cytochrome-C inducible by calcium and inhibited by cyclosporine-A [Eskes et al., 1998]. Bax regulates another MPTP that permits cytochrome-C release by Mg^{2+} ions but insensitive to cyclosporine-A inhibition. Cyclosporines may manipulate the mitochondrial membrane potential and inhibit cell-mediated mineralization by blocking the MPTP [Klein et al., 1997a]. Therefore, it is possible that the mitochondrial machinery used in apoptosis could serve also in initiation of cell-mediated mineralization, whether using cyclosporine-A sensitive MPTP or Mg^{2+} sensitive MPTP in which Bax is involved. Interestingly, an MPTP protein complex that involves ANT (mitochondrial adenine nucleotide translocator) that may form pores on its own which are inhibited by ATP and/or Bcl-2 but their permeability is enhanced by Bax [Brenner et al., 2000]. Thus in mineralizing cells, (whether in bone or in arterial walls) differences in fine

tuning under LDL may occur that determine where mineralization (mitochondrial calcium release) prevails over apoptosis (cytochrome-C release) or vice-versa. It can be concluded that LDL (in an oxidation level yet to be determined) can kill osteoblastic cell lines by an apoptotic mechanism involving at least partially the Bcl-2 system. It is also worthwhile to examine additional mechanism for this killing effect since it could play a role in OP in parallel to atherosclerosis under mechanisms that share signaling pathways, and therefore therapeutic molecular targets.

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