Journal of Cellular Biochemistry 96:198–208 (2005)

Cholestane-3β,5α,6β-Triol Inhibits Osteoblastic Differentiation and Promotes Apoptosis of Rat Bone Marrow Stromal Cells

Hongmei Liu,[1,2] Lan Yuan,[3] Shanjin Xu,[1] Kui Wang,[1] and Tianlan Zhang[1]*

[1]Department of Chemical Biology, Peking University School of Pharmaceutical Sciences, Beijing 100083, PR China

[2]Department of Chemistry, Huazhong University of Science and Technology, Wuhan, Hubei 430074, PR China

[3]Peking University Medical and Healthy Analysis Center, Beijing 100083, PR China

Abstract Converging lines of evidence suggest that oxidized lipids, long recognized as a risk factor in atherogenesis, also contribute to osteoporosis, but the underlying mechanism is not understood in detail. The effect of atherogenesis related factors including oxysterols on the differentiation and survival of marrow stromal cells (MSCs) would be very important in understanding the link between atherosclerosis and osteoporosis. In the present study, the effect of oxysterol cholestane-3β,5α,6β-triol (Triol) on osteoblastic differentiation and apoptosis of primary rat bone MSCs as well as the related mechanisms were studied. Triol inhibited MSCs osteoblastic differentiation as demonstrated by inhibition of alkaline phosphatase activity, osteocalcin secretion, and matrix mineralization. In the other aspect, Triol promoted MSCs apoptosis, as characterized by condensed or fragmented nuclei as well as active externalization of phosphatidyl serine to the cell surface. In addition, Triol was found to induce increases of intracellular Ca^{2+} and Ca^{2+}-dependent reactive oxygen species generation in MSCs. These effects were involved in the action of Triol on apoptosis, but not on osteoblastic differentiation of MSCs. These results suggested that Triol might contribute to the decreased bone formation by inhibition of osteoblastic differentiation and promotion of apoptosis of MSCs, providing insights about common factors underlying the pathogenesis of atherosclerosis and osteoporosis. J. Cell. Biochem. 96: 198–208, 2005. © 2005 Wiley-Liss, Inc.

Key words: cholestane-3β,5α,6β-triol; marrow stromal cells; differentiation; apoptosis; intracellular Ca^{2+}; reactive oxygen species

The association between osteoporosis and atherosclerosis has been reported widely in the elder population, but its cause–effect relation is open to be answered [McFarlane et al., 2004]. Recently, converging lines of evidence suggest that oxidized lipids, long recognized as a risk factor in atherogenesis, also contribute to osteoporosis development [Parhami et al., 1997, 1999, 2000]. In age-related osteoporosis, the decreased number and osteogenic activity of osteoblasts, in part, lead to decreased bone formation [Egrise et al., 1992; Bergman et al., 1996; Mullender et al., 1996]. Since osteoblasts matured from osteoprogenitor cells arising from marrow stromal cells (MSCs) [Pittenger et al., 1999], elucidation of the factor and molecular mechanisms involved in the differentiation and survival of MSCs would be very important in understanding the osteoporotic disease process. In vitro studies indicated that oxidized lipids, especially oxidized low density lipoprotein (LDL), inhibited bone cell osteoblastic differentiation in certain circumstances [Parhami et al., 1997, 1999]. However, the role of oxidized lipids in osteoblastic differentiation is still not well documented.

Oxysterols, the oxidation products of cholesterol, were found in the circulation and in

Grant sponsor: The National Natural Science Foundation of China; Grant number: 20031010.

*Correspondence to: Tianlan Zhang, Department of Chemical Biology, Peking University School of Pharmaceutical Sciences, 38 Xueyuan Road, Beijing 100083, PR China. E-mail: tlzhang@bjmu.edu.cn

Received 2 January 2005; Accepted 10 March 2005

DOI 10.1002/jcb.20510

© 2005 Wiley-Liss, Inc.

human and animal tissue [Salonen et al., 1997; Zieden et al., 1999]. On the basis of animal experimental models and human clinical studies, oxysterols have been shown to have potent effects in atherosclerotic plaque formation [Brown and Jessup, 1999]. The contents of oxysterols were much higher in oxidized LDL, and they have been shown to be active components of oxidized LDL [Colles et al., 2001]. Since oxidized LDL inhibited osteoblastic differentiation of osteoprogenitor cells [Parhami et al., 1999], we hypothesized that some oxysterols generated by osteoprogenitor cells, as well as those carried by blood to bone vessels, might affect osteoblastic differentiation or survival of these cells, and consequently the bone formation. Cholestane-3β,5α,6β-triol (Triol) appeared to be one of the most angiotoxic oxysterols tested in vivo [Imai et al., 1980; Peng et al., 1985; Stiko et al., 1996]. In the present study, we studied the effect of Triol on osteoblastic differentiation and apoptosis of rat bone MSCs as well as the related mechanisms.

MATERIALS AND METHODS

Cell Culture and Treatment

Rat bone MSCs were prepared and cultured as described by Maniatopoulos et al. [1988]. Briefly, the femurs were removed aseptically from young adult male SD rats (approximately 150 g) and cleaned of soft tissues. The epiphysis were removed and the marrow was flushed out with a syringe containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and the antibiotic mixture (100 U/ml penicillin; 100 μg/ml streptomycin). A single cell suspension was prepared by repeated pipetting. Cells were cultured in DMEM supplemented with 15% FBS, antibiotics, 10^{-8}M dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml of freshly-prepared ascorbic acid (approximately 284 μM vitamin C). After 4 days, non-adhering hematopoetic cells were discarded from the adherent cells representing bone MSCs. The cell cultures were kept up to indicated days, with total renewal of the culture medium every 2 or 3 days.

Cells were treated with different concentration of Triol (Sigma-Aldrich, St. Louis, MO) for indicated time, after pretreating with Ca^{2+} channel blocker, verapamil (1 μM), or antioxidant, vitamin C plus vitamin E (VC + VE, 25 μM) for 30 min or 2 h. The effects of Triol treatment were studied by the procedures given in the following sections.

Cell Viability Assay

On day 4 of culture, MSCs were treated with varied concentration of Triol. After 1, 2, 3, 10, or 17 days, cell viability was evaluated using the MTT assay [Denizot and Lang, 1986]. The cells were incubated with 0.5 mg/ml MTT (Sigma-Aldrich) for 4 h at 37°C; the solution was then removed and formazan salts dissolved with dimethyl sulphoxide, and the absorbance at 570 nm of the resulting solution was measured.

Measurement of Osteoblastic Differentiation Markers

At 7, 14, and 21 days of culture, the cell conditioned media were collected and osteocalcin secretion was measured by radioimmunoassay (RIA) employing [^{125}I] RIA kit (East Asia Institute of Immune Technology, Beijing, China). Alkaline phosphatase (ALP) activity of whole cell extracts was measured as described previously [Parhami et al., 1997] using an ALP activity assay kit. One unit was defined as the activity producing 1 μmol of *p*-nitrophenol within 30 min. Matrix mineralization was detected by von Kossa staining (30 min, 5% silver nitrate) [Parhami et al., 1997]. Alternatively, calcium deposition in the matrix was quantitated by the method described by Wada et al. [1999]. ALP activity, osteocalcin secretion, and calcium deposition were all normalized to total protein content of the cell layer determined by Lowry method [Lowry et al., 1951].

Detection of Apoptosis: Nuclear Morphology

MSCs were stained at 37°C for 30 min with 5 μg/ml Hoechst 33258 (Sigma-Aldrich) in PBS. Apoptotic cells were characterized by condensed or fragmented nuclei visualized using laser scanning confocal microscopy (Leica, TCS NT type, Gemany).

FITC-Labeled Annexin V/Propidium Iodide Double Staining

FITC-labeled annexin V/propidium iodide (PI) double staining was used to evaluate the percent of apoptotic cells. Active externalization of phosphatidyl serine to the cell surface is an early event in apoptotic cell death, and annexin V binding to phosphatidyl serine was used as a marker of early apoptotic events that

precede nuclear changes. Cells were also co-stained with PI as a marker of cell membrane permeability, which occurs during the later stages of apoptosis and in necrosis. The double staining was performed using a commercially available kit (Boehringer Ingelheim, Heidelberg, Germany) and then analyzed with bivariate flow cytometry (Becton-Dickinsin, San Jose, CA).

Measurement of Intracellular Ca^{2+}

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) in single cell was measured using fluo-3 as fluorescent Ca^{2+} probe. MSCs cultured in Petri dish (Costar, Cambridge, MA) were loaded with 10 μM fluo-3-AM (Molecular Probes, Eugene, OR) for 1 h at 37°C in modified Kreb's Ringer buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 6 mM glucose, 1.2 mM $MgSO_4$, and 1 mM $CaCl_2$). The Petri dish was placed on the stage of laser scanning confocal microscopy and Fura-3 fluorescence in single cell was monitored in real-time. Data from all measurements were presented as fluo-3 fluorescence intensity and were directly representative of changes in $[Ca^{2+}]_i$. Results shown in the present study were representative of multiple independent single cell recordings (~30).

Determination of Intracellular Reactive Oxygen Species

The level of reactive oxygen species (ROS) in MSCs was determined on the basis of the fluorescent signal generated by 2,7-dichlorofluorescein diacetate (2,7-DCFH-DA) upon reaction with hydroxyl radical, hydrogen peroxide, or peroxynitrite. The treated cells were loaded with 5 μg/ml 2,7-DCFH-DA (Molecular Probes) for 20 min at room temperature in modified Kreb's Ringer buffer. With laser scanning confocal microscopy, the fluorescence intensity of eight fields per dish was measured and processed with the software provided with the microscopy system. Three parallel experiments were performed. Results were shown as the mean ± SD of fluorescence intensity.

Statistical Analysis

Results from a representative of three independent experiments are shown as mean ± SD. Means were compared by one-way ANOVA, with comparison of different groups by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered significant.

RESULTS

Effect of Triol on Cell Viability of MSCs

As estimated by MTT assay, the effect of Triol on cell viability of MSCs was concentration-dependent (Fig. 1). No significant effect was observed after 1, 2, 3, 10, or 17 days treatment with 1 μM Triol. With 5 μM Triol, cell viability increased and reached a maximum at 122.7% of control after treatment for 2 days, but decreased after 10 days. With higher concentration (15 ~ 50 μM) of Triol, however, cell viability significantly decreased after treatment for 1 or 2 days; and in these cases, all cells died after 10 or 17 days, or even 3 days in the case of 50 μM.

Effect of Triol on Osteoblastic Differentiation of MSCs

Marrow stromal cells (MSCs) cultured in medium supplemented with dexamethasone, β-glycerophosphate, and ascorbic acid can spontaneously differentiate into osteoblast [Maniatopoulos et al., 1988; Malaval et al., 1994]. In the present study, to assess the effect of Triol on osteoblastic differentiation of MSCs, ALP activity, osteocalcin secretion, and matrix mineralization were measured as indices of differentiation.

Induction of ALP activity is an early marker of osteoblastic differentiation and plays an important role in eventual mineralization process [Stein and Lian, 1993; Malaval et al., 1994]. In control MSCs, ALP activity peaked on day 14 of culture at 11.19 ± 0.27 U/mg protein, and then declined. Triol treatment resulted in a significant decrease in ALP activity in a time- and

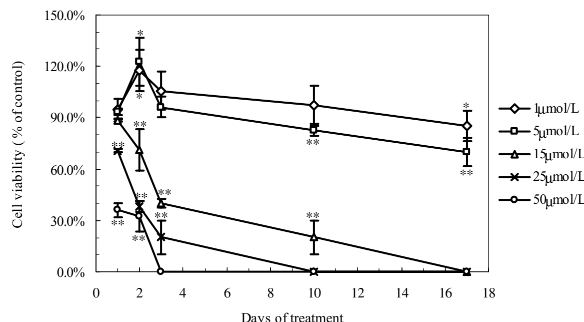

Fig. 1. Effect of Triol on the cell viability of MSCs (mean ± SD, n = 6). MSCs on day 4 of culture were treated with different concentration of Triol. After treatment for 1, 2, 3, 10, and 17 days, the cell viability was determined by MTT assay. Similar results were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.01$; compared with control.

dose-dependent manner (Fig. 2A,B). Moreover, ALP activity also significantly decreased when Triol was applied to MSCs at different points (from day 4 through 7 of culture, from day 11 through 14 of culture, and from day 18 through 21 of culture) during culture (Fig. 2C).

Osteocalcin is a more specific and relatively late stage marker of osteoblastic differentiation [Malaval et al., 1994]. In control MSCs, osteocalcin secretion increased gradually to maximum levels of 15.1 ± 2.7 nmol/mg protein on day 21 of culture. Similar to ALP activity,

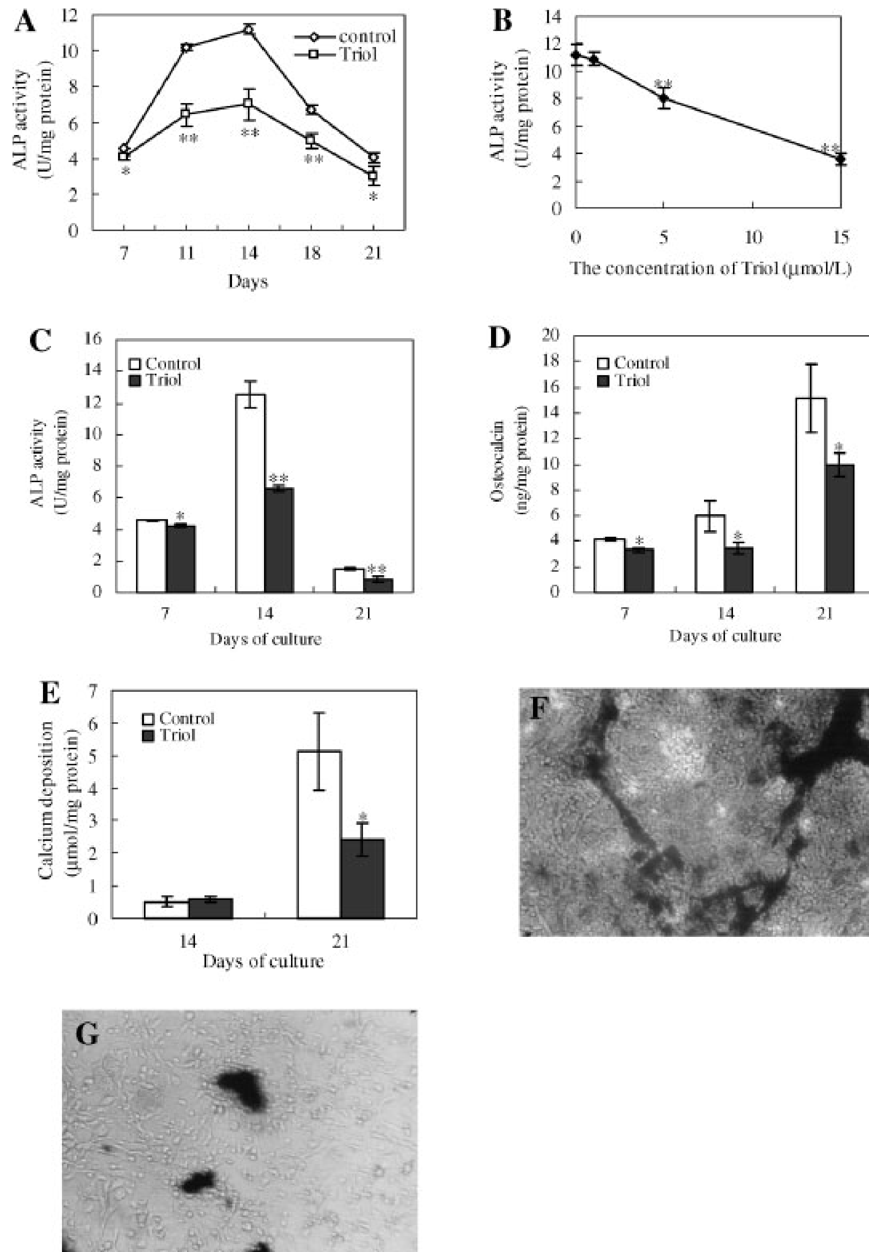

Fig. 2. Effect of Triol on osteoblastic differentiation of MSCs (mean ± SD, n = 4). **A**: Temporal change of ALP activity in MSCs treated with 5 μM Triol. ALP activity was assayed on day 7, 11, 14, 18, and 21 of culture in the cells treated without or with 5 μM Triol from day 4 of culture. **B**: Effect of different dose of Triol on ALP activity. ALP activity was assayed on day 14 of culture after the cells were exposed to different dose of Triol for the last 3 days. ALP activity (**C**) and osteocalcin secretion (**D**) were measured on day 7, 14, and 21 of culture after the cells were treated without or with 5 μM Triol for the last 3 days. **E**: Calcium deposition was determined on day 14 and 21 of culture in the cells treated without or with 5 μM Triol from day 4 of culture. Von Kossa staining was performed on day 21 of culture in the cells treated without (**F**, ×180) or with (**G**, ×180) 5 μM Triol from day 4 of culture. Similar results were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.01$; compared with control.

osteocalcin secretion significantly reduced after treatment with Triol at different points during culture (Fig. 2D).

Matrix mineralization, as measured by calcium deposition, was undetectable in control MSCs on day 7 of culture, low (0.51 ± 0.17 μmol/mg protein) on day 14 of culture, and increased almost 10 fold to 5.14 ± 1.19 μmol/mg protein on day 21 of culture. Triol treatment markedly suppressed the increase of calcium deposition on day 21 of culture (Fig. 2E). In consistence with these results, von Kossa staining showed that there were large amounts of mineral on day 21 of culture in control MSCs (Fig. 2F), and few in cells treated with Triol (Fig. 2G).

To clarify the roles of Ca^{2+} and ROS in Triol-induced inhibition of osteoblastic differentiation, MSCs were pretreated with Ca^{2+} channel blocker, verapamil, and antioxidants, VC + VE, before exposure to Triol. However, the results showed that both pretreatments failed to significantly block the inhibitory effect of Triol on ALP activity, osteocalcin secretion, and calcium deposition (Table I).

Effect of Triol on Apoptosis of MSCs

By using confocal microscopy and Hoechst 33258 staining, apoptosis of MSCs was visualized. MSCs on day 21 of culture without Triol were normal in nuclear morphology in monolayer (Fig. 3A), but with several condensed nuclei in multilayer (Fig. 3B). More condensed nuclei, and especially many fragmented nuclei, were observed in the cells treated with Triol (Fig. 3C).

To evaluate the apoptosis of MSCs, FITC-labeled annexin V/PI double staining and flow cytometric analysis were employed. As shown in Figure 4, Triol had little effect on apoptosis at lower level (5 μM), while it significantly increased both the early apoptotic cells and late apoptotic or necrotic cells at higher level (15 ~ 25 μM). Furthermore, VC + VE and verapamil pretreatment significantly reduced the early apoptosis promoted by 25 μM Triol (Table II), indicating that intracellular Ca^{2+} and ROS might be involved in the action of Triol on MSCs apoptosis.

Effect of Triol on $[Ca^{2+}]_i$ in MSCs

In MSCs on day 14 of culture, as shown by Fluo-3 fluorescence, addition of 50 μM Triol immediately caused a dramatic but transient $[Ca^{2+}]_i$ increase (Fig. 5A), which was almost completely eliminated by verapamil (1 μM) pretreatment (Fig. 5B). A slower $[Ca^{2+}]_i$ increase was detected with 25 μM Triol (Fig. 5C). In addition, Triol at lower level (5 μM) had no influence on $[Ca^{2+}]_i$ after addition for half an hour, but induced a significant increase of $[Ca^{2+}]_i$ after addition for 24 h (data not shown).

When MSCs on day 21 of culture were treated with 50 μM Triol, $[Ca^{2+}]_i$ changed by different trend, depending on basal $[Ca^{2+}]_i$. In those cells with a lower basal $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ increased immediately, but followed by a gradual decline to a higher basal line (Fig. 5D). In those cells with a higher basal $[Ca^{2+}]_i$, Ca^{2+} oscillations were observed, which last for about 4 min (Fig. 5E). Similarly, verapamil pretreatment significantly attenuated the increase or oscillations of $[Ca^{2+}]_i$ (Fig. 5F).

Effect of Triol on Intracellular ROS Generation in MSCs

As determined by DCF fluorescence, in MSCs on day 14 of culture, a significant increase in ROS level was detectable after 1 h, and reached

TABLE I. Influence of Ca^{2+} Channel Blocker, Verapamil, and Antioxidant, VC + VE, on Triol-Induced Inhibition of ALP Activity, Osteocalcin Secretion, and Calcium Deposition in MSCs

Group	ALP activity (U/mg protein)	Osteocalcin secretion (μmol/mg protein)	Calcium deposition (ng/mg protein)
Control	12.51 ± 0.87	14.88 ± 1.64	5.14 ± 1.19
Verapamil	11.86 ± 0.70	14.28 ± 1.56	4.88 ± 0.37
VC + VE	11.26 ± 0.75	14.10 ± 0.21	4.39 ± 0.66
Triol	6.60 ± 0.20*	9.15 ± 0.33*	2.44 ± 0.50*
Verapamil + Triol	6.60 ± 0.49	8.48 ± 0.55	2.69 ± 0.24
VC + VE + Triol	6.69 ± 0.41	8.94 ± 0.58	2.83 ± 0.18

MSCs on day 11 of culture were pretreated with 1 μM verapamil or 25 μM VC + VE for 2 h, and then exposed to 15 μM Triol for an additional 3 days (mean ± SD, n = 4). Similar results were obtained in three independent experiments.
*$P < 0.01$, compared with control.

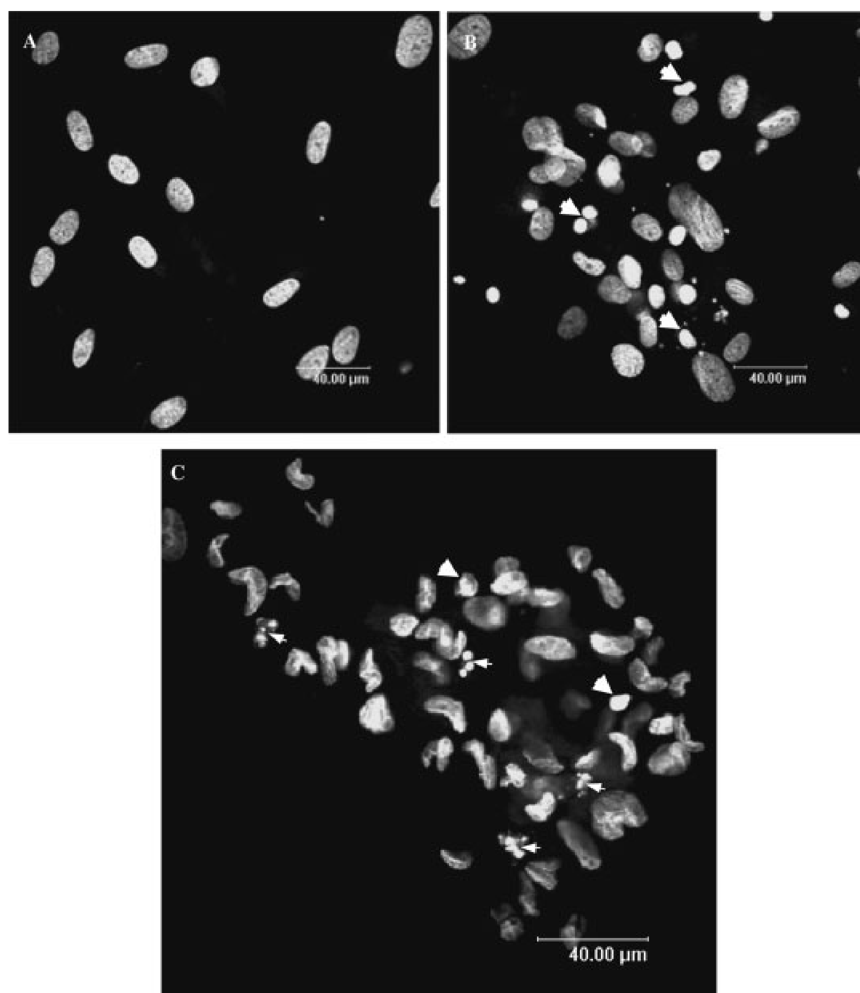

Fig. 3. Detection of apoptosis in MSCs on day 21 of culture by Hoechst 33258 staining and laser scanning confocal microscopy. **A**: The monolayer cells showed normal morphology nuclei. **B**: Several condensed nuclei (large arrow) appeared in multilayer of MSCs. **C**: After treatment with 25 μM Triol for the last 24 h, the multilayer cells exhibited much more condensed or fragmented nuclei (small arrow). One representative experiment out of three is shown.

up to 11 fold of control (103.0 ± 39.5 vs. 9.3 ± 4.6) after 6 h incubation with 50 μM Triol (Fig. 6A). This effect of Triol on ROS generation was concentration-dependent over the range $5 \sim 50$ μM (Fig. 6B). Moreover, VC + VE and verapamil both significantly decreased Triol-induced ROS generation (Fig. 6C). The effect of Triol on ROS generation in MSCs on day 21 of culture was similar to that in cells on day 14 of culture (data not shown).

DISCUSSION

Triol Inhibited Osteoblastic Differentiation of MSCs

Our results showed that during osteoblastic differentiation of MSCs in the presence of dexamethasone, β-glycerophosphate, and ascorbic acid, the activity of ALP peaked early, and then declined, while osteocalcin secretion was increased with more advanced osteoblast. This pattern was consistent with the previous results reported by Malaval et al. [1994]. On the basis of this pattern, Triol treatment inhibited the differentiation of MSCs into functional osteoblastic cells, as revealed by the decrease in ALP activity, osteocalcin secretion, and matrix mineralization. ALP, the early marker of osteoblastic phenotype, participates in the mineralization of osteoblasts by providing phosphate for hydroxyapatite formation [Anderson, 1995]. Inhibition of ALP inhibits mineralization of osteoblast cultures, and its impaired expression in hypophosphatasia in human and in

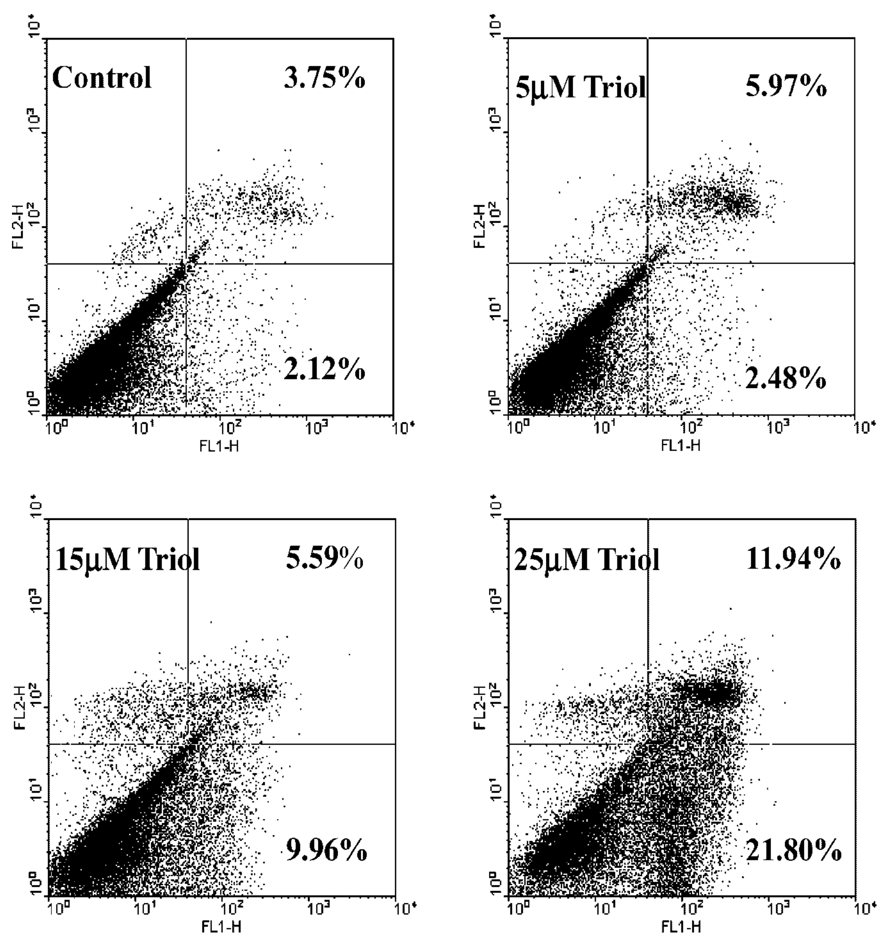

Fig. 4. Dot plots of FITC-labeled annexin V green fluorescence (FL1-H) versus PI red fluorescence (FL2-H) of MSCs on day 21 of culture. Cells were treated without or with different dose of Triol for the last 24 h. The lower left quadrant of each panels contains the viable cells (double negative), the lower right quadrant shows the early apoptotic cells (annexin V^+/PI^-), the upper right quadrant represents the late apoptotic or necrotic cells (annexin V^+/PI^+), and the upper left quadrant contains necrotic cells (annexin V^-/PI^+). One representative experiment out of three is shown.

tissue nonspecific ALP knockout mice is associated with defects in bone mineralization [Whyte, 1994; Fedde et al., 1999; Wennberg et al., 2000; Anderson et al., 2004]. Osteocalcin, essential for proper differentiation and mineralization of osteoblasts, is the most specific marker of the osteoblastic phenotype [Malaval et al., 1994]. Taken together, it could be concluded that Triol inhibited osteoblastic differentiation of MSCs by interfering with the key differentiation steps. This result was consistent with the previous reports that oxidized LDL inhibited osteoblastic differentiation of osteoprogenitor cells [Parhami et al., 1999]. Thus, we speculated that some oxysterols including Triol might be responsible for the effect of oxidized LDL on osteoblastic differentiation.

TABLE II. Effect of Verapamil and VC + VE on Triol-Promoted Apoptosis in MSCs

Group	Early apoptotic cells (%)	Late apoptotic cells (%)
Control	2.12 ± 0.15	3.75 ± 2.08
Verapamil	3.19 ± 1.55	3.04 ± 0.45
VC + VE	1.58 ± 0.50	3.80 ± 0.99
Triol	21.80 ± 0.74*	11.94 ± 1.22*
Verapamil + Triol	18.20 ± 0.53**	12.14 ± 0.49
VC + VE + Triol	17.48 ± 1.20**	10.94 ± 0.87

Cells on day 20 of culture were pretreated for 2 h with 1 μM verapamil or 25 μM VC + VE, and then exposed to 25 μM Triol for an additional 24 h. The apoptosis of MSCs was detected by FITC-labeled annexin V/PI double staining and flow cytometric analysis (mean ± SD, n = 3). Similar results were obtained in three independent experiments.
*$P < 0.01$, compared with control.
**$P < 0.05$, compared with cells treated with Triol alone.

However, Kha et al. [2004] recently reported that the combination of oxysterols, 22(R)-hydroxycholesterol (22R) + 20(S)-hydroxycholesterol (20S) or 22(S)-hydroxycholesterol (22S) + 20S, could completely enhance osteoblastic differentiation of osteoprogenitor cells, while individual oxysterols (22R, 20S, 22S) were only able to

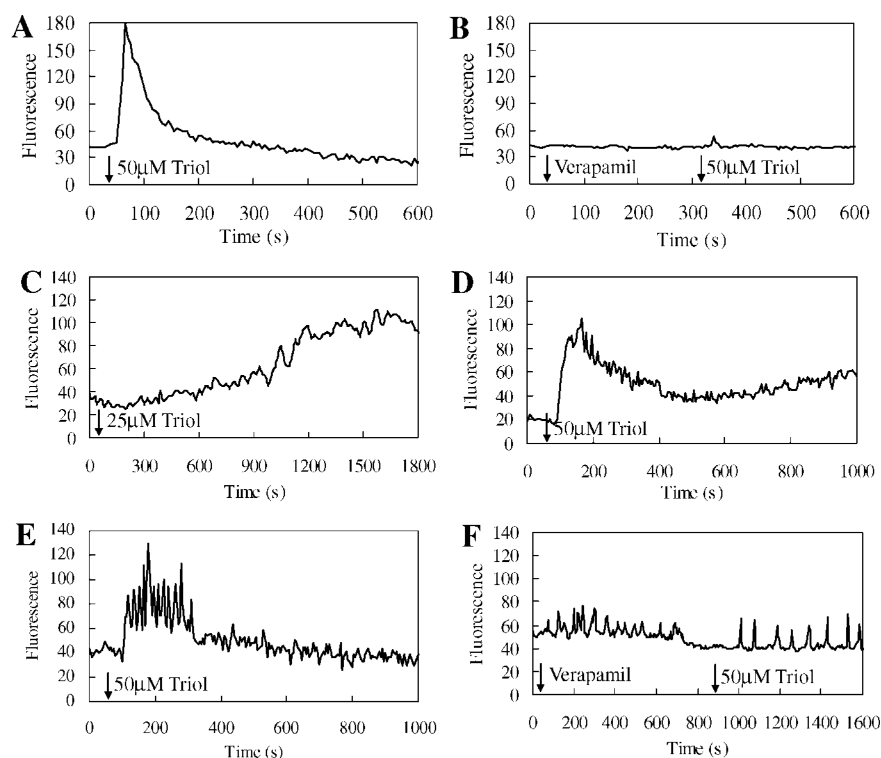

Fig. 5. Effect of Triol on $[Ca^{2+}]_i$ in MSCs on day 14 of culture (**A–C**) or on day 21 of culture (**D–F**). The arrows indicated the time of addition of Triol or 1 μM verapamil. One representative out of multiple independent single cell recordings (~30) is shown.

increase ALP activity without inducing osteocalcin gene expression and mineralization. Taking their results and ours into account, it is likely that the effect of oxysterols on osteoblastic differentiation is specific, and varies with different oxysterols.

Many studies explained the mechanism by which oxysterols induced cell responses on the basis of the intracellular signaling molecules such as intracellular Ca^{2+} and ROS [Panini and Sinensky, 2001]. In the present experiments, we evaluated the roles of these two factors in the response of MSCs to Triol.

An elevation of $[Ca^{2+}]_i$ has been reported to be involved in neuronal differentiation [Gu and Spitzer, 1997; Carey and Matsumoto, 1999]. In this study, Triol was found to induce an elevation of $[Ca^{2+}]_i$ in MSCs, which was completely eliminated by Ca^{2+} channel blocker, verapamil, suggesting that the signal was mainly due to the opening of Ca^{2+} channels and then the influx of extracellular Ca^{2+}. On the other hand, ROS, such as H_2O_2, have been reported to be able to inhibit osteoblastic differentiation of primary rabbit bone MSCs [Bai et al., 2004]. In the present study, Triol induced ROS generation in MSCs in a time- and concentration-dependent manner, and this effect was significantly depressed by antioxidant VC + VE and Ca^{2+} channel blocker verapamil. These results revealed that ROS generation induced by Triol was Ca^{2+}-dependent. However, our data also revealed that inhibition of the Ca^{2+} influx by verapamil and inhibition of ROS generation by both VC + VE had no significant effect on Triol-induced inhibition of ALP activity, osteocalcin secretion, and mineralization. This indicated that intracellular Ca^{2+} and ROS might not be involved in the mechanism by which Triol inhibited osteoblastic differentiation of MSCs.

Recent studies have shown that mitogen-activated protein kinases (MAPK) pathway contributed to the decreased osteoblastic differentiation. For example, extracellular signal-regulated kinase (ERK) activation was required for H_2O_2-induced inhibition of osteoblastic differentiation in rabbit bone MSCs [Bai et al., 2004]. It was worth to note that oxidized LDL inhibited osteoblastic differentiation of osteoprogenitor cells through a MAPK-dependent pathway [Parhami et al., 1999]. Therefore,

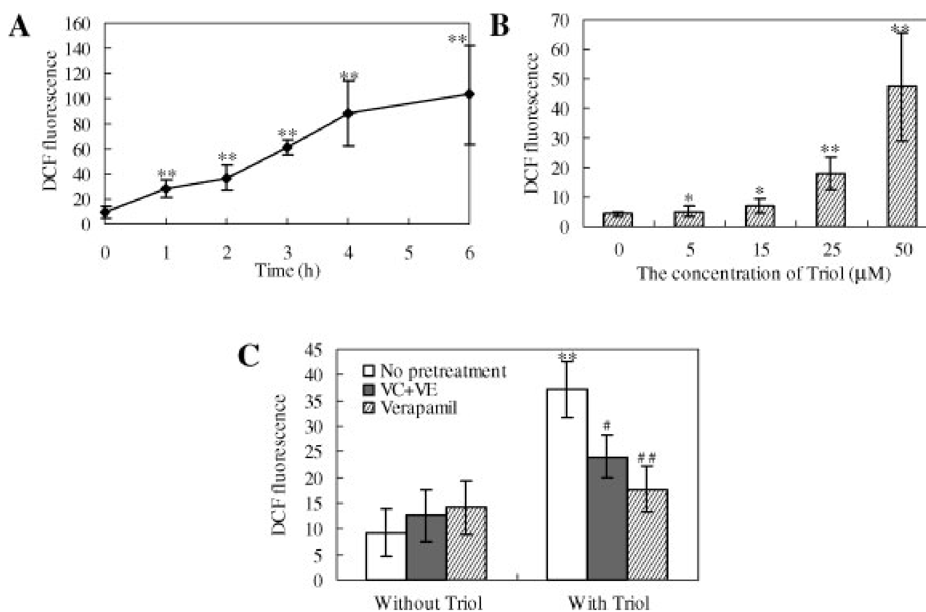

Fig. 6. Effect of Triol on ROS generation in MSCs on day 14 of culture (mean ± SD, n = 8). **A**: Temporal change of ROS generation in MSCs treated with 50 μM Triol. **B**: ROS generation in MSCs treated with different concentration of Triol for 6 h. **C**: Effect of VC + VE and verapamil on ROS generation induced by Triol. Cells were pretreated for 2 h with 25 μM VC + VE or 1 μM verapamil, followed by addition of 15 μM Triol for 6 h. *, $P < 0.05$; **, $P < 0.01$; compared with control. #, $P < 0.05$; # #, $P < 0.01$; compared with cells treated with Triol alone. Similar results were obtained in three independent experiments.

whether MAPK were associated with the inhibitory effect of Triol on osteoblastic differentiation of MSCs would have our attention in further research.

Triol Promoted MSCs Apoptosis by a Ca^{2+}- and ROS-Dependent Mechanism

Other than the decreased osteoblastic differentiation, MSCs death might be another reason for the decreased bone formation. Our data revealed that Triol promoted apoptosis of MSCs as characterized by condensed or fragmented nuclei as well as active externalization of phosphatidyl serine to the cell surface. Furthermore, the effect of Triol on apoptosis was attenuated by inhibiting Ca^{2+} influx with verapamil and ROS increase with VC + VE both. These results suggested that, in contrast to the mechanism by which Triol inhibited differentiation of MSCs, the influx of extracellular Ca^{2+} and the increased ROS generation might be involved in the Triol-promoted apoptosis. There was some evidence supporting that the Ca^{2+} influx and ROS generation were required for oxysterol-induced apoptosis in cultured cells including vascular endothelial cells, smooth muscle cells, and macrophages [Lizard et al., 1998; Ares et al., 2000; Rusinol et al., 2000; Lee and Chau, 2001; Spyridopoulos et al., 2001; Uemura et al., 2002]. In these cells, following exposure to oxysterols, large increases in $[Ca^{2+}]_i$ and intracellular ROS were observed, and both these increases and apoptotic response were blocked by the Ca^{2+} chelator, Ca^{2+} channel blockers, or antioxidants (e.g., VE). In addition, as mentioned above, the present data also demonstrated that ROS generation was dependent on the Ca^{2+} influx induced by Triol in MSCs. In summary, all these data suggested a requirement for the Ca^{2+} influx and Ca^{2+}-dependent ROS generation for Triol-enhanced apoptosis in MSCs.

We previously reported that Triol promoted vascular smooth muscle cells calcification, contributing to vascular mineralization in atherosclerosis [Liu et al., 2004]. The present results demonstrated that, under present condition, Triol inhibited osteoblastic differentiation and promoted apoptosis of MSCs. Furthermore, Triol was found to induce increases of intracellular Ca^{2+} and intracellular Ca^{2+}-dependent ROS generation in MSCs, and these effects were involved in the action of Triol on apoptosis, but not on osteoblastic differentiation of MSCs. All these effects of Triol on MSCs might contribute to the decreased bone formation in osteoporosis.

Taken together, our study may provide insights about common factors underlying the pathogenesis of atherosclerosis and osteoporosis. Further studies are required to directly test the hypothesis that oxidized lipids including oxysterols in vivo regulate bone formation.

REFERENCES

Anderson HC. 1995. Molecular biology of matrix vesicles. Clin Orthop 314:266–280.

Anderson HC, Sipe JB, Hessle L, Dhanyamraju R, Atti E, Camacho NP, Millan JL. 2004. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. Am J Pathol 164: 841–847.

Ares MP, Pörn-Ares MI, Moses S, Thyberg J, Juntti-Berggren L, Berggren PO, Hultgårdh-Nilsson A, Kallin B, Nilsson J. 2000. 7β-hydroxycholesterol induces Ca^{2+} oscillations, MAP kinase activation and apoptosis in human aortic smooth muscle cells. Atherosclerosis 153: 23–35.

Bai XC, Lu D, Bai J, Zheng H, Ke ZY, Li XM, Luo SQ. 2004. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB. Biochem Biophys Res Commun 314:197–207.

Bergman RJ, Gazit D, Kahn AJ, Gruber H, McDougall S, Hahn TJ. 1996. Age-related changes in osteogenic stem cells in mice. J Bone Miner Res 11:568–577.

Brown AJ, Jessup W. 1999. Oxysterols and atherosclerosis. Atherosclerosis 142:1–28.

Carey MB, Matsumoto SG. 1999. Spontaneous calcium transients are required for neuronal differentiation of murine neural crest. Dev Biol 215:298–313.

Colles SM, Maxson JM, Carlson SG, Chisolm GM. 2001. Oxidized LDL-induced injury and apoptosis in atherosclerosis. Potential roles for oxysterols. Trends Cardiovasc Med 11:131–138.

Denizot F, Lang R. 1986. Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89:271–277.

Egrise D, Martin D, Vienne A, Neve P, Schoutens A. 1992. The number of fibroblastic colonies formed from bone marrow is decreased and the in vitro proliferation rate of trabecular bone cells increased in aged rats. Bone 13: 355–361.

Fedde KN, Blair L, Silverstein J, Coburn SP, Ryan LM, Weinstein RS, Waymire K, Narisawa S, Millan JL, MacGregor GR, Whyte MP. 1999. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. J Bone Miner Res 14:2015–2026.

Gu XN, Spitzer NC. 1997. Breaking the code: Regulation of neuronal differentiation by spontaneous calcium transients. Dev Neurosci 19:33–41.

Imai H, Werthessen NT, Subramanyan V, LeQuesne PW, Soloway AH, Kanisawa M. 1980. Angiotoxicity of oxygenated sterols and possible precursors. Science 207: 651–653.

Kha HT, Basseri B, Shouhed D, Richardson J, Tetradis S, Hahn TJ, Parhami F. 2004. Oxysterols regulate differentiation of mesenchymal stem cells: Pro-bone and anti-fat. J Bone Miner Res 19:830–840.

Lee T, Chau L. 2001. Fas/Fas ligand-mediated death pathway is involved in oxLDL-induced apoptosis in vascular smooth muscle cells. Am J Physiol Cell Physiol 280:C709–C718.

Liu H, Yuan L, Xu S, Zhang T, Wang K. 2004. Cholestane-3β, 5α, 6β-triol promotes vascular smooth muscle cell calcification. Life Sci 76:533–543.

Lizard G, Gueldry S, Sordet O, Monier S, Athias A, Miguet C, Bessede G, Lemaire S, Solary E, Gambert P. 1998. Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. FASEB J 12:1651–1663.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Malaval L, Modrowski D, Gupta AK, Aubin JE. 1994. Cellular expression of bone-related proteins during in vitro osteogenesis in rat bone marrow stromal cell cultures. J Cell Physiol 158:555–572.

Maniatopoulos C, Sodek J, Melcher AH. 1988. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res 254:317–330.

McFarlane SI, Muniyappa R, Shin JJ, Bahtiyar G, Sowers JR. 2004. Osteoporosis and cardiovascular disease: Brittle bones and boned arteries, is there a link? Endocrine 23:1–10.

Mullender MG, van der Meer DD, Huiskes R, Lips P. 1996. Osteocyte density changes in aging and osteoporosis. Bone 18:109–113.

Panini SR, Sinensky MS. 2001. Mechanisms of oxysterol-induced apoptosis. Curr Opin Lipidol 12:529–533.

Parhami F, Morrow AD, Balucan J, Leitinger N, Watson AD, Tintut Y, Berliner JA, Demer LL. 1997. Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. Arterioscler Thromb Vasc Biol 17: 680–687.

Parhami F, Jackson SM, Le V, Balucan JP, Tintut Y, Territo M, Demer LL. 1999. Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. J Bone Min Res 14:2067–2078.

Parhami F, Garfinekel A, Demer LL. 2000. Role of lipids in osteoporosis. Arterioscler Thromb Vasc Biol 20:2346–2348.

Peng SK, Taylor CB, Hill JC, Morin RJ. 1985. Cholesterol oxidation derivatives and arterial endothelial damage. Atherosclerosis 54:121–133.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cell. Science 284:143–147.

Rusinol AE, Yang L, Thewke D, Panini SR, Kramer MF, Sinensky MS. 2000. Isolation of a somatic cell mutant resistant to the induction of apoptosis by oxidized low density lipoprotein. J Biol Chem 275:7296–7303.

Salonen JT, Nyyssonen K, Salonen R, Porkkala-Sataho E, Tuomainen T-P, Diczfalusy U, Björkhem I. 1997. Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation 95:840–845.

Spyridopoulos I, Wischhusen J, Rabenstein B, Mayer P, Axel DI, Fröhlich KU, Karsch KR. 2001. Alcohol enhances oxysterol-induced apoptosis in human endothelial cells by a calcium-dependent mechanism. Arterioscler Thromb Vasc Biol 21:439–449.

Stein G, Lian J. 1993. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14:424–442.

Stiko A, Regnström J, Shah PK, Cercek B, Nilsson J. 1996. Active oxygen species and lysophosphatidylcholine are involved in oxidized low density lipoprotein activation of smooth muscle cell DNA synthesis. Arterioscler Thromb Vasc Biol 16:194–200.

Uemura M, Manabe H, Yoshida N, Fujita N, Ochiai J, Matsumoto N, Takagi T, Naito Y, Yoshikawa T. 2002. Alpha-tocopherol prevents apoptosis of vascular endothelial cells via a mechanism exceeding that of mere antioxidation. Eur J Pharmacol 456:29–37.

Wada T, McKee MD, Steitz S, Giachelli CM. 1999. Calcification of vascular smooth muscle cell cultures inhibited by osteopontin. Circ Res 84:166–178.

Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millan JL. 2000. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res 15:1879–1888.

Whyte MP. 1994. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr Rev 15:439–461.

Zieden B, Kaminskas A, Kristenson M, Kucinskiene Z, Vessby B, Olsson AG, Diczfalusy U. 1999. Increased plasma 7 beta-hydroxycholesterol concentrations in a population with a high risk for cardiovascular disease. Arterioscler Thromb Vasc Biol 19:967–971.