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HDL_3 Reduces the Association and Modulates the Metabolism of Oxidized LDL by Osteoblastic Cells: A Protection Against Cell Death

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ABSTRACT

Oxidized low density lipoproteins (OxLDL) are known to promote atherosclerosis, but it is only recently that OxLDL have been associated with alterations of the functions of bone-forming osteoblasts and osteoporosis. Although high density lipoproteins (HDL) are recognized for their anti-atherogenic action, there is less information about their ability to protect against osteoporosis. Therefore, we investigated the capacity of HDL_3 to prevent the cell death induced by OxLDL in human osteoblastic cells. Simultaneous exposure of the cells to HDL_3 and OxLDL abolished the reduction of cell viability monitored by MTT activity measurement and the induction of apoptosis determined by annexin V staining indicating that HDL_3 prevent the apoptosis of osteoblasts induced by OxLDL. This protection correlated with the displacement by HDL_3 of OxLDL association to osteoblasts, signifying that OxLDL binding and/or internalization are/is necessary for their cytotoxic effects. We also found that exposition of osteoblastic cells to HDL_3 prior to incubation with OxLDL reduced cell death and preserved the lysosomal integrity. This protection was correlated with an increase of SR-BI expression, a modification of OxLDL metabolism with less global uptake of OxLDL and greater selective uptake of cholesterol from OxLDL. These results strongly suggest that, as for atherosclerosis, HDL may exert beneficial actions on bone metabolism. J. Cell. Biochem. 105: 1374–1385, 2008.

KEY WORDS: OSTEOBLAST; HIGH DENSITY LIPOPROTEIN; LOW DENSITY LIPOPROTEIN; OxLDL; CHOLESTEROL; EFFLUX; CELL DEATH

The bone is a living tissue that is continuously being remodeled. This process implies, first, the breaking down of the bone by the action of the osteoclasts [Rodan and Martin, 2000]. In a second step, differentiated osteoblastic cells are recruited to the resorption lacunae where they secrete a variety of proteins such as type I collagen and noncollagenous proteins to generate a new bone matrix [Rodan and Martin, 2000]. Aside from their role in the reconstruction of bone matrix, osteoblasts are also involved in the regulation of osteoclastic differentiation and bone resorption, given their capacity to secrete a variety of factors necessary for osteoclastogenesis regulation such as macrophage-colony stimulating factor (M-CSF) [Kodama et al., 1991] and osteoprotegerin (OPG) [Akatsu et al., 1998; Udagawa et al., 2000]. Therefore, adequate osteoblastic proliferation/apoptosis, differentiation and secretory functions are essential not only for bone formation but also for the regulation of bone resorption process.

The loss of equilibrium between osteoblastic and osteoclastic functions, in favor of increased osteoclast-mediated bone resorption over bone formation by osteoblasts, results in osteoporosis that is characterized by reduced bone mass, lower mineral density, and thereby higher bone fragility and susceptibility to fractures. Although the exact etiology of osteoporosis remains to be clarified, in numerous cases growing evidence suggests that atherogenic conditions are positively associated with osteoporosis development. Individuals with lower bone density and osteoporosis also present higher lipid levels [Laroche et al., 1994; Uyama et al., 1997; Barengolts et al., 1998], a condition considered as an important atherogenic risk factor [Castelli et al., 1997]. Other pieces of evidence suggesting a possible role of blood cholesterol in bone metabolism regulation include the observation of reduced bone formation in atherosclerosis-susceptible C57BL/6 mice maintained under an atherogenic high-fat diet [Parhami et al., 2001].

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Although, these studies suggest the existence of one or several contributory factors for the parallel development of atherosclerosis and osteoporosis, it is only recently that OxLDL were identified as one of these possible factors. OxLDL have been shown to affect the differentiation of cells towards an osteoblastic phenotype. Indeed, calcifying vascular cells (CVC), isolated and cloned from a subpopulation of aortic medial smooth muscle cells, undergo osteoblastic differentiation after an exposition to OxLDL or acetylated LDL [Parhami et al., 1997]. In contrast, OxLDL and oxidative stress inhibit osteogenic differentiation of mesenchymal stem cells and preosteoblasts [Parhami et al., 1997; Mody et al., 2001]. Moreover, Tintut et al. [2004] have reported increased levels of lipid hydroperoxides in bone marrow of hyperlipidemic mice. The potential implication of OxLDL in the development of osteoporosis is further supported by the demonstration that OxLDL caused cell death in osteoblasts [Klein et al., 2003; Hirasawa et al., 2007]. Furthermore we have reported that OxLDL are poorly degraded by osteoblasts, that they accumulate in the lysosomal compartment, induce the loss of the lysosomal integrity and elicit cell death. This was supported by the demonstration that chloroquine, an inhibitor of lysosomal activity, accentuated cell death induced by OxLDL [Brodeur et al., 2008a].

It is well established that cardiovascular diseases are directly related to plasma concentrations of LDL-cholesterol [Castelli et al., 1997] and inversely associated with high density lipoproteins (HDL)-cholesterol [Gordon and Rifkind, 1998]. However, the potential of HDL to protect against osteoporosis is somewhat controversial. Indeed, a positive correlation has been found between HDL and bone mineral density [Yamaguchi et al., 2002; Solomon et al., 2005; Dennison et al., 2007] and a negative correlation was reported with bone remodeling markers [Majima et al., 2008], as well as with the risk of fractures [Martínez-Ramírez et al., 2007], while two studies found no relationship [Poli et al., 2003; Cui et al., 2005] and one group reported an inverse relationship [D'Amelio et al., 2001]. Although the mechanisms of protection afforded by HDL on cardiovascular diseases are not clearly identified, it was attributed to various characteristics of these lipoprotein particles. Classically, the atheroprotective mechanism of HDL is thought to be related to its role in reverse transport of cholesterol from peripheral tissues to liver [Kwiterovich, 1998]. HDL_3 have been shown to inhibit apoptosis induced by OxLDL in the monocytic RAW264.7 cells by promoting cholesterol efflux [Jiang et al., 2006]. Another important characteristic of HDL is their capacity to prevent the oxidative modification of LDL since it was demonstrated that lipophilic antioxidants carried by HDL can interrupt LDL oxidation cascade [Navab et al., 2004], thereby preventing the formation of proatherogenic OxLDL. In addition, HDL can deliver antioxidants such as vitamin E to cells [Mardones and Rigotti, 2004], and vitamin E has been shown to protect from OxLDL-induced cell death in endothelial cells [Vieira et al., 1998] and coronary smooth muscle cells [de Nigris et al., 2000]. Furthermore, several studies have demonstrated that HDL inhibit cell signaling mediated by OxLDL and thus offset several adverse biological effects [Suc et al., 1997; Robbesyn et al., 2003; Lee et al., 2005; Schmidt et al., 2006]. At the level of endothelium, it was demonstrated that the protective effect of HDL depends on stimulation of nitric oxide (NO) formation which regulates vasoreactivity [Rämet et al., 2003]. This induction of NO production comes from the activation of phosphoinositol-3 kinase which induces the AKT pathways [Ozaki et al., 2003]. The activation of this survival pathway by HDL leads also to the inhibition of the apoptosis of endothelial cells by inhibiting the mitochondrial apoptosis pathway [Nofer et al., 2001; von Eckardstein et al., 2005]. Moreover, Klein et al. [2006] have also demonstrated that native LDL induce SaOS cell death by the inhibition of AKT, suggesting that by their ability to activate this pathway, HDL could counteract this effect.

Our aim was to investigate if and how HDL_3 have the ability to counteract the osteoblastic death induced by OxLDL. Despite the fact that the protective effects of HDL have been related to numerous properties, the impact of HDL on the cellular metabolism of OxLDL has not been investigated. This issue is particularly important given the demonstration that OxLDL accumulation in cells leads to detrimental effects [Li et al., 1998]. In accordance, we have reported that OxLDL accumulation in osteoblast lysosomes leads to apoptosis, which further indicates that OxLDL internalization is a process particularly important in osteoblasts exposed to OxLDL [Brodeur et al., 2008a]. Recently, we have demonstrated that osteoblastic cells express the scavenger receptor class B type I (SR-BI) and the cluster of differenciation-36 (CD36) [Brodeur et al., 2008b]. Both receptors bind OxLDL and HDL, suggesting therefore that HDL could inhibit the apoptosis induced by OxLDL by inhibiting their internalization and thereby, their metabolism. Here, using human osteoblastic cells, we report that OxLDL binding and/or internalization are/is necessary for OxLDL cytotoxic effects. We also show that exposition of osteoblastic cells to HDL_3 prior to incubation with OxLDL reduces cell death and preserves lysosomal integrity. Moreover we demonstrate that this protection is correlated with an increase of SR-BI expression and a modification of OxLDL metabolism as illustrated by less OxLDL global uptake but greater cholesteryl ester selective uptake from OxLDL.

EXPERIMENTAL PROCEDURES

MATERIALS

The human osteoblast-like cell line MG-63 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Normal human osteoblasts (hOB) were purchased from PromoCell (Heidelberg, Germany). Dulbecco's Modified Eagle's Medium-F12 without phenol was bought from Sigma (St. Louis, MO). Glutamine, Trypsin-EDTA and penicillin–streptomycin solutions were purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada). Fetal bovine serum (FBS) was purchased from Cansera (Etobicoke, Ontario, Canada). ^{125}Iodine (as sodium iodide, 100 mCi/mmol) was bought from ICN Canada (Montreal, Quebec, Canada), while [1,2-^{3}H(N)]-cholesterol (40–60 Ci/mmol) came from Perkin Elmer Life Sciences (Woodbridge, Ontario, Canada). 1,2-[^{3}H]-cholesteryl oleate (50 mCi/mmol) was bought from Amersham Pharmacia Biotech (Laval, Quebec, Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT), fatty acid-free (FFA) and regular bovine serum albumin (BSA) (fraction V), acridine orange, and β-cyclodextrin were purchased from Sigma (Oakville, Ontario, Canada).

OSTEOBLAST CELL CULTURE

Human osteoblasts (MG-63 and hOB) were grown in Dulbecco's Modified Eagle's Medium-F12 without phenol (DMEM-F12). The media was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cells were cultured in 5% CO_2 at 37°C and were harvested once a week with Trypsin-EDTA. For experiments, cells were trypsinized, seeded at 2,000 cells/cm^2 and cultured for 5 days prior to the assays.

ISOLATION AND RADIOLABELING OF LIPOPROTEINS

Lipoproteins were isolated from human plasma obtained from Bioreclamation (Hicksville, NY). Before the isolation, the plasma was adjusted to 0.01% ethylenediamine tetraacetate (EDTA), 0.02% sodium azide and 10 μM phenylmethylsulfonylfluoride (PMSF). Human LDL (d = 1.025–1.063 g/ml), and HDL_3 (density 1.125–1.21 g/ml) were prepared by ultracentrifugation as described by Brissette et al. [1996]. Lipoproteins were iodinated by a modification [Langer et al., 1972] of the iodine monochloride method of McFarlane [1948]. Briefly, 1 mCi of sodium ^{125}iodide was used to iodinate 2.5 mg of lipoprotein in the presence of 30 nmol of iodine monochloride in 0.5 M glycine–NaOH, pH 10. Free iodine was removed by gel filtration on Sephadex G-25 followed by dialysis in Tris-buffered saline (TBS). The specific radioactivity ranged from 100,000 to 250,000 cpm/μg protein.

LIPOPROTEIN MODIFICATION

LDL preparations were dialyzed against TBS to remove EDTA before oxidation. Highly OxLDL (H-OxLDL) were prepared as described by Lougheed and Steinbrecher [1996]. LDL (200 μg of protein/ml in TBS) was incubated with 5 μM $CuSO_4$ for 20 h at 37°C. Oxidation was stopped by the addition of EDTA (final concentration of 100 μM) and butylated hydroxytoluene (40 μM final) and the OxLDL were concentrated to 15–20 mg/ml using Centriplus-100 ultrafiltration devices (Amicon, Oakville, Ont.). H-OxLDL typically resulted in a 2.8-fold increase in the electrophoretic mobility relative to native LDL on 0.5% agarose/barbital gels. Mildly OxLDL (M-OxLDL) were obtained after 4 h of incubation under similar conditions and showed a 1.5-fold increase in the electrophoretic mobility relative to native LDL.

COMPETITION ASSAYS

Unlabeled HDL_3, LDL and H- or M-OxLDL were used as competitors for ^{125}I-OxLDL association with cells. Cells were washed twice with 1 ml of phosphate-buffered saline (PBS) and incubated for 2 h at 37°C with 20 μg of protein/ml of ^{125}I-OxLDL in a total volume of 250 μl containing 125 μl of culture medium (2×), 4% BSA, pH 7.4 (total binding). ^{125}I-OxLDL were incubated with cells in the presence of increasing concentrations of unlabeled competitors (0–800 μg/ml). At the end of the incubation, the cells were washed once with 1 ml of PBS containing 0.2% BSA (PBS-BSA) followed by one wash with 1 ml of PBS. The cells were then solubilized in 1.5 ml of 0.2 N NaOH. Radioactivity counts in the homogenates were obtained with a Cobra II counter (Canberra-Packard). The 100% specific association value was calculated by subtracting the non-specific association data (obtained by the addition of 2 mg of protein/ml of the proper unlabeled OxLDL) from the total association.

MTT ACTIVITY ASSAYS

MTT activity was determined in 96-well plates (Sarstedt) by microtiter tetrazolium assay after incubation of cells with lipoproteins during 24, 48 h in DMEM-F12 supplemented with 2% of human serum deficient in lipoproteins (LPDS). Briefly, MTT reagent was added to the media at a final concentration of 0.5 mg/ml. Four hours later, formazan crystals generated by cellular reduction of the MTT reagent were dissolved in dimethylsulfoxide (DMSO) for 30 min at 37°C and the absorbance was determined at 575 nm. Results are expressed as the percentage of MTT values of treated versus control conditions.

CHOLESTEROL EFFLUX ASSAY

Cells at 80% confluence were labeled with 1 μCi [1,2-^{3}H(N)]-cholesterol for 24 h at 37°C and then equilibrated with 1 ml of DMEM-F12–0.2% BSA for 24 h at 37°C. Efflux assay was performed in the absence or presence of lipoproteins or β-cyclodextrin for 4 h at 37°C. At the end of the incubation, the medium was harvested and the cells were solubilized in 1.5 ml of 0.1 N NaOH, assayed for protein content and counted for radioactivity in beta-counter (Wallach-Fisher). Percentage of efflux was calculated by subtracting the radioactive counts in the absence of HDL_3 from the radioactive counts in the presence of HDL_3 and then divided by the sum of the radioactive counts in the media plus the cell fraction.

CELLULAR LIPOPROTEIN ASSOCIATION ASSAYS

Cellular association assays of ^{125}I-lipoprotein or ^{3}H-CE-lipoprotein (20 μg of protein/ml) were conducted essentially as for competition studies with the use of a beta-counter (Wallach-Fisher) if cells were incubated with [^{3}H]CE-lipoproteins. The results are expressed in μg of lipoprotein protein/mg of cell protein. To compare the association of lipoproteins labeled in protein (^{125}I) or in CE (^{3}H), the association data of ^{3}H-CE-lipoprotein were also estimated as μg of lipoprotein protein/μg of lipoprotein protein (apparent uptake). To achieve this, the specific activity of ^{3}H-CE-lipoprotein was expressed in counts per μg of lipoprotein protein. The specific association was calculated by subtracting the non-specific association from the total association. Selective uptake of CE is observed when specific [^{3}H]CE-lipoproteins association minus specific ^{125}I-lipoprotein association is greater than zero.

EVALUATION OF LYSOSOMAL INTEGRITY BY ACRIDINE ORANGE STAINING

MG-63 cells incubated in presence or absence of HDL_3 (200 μg/ml) for 24 h were exposed or not to OxLDL (200 μg/ml) and were vitally stained with an acridine orange (AO) solution at 5 μg/ml of complete medium for 30 min at 37°C. The intensities of red and green AO fluorescence were then examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) with a Plan-Apochromatic 60× oil N.A. 1.4 objective lens.

IMMUNOBLOTTING

Total proteins of MG-63 cells were extracted by ice cold lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% IGEPAL and 60 mM Octylglucoside) containing 1 mM Na_2VO_3, 0.1 mM PMSF, 10 μg/ml Leupeptin, 0.5 mM Benzamidin and 1 μg/

ml Aprotinin. Proteins were separated on 8% reducing SDS–PAGE and transferred to PVDF membranes. Immunodetections were performed with anti-SR-BI and CD36 polyclonal antibody (Novus Biologicals, Littleton, CO) or anti-Actin monoclonal antibody (Sigma) followed by enhanced chemiluminescence detection on Kodak Biomax ML film.

OTHER METHODS

Protein content was determined by the method of Lowry et al. [1951] with BSA as standard. Student's *t*-test or ANOVA test was used to obtain statistical comparison of the data. Differences were considered significant at $P < 0.05$.

RESULTS

HDL OFFSET CELL DEATH INDUCED BY OXLDL

We previously demonstrated that high concentrations of OxLDL induced osteoblastic cell death [Brodeur et al., 2008a]. To evaluate the potential of HDL_3 to inhibit apoptosis, we exposed MG-63 cells to 200 μg/ml of OxLDL in the absence or presence of increasing concentrations of HDL_3 (20–200 μg/ml). Thereafter, osteoblastic cell viability was evaluated by MTT assays. The results presented in Figure 1 show that after 24 h of incubation in the absence of HDL_3, M-OxLDL and H-OxLDL reduced MTT activity by 40% and 50%, respectively. This reduction was accentuated to 90% after 48 h (Fig. 1A,B), indicating that OxLDL decreased MG-63 cell viability in a time-dependent manner. This reduction of MTT activity induced by M-OxLDL after 24 h was completely abolished in the presence of 20 μg/ml of HDL_3 (Fig. 1A). Higher concentrations of HDL_3 also offset the reduced MTT activity induced by M-OxLDL in favor of an increased activity (ANOVA, $P < 0.0001$; Dunnett's $P < 0.001$ from 20 μg/ml) (Fig. 1A). This higher activity suggests that in presence of HDL_3, M-OxLDL recover their ability to induce cell proliferation as expected from the demonstration that OxLDL have a mitogenic action on osteoblasts at low concentrations [Brodeur et al., 2008a]. Similarly, HDL_3 also inhibited the decrease of MTT activity induced by H-OxLDL, but a higher concentration of HDL_3 (50 μg/ml) was needed to completely rescue the effect of H-OxLDL on MTT activity (ANOVA, $P < 0.0001$; Dunnett's $P < 0.001$ from 100 μg/ml) (Fig. 1B). The protecting effect of HDL_3 on the reduction of MTT

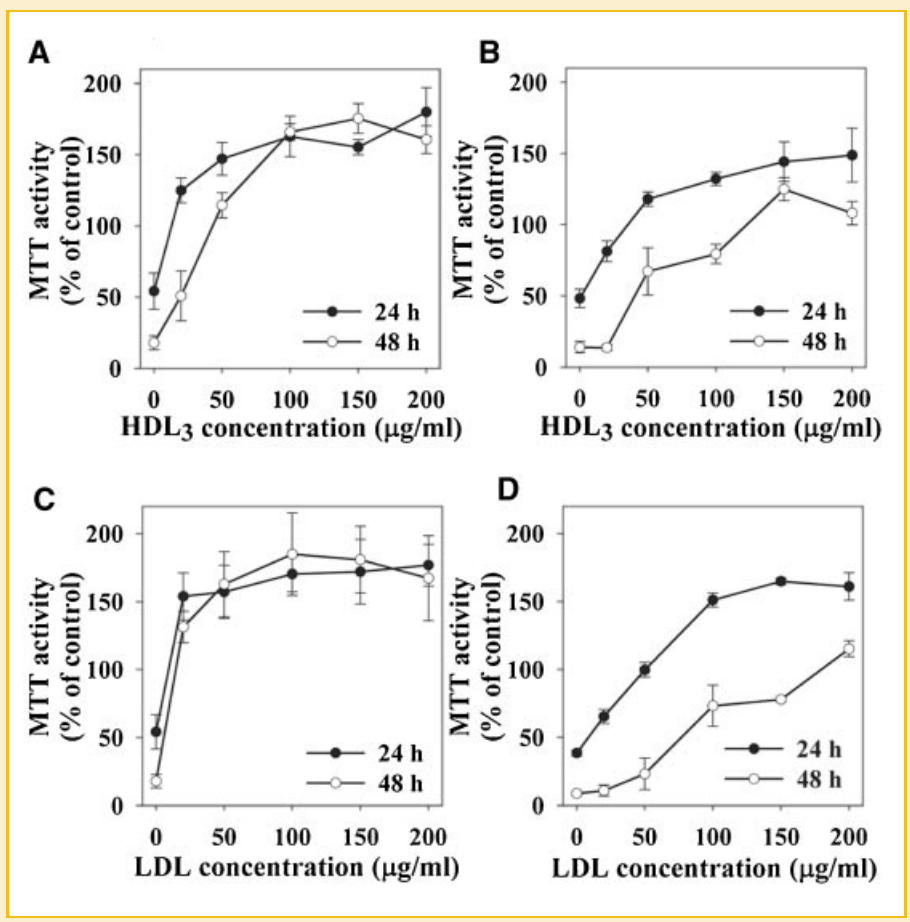

Fig. 1. Effect of HDL_3 and LDL on OxLDL-induced MG-63 cell death. MG-63 cells were incubated with 200 μg/ml of M-OxLDL (A,C) or H-OxLDL (B,D) in the absence or presence of increasing concentrations (20–200 μg of protein/ml) of native HDL_3 (A,B) or LDL (C,D) during 24 and 48 h in DMEM-F12 supplemented with 2% LPDS. Cell viability was determined by MTT assays and is expressed as the mean ± SEM of the relative MTT activity compared to control condition of five experiments conducted each in triplicate. Statistical differences were determined by one-way ANOVA.

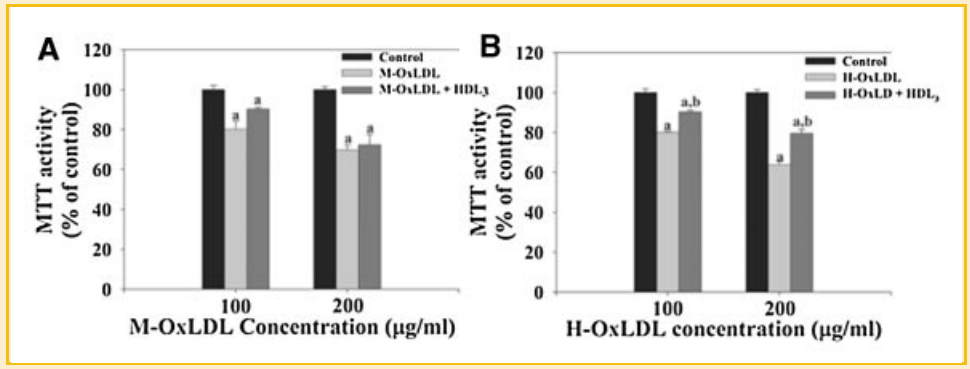

Fig. 2. Effect of HDL_3 on OxLDL-induced hOB cell death. hOB cells were incubated with 100 or 200 µg/ml of M-OxLDL (A) or H-OxLDL (B) in the absence (control) or presence of HDL_3 (200 µg of protein/ml) during 24 in DMEM-F12 supplemented with 2% LPDS. Cell viability was determined by MTT assays and is expressed as the mean ± SEM of the relative MTT activity compared to control condition of four experiments conducted each in triplicate. [a]Significantly different ($P < 0.05$) from the results obtained from control. [b]Significantly different ($P < 0.005$) from the results obtained with OxLDL alone.

activity caused by OxLDL was also apparent after 48 h, however higher concentrations of HDL_3 were required (ANOVA, $P < 0.0001$; Dunnett's $P < 0.001$ from 50 µg/ml) (Fig. 1A,B). Together, these results indicate that HDL_3 protect MG-63 cells from death induced by OxLDL. Furthermore, the addition of LDL protected the cells from M-OxLDL-induced death (Fig. 1C). This protective effect was already evident at the low concentration of 20 µg/ml of LDL and was maintained during 48 h (ANOVA, $P < 0.0001$; Dunnett's $P < 0.001$ from 20 µg/ml). Similarly, LDL also prevented the reduction of MTT activity induced by 200 µg/ml of H-OxLDL, but as for HDL_3, higher concentrations of LDL were needed to reduce the effect of H-OxLDL on MTT activity (ANOVA, $P < 0.0001$; Dunnett's $P < 0.001$ from 20 µg/ml for 24 h and from 100 µg/ml for 48 h) (Fig. 1D).

To establish that the toxic effect of OxLDL and protection by HDL_3 were not restricted to the MG-63 cell line, we used primary normal human osteoblasts (hOB). We reported for the first time that OxLDL decreased the viability of primary human osteoblasts (Fig. 2). Moreover, HDL_3 retained their ability to counteract the cytotoxicity of H-OxLDL while this protecting effect was not significant for the M-OxLDL (Fig. 2).

To further characterize if the inhibition of HDL_3 on the reduction of MTT activity induced by OxLDL results from the inhibition of apoptosis induced by these modified lipoproteins, MG-63 cells were labeled with annexin-V/FITC and propidium iodide (PI). Figure 3 shows that H-OxLDL induced apoptosis of MG-63 cells since after 48 h approximately 50% of cells treated with H-OxLDL are annexin-V-positive and gradually move toward the upper right quadrant, typical of a late apoptotic or necrotic stage (Fig. 3). In accordance with MTT results, externalization of phosphatidylserine (PS) residues induced by H-OxLDL was reduced by the presence of HDL_3 at 200 µg/ml (Fig. 3), indicating that HDL_3 inhibited the apoptosis induced by OxLDL. As for HDL_3, the results demonstrated that LDL inhibited the apoptosis induced by OxLDL (Fig. 3). Similar results were also obtained with M-OxLDL (data not shown).

We next incubated cells with increasing concentrations of HDL_3 (0–200 µg/ml) alone to evaluate if their protecting action was due to a direct mitogenic action. The results indicated that none of the concentrations of HDL_3 used affects MTT activity or DNA synthesis as demonstrated by ^{3}H-thymidine incorporation assays in MG-63 cells upon 72 h of treatment (data not shown). Therefore, HDL_3 have no direct effect on MG-63 cell viability and proliferation and they offset the apoptotic effects of OxLDL by one or many indirect mechanism(s).

In the above-reported experiments (coincubation of cells with HDL_3 and OxLDL), the protective effect of HDL_3 might have resulted

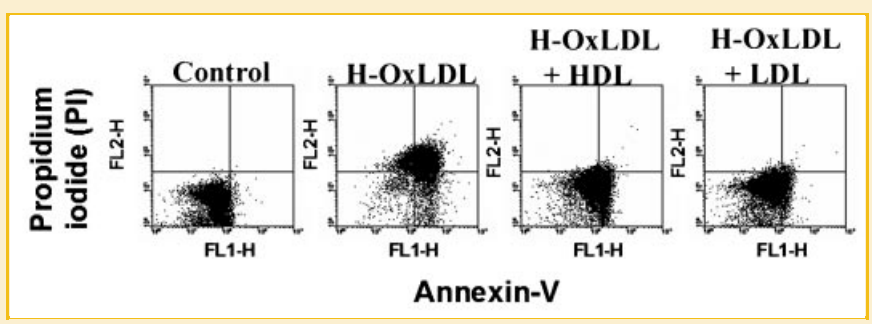

Fig. 3. Effect of HDL_3 on OxLDL-induced osteoblastic apoptosis. MG-63 cells seeded in 6-well plates were incubated during 48 h in DMEM-F12 supplemented with 2% LPDS without (control) or with 200 µg/ml of H-OxLDL in the presence or absence of 200 µg/ml of HDL_3. The cells were then labeled with Annexin-V-FITC and propidium iodide. Representative results of three different experiment examined with a FACScan (Becton Dickinson) are shown.

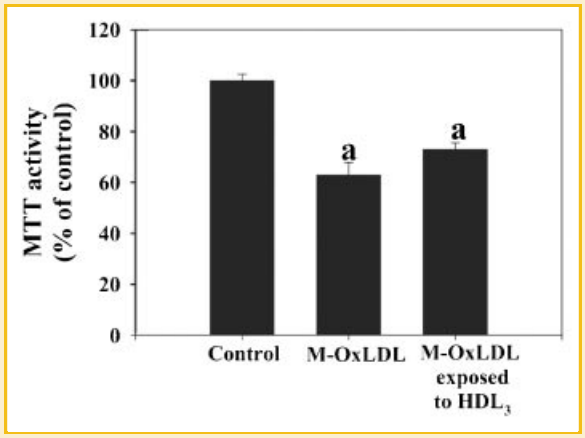

Fig. 4. Impact of pre-incubation of OxLDL with HDL_3. M-OxLDL (200 μg/ml) was pre-incubated for 24 h at 37°C without or with HDL_3 (200 μg/ml) in DMEM-F12 supplemented with 2% LPDS, separated from HDL_3 by ultracentrifugation, and dialyzed. This re-isolated OxLDL fraction was added (200 μg/ml) to the culture medium and the toxicity evaluated after 24 h incubation. Cell viability was determined by MTT assays and is expressed as the mean ± SEM of the relative MTT activity compared to control condition of four experiments conducted each in triplicate. [a]Significantly different ($P < 0.05$) from the results obtained from control (no OxLDL).

from the interaction of HDL_3 with OxLDL, leading to reduced amounts of cytotoxic compounds in OxLDL [Mackness and Durrington, 1995]. To test this possibility, M-OxLDL (200 μg/ml) were preincubated with HDL_3 (200 μg/ml) for 24 h and separated again. The cytotoxic effect of the M-OxLDL preincubated with HDL_3 was not significantly different from the cytotoxic effect of M-OxLDL preincubated under the same conditions but without HDL_3 (Fig. 4). Therefore, the protective effect of HDL_3 cannot be attributed to the interaction of OxLDL with HDL_3 (nor to the inactivation or extraction of toxic compounds by HDL_3).

COMPETITION ASSOCIATION ASSAYS

To determine if the protective effect of HDL_3 and LDL resulted from a competition for the association of OxLDL to MG-63 cells, we conducted association assays with ^{125}I-M- or ^{125}I-H-OxLDL in the presence of competitors. Association of both radioactive ligands was efficiently displaced (>90%) by their corresponding unlabeled counterpart (Fig. 5). The results also demonstrated that H-OxLDL competed for approximately 70% of M-OxLDL association (Fig. 5A) and similarly M-OxLDL efficiently reduced ^{125}I-H-OxLDL association (approximately 80%) (Fig. 5B). HDL_3, a well-known ligand of scavenger receptors of class B (SR-BI & CD36), efficiently decreased M- and H-OxLDL association by approximately 60% and 40%, respectively. LDL particles seem also to share a common binding site with OxLDL in MG-63 cells, as they decreased OxLDL association by approximately 40% (Fig. 5). Taken together, these results indicate that HDL_3 and LDL have both the ability to reduce the association of OxLDL with MG-63 cells.

EVALUATION OF CHOLESTEROL EFFLUX AS A PROTECTIVE MECHANISM AGAINST OXLDL-INDUCED CELL DEATH

We next conduct cholesterol efflux experiments to demonstrate that this process is operative in MG-63 cells. The results presented in Figure 6A show that HDL_3 induced cholesterol efflux from the cells in a dose-dependent manner. In order to define if the protection against cell death brought by HDL_3 is related to cholesterol efflux, we determined the capacity of β-cyclodextrin, a known acceptor of cholesterol [Kilsdonk et al., 1995], to promote cholesterol efflux from osteoblastic cells and to prevent cell death induced by OxLDL. We observed that β-cyclodextrin dose-dependently induced cholesterol efflux from MG-63 cells (Fig. 6A). The results presented in Figure 6B demonstrates that β-cyclodextrin has no impact on MTT activity of MG-63 cells incubated in presence of native LDL (100 μg/ml) after 24 h of incubation. In contrast, under conditions where OxLDL favor cell proliferation, treatments with M-OxLDL (100 μg/ml) in the presence of β-cyclodextrin reduce MTT activity of osteoblasts

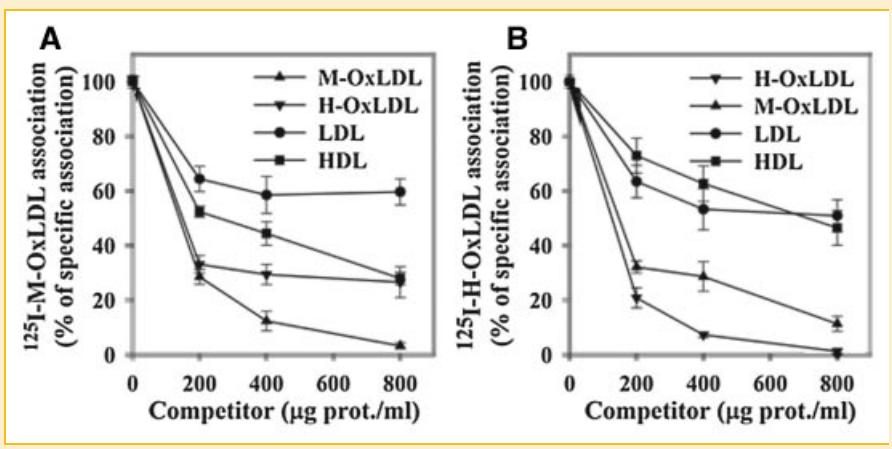

Fig. 5. Competition curves of ^{125}I-mildly-OxLDL (A) and ^{125}I-highly-OxLDL (B) association with MG-63 cells by unlabeled OxLDL, LDL and HDL_3. ^{125}I-lipoproteins at 20 μg/ml were incubated with MG-63 cells during 2 h at 37°C in 12-well plates in the presence of the indicated concentrations of either unlabeled OxLDL, LDL or HDL_3. The value without competitor in each experiment was set as 100%. Each point represents the mean ± SEM derived from three to four experiments conducted in duplicate.

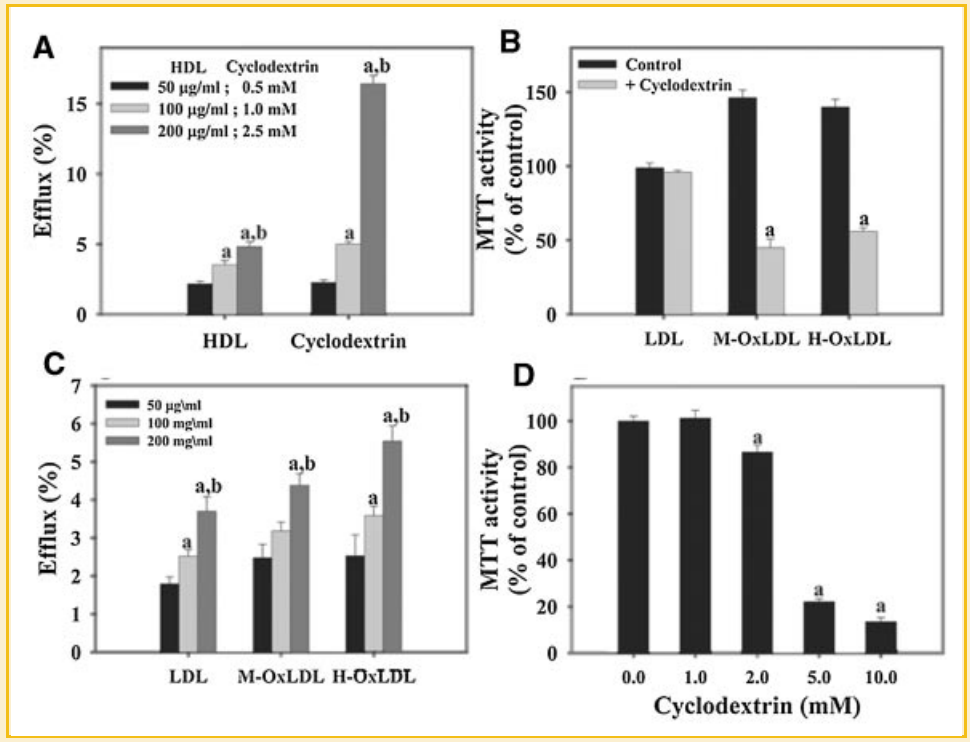

Fig. 6. Importance of cholesterol efflux in the protecting effect of HDL_3. Cholesterol efflux (A): Efflux assays were performed in the absence or presence of 50, 100, and 200 µg/ml HDL_3 or 0.5, 1.0, and 2.5 mM of β-cyclodextrin during 4 h at 37°C as described in Experimental Procedures Section. Each result represents the mean percentage ± SEM of three to four experiments each conducted in triplicate. [a]Significantly different ($P < 0.05$) from the results obtained with 50 µg/ml or 0.5mM. [b]Significantly different ($P < 0.005$) from the results obtained with 100 µg/ml or 1.0 mM. MTT activity in the presence of β-cyclodextrin (B): MG-63 cells were incubated with native LDL and OxLDL (200 µg/ml) during 24 h in DMEM-F12 containing 2% LPDS and supplemented or not with 1 mM of β-cyclodextrin. MTT activity is expressed as the mean ± SEM of five experiments conducted each in triplicate. [a]Significantly different ($P < 0.05$) from the results obtained with lipoproteins alone. Cholesterol efflux (C): Efflux assays were performed in the absence or presence of native LDL and OxLDL as described in Experimental Procedures Section. Each result represents the mean percentage ± SEM of three to four experiments each conducted in triplicate. [a]Significantly different ($P < 0.05$) from the results obtained with 50 µg/ml. [b]Significantly different ($P < 0.05$) from the results obtained with 100 µg/ml. Impact of the β-cyclodextrin on cell viability (D): MG-63 cells were incubated with β-cyclodextrin during 24 h in DMEM-F12 containing 2% LPDS. MTT activity is expressed as the mean ± SEM of five experiments conducted each in triplicate. [a]Significantly different ($P < 0.05$) from the results obtained without β-cyclodextrin.

(Fig. 6B). Similarly, the addition of H-OxLDL in the presence of β-cyclodextrin prevented the rise of cellular MTT activity induced by OxLDL and rather accentuated the loss of MTT activity in MG-63 cells (Fig. 6B). Similar results were obtained after 48 h (data not shown). Overall, these results suggest that the protective effect of HDL_3 is not a consequence of their cholesterol efflux ability.

As simultaneous incubation of cells with OxLDL and β-cyclodextrin accentuated the loss of MTT activity, we speculated that OxLDL per se may promote cholesterol efflux. In accordance, we found that native LDL and OxLDL also induced cholesterol efflux from MG-63 cells to a similar extent to HDL_3 (Fig. 6C). Since OxLDL and β-cyclodextrin both induced cholesterol efflux, it became possible that together these efflux mediators decreased cell cholesterol in an additive manner which to a point causes cell death. In accordance, we found that 1 mM of β-cyclodextrin alone promoting a cholesterol efflux of 5% had no effect on MTT activity while concentrations of 2 mM and above associated with higher cholesterol efflux lead to a reduction of the MTT activity (Fig. 6D).

EFFECT OF PRE-INCUBATION OF CELLS WITH HDL_3 ON OXLDL INDUCED CELL DEATH

In order to determine if HDL could prevent OxLDL-induced cell death via other mechanisms beside the reduction of OxLDL association to MG-63 cells, we next investigated the impact of a HDL_3 pre-treatment on the cell death induced by OxLDL. MG-63 cells were incubated during 24 h with 200 µg/ml of HDL_3 and thereafter, the media was removed and cells were treated with increasing concentrations of OxLDL (20–200 µg/ml). Figure 7A shows that the decrease of MTT activity induced by M-OxLDL after 24 h was partially counterbalanced by pre-incubation of MG-63 cells with HDL_3, preventing by approximately 30% the reduction of MTT activity caused by 150–250 µg/ml of M-OxLDL (ANOVA, $P < 0.0001$). This protecting effect also occurred when cells were exposed during 48 h to M-OxLDL (ANOVA, $P < 0.0001$) (Fig. 7B). However, the results demonstrated that pre-incubation of osteoblasts with HDL_3 does not prevent the reduction of MTT activity induced by H-OxLDL (Fig. 7C,D).

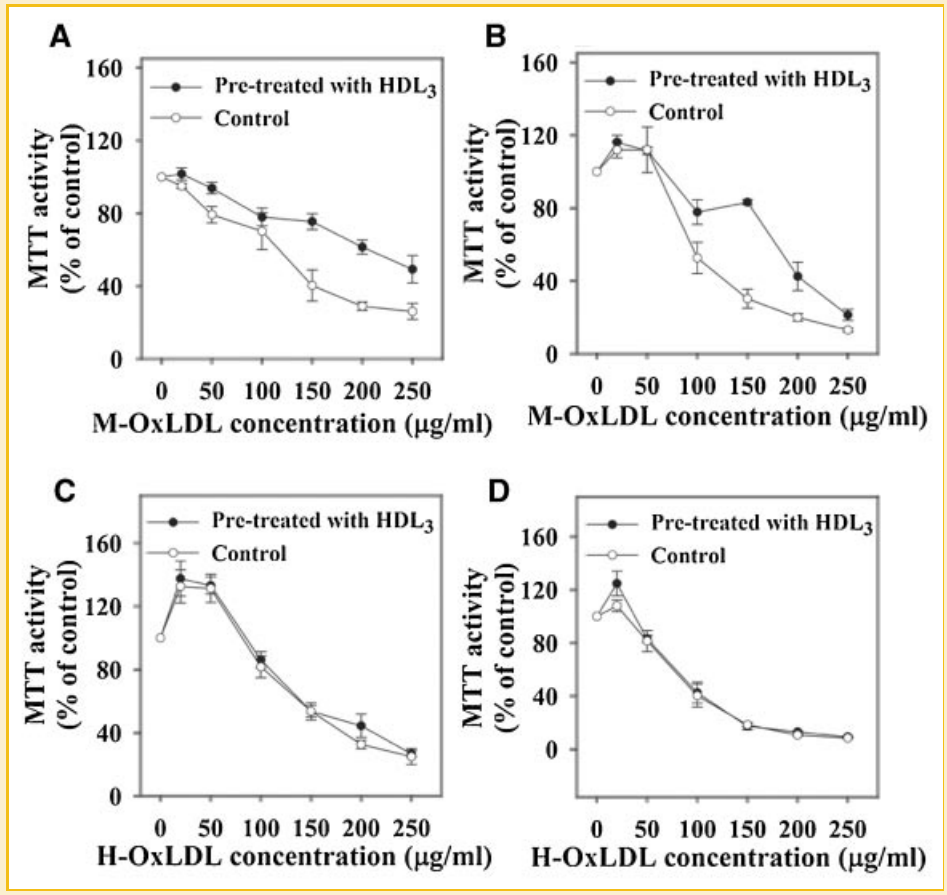

Fig. 7. Effect of HDL_3 pre-treatment on OxLDL-induced osteoblastic cell death. MG-63 cells were incubated in absence (open circle) or presence (close circle) of 200 µg/ml of HDL_3 during 24 h in DMEM-F12 supplemented with 2% LPDS and then incubated with increasing concentrations (20–250 µg of protein/ml) of M-OxLDL (A,B) or H-OxLDL (C,D) during 24 h (A, C) and 48 h (B,D) in DMEM-F12 supplemented with 2% LPDS. Cell viability was determined by MTT assays and is expressed as the mean ± SEM of four experiments conducted each in triplicate. Statistical differences were determined by one-way ANOVA.

IMPACT OF HDL ON THE METABOLISM OF OXLDL BY OSTEOBLASTIC CELLS

We previously demonstrated that the accumulation of OxLDL in lysosome reduced the MG-63 cell viability by causing lysosomal membrane damage [Brodeur et al., 2008a]. We therefore evaluated if a pre-incubation of MG-63 cells with HDL_3 was associated with a modification of OxLDL metabolism. To this end, cells were pre-treated with HDL_3 (50–200 µg/ml) for 24 h and thereafter the expression of scavenger receptors of class B, previously shown to be expressed by osteoblastic cells [Brodeur et al., 2008b], was evaluated. Results presented in Figure 8 show that HDL_3 do not modulate the expression of CD36 in its mature form (85 kDa). We have also detected a 70 kDa protein (probably another glycosylated form of CD36) that was again not modulated. However, we showed that HDL_3 increased by 1.5-fold (50 µg/ml) and 1.8-fold (100 and 200 µg/ml) the expression level of SR-BI. To determine if this higher expression of SR-BI has an impact on OxLDL metabolism, selective uptake experiments were conducted. Results presented in Figure 9 demonstrated that HDL_3 pre-treatment (200 µg/ml for 24 h) had no effect on protein association of M-OxLDL but reduced ^{125}I-H-OxLDL

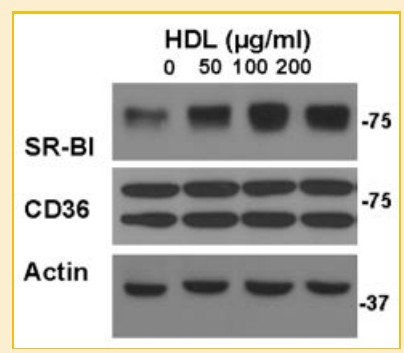

Fig. 8. Impact of HDL_3 on SR-BI and CD36 expression in MG-63 cell. MG-63 cells were incubated in the absence or presence of increasing concentrations of HDL_3 (50–200 µg/ml) during 24 h in DMEM-F12 supplemented with 2% LPDS and then total proteins of osteoblastic cells were extracted as described in Experimental Procedures Section. Representative results are shown for 50 µg of proteins subjected to SDS-PAGE and immunodetection with anti-SR-BI, CD36 or actin antibodies followed by enhanced chemiluminescence revelation.

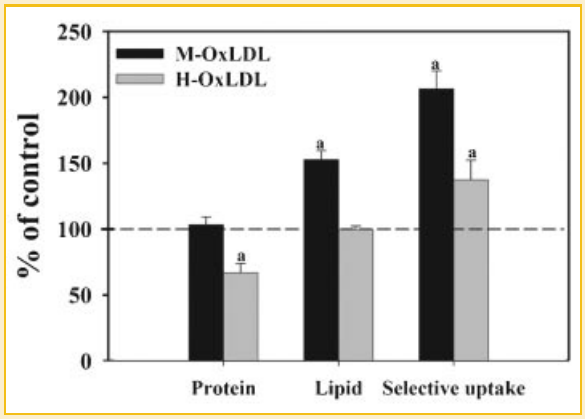

Fig. 9. Effect of HDL_3 on ^{125}I-lipoprotein association, ^3H-CE-lipoprotein association and ^3H-CE-lipoprotein association due to cholesteryl ester selective uptake in MG-63 cells. MG-63 cells were incubated in the absence or presence of HDL_3 (200 μg/ml) during 24 h in DMEM-F12 supplemented with 2% LPDS. Then cells were incubated with radiolabeled lipoproteins (20 μg/ml) during 4 h at 37°C in 12-well plates. Nonspecific radioactive lipoprotein association was determined by the addition of unlabeled lipoprotein at 1.5 mg of protein/ml and values were subtracted from total association. ^3H-CE-lipoprotein association was calculated as apparent protein association since its specific activity was established as cpm/μg of lipoprotein protein. To determine the amount of CE association due to selective uptake, protein association was subtracted from CE association, both in μg of protein/mg of cell protein. Results are shown as the mean ± SEM of four experiments conducted in duplicate. Statistical differences were determined with a *t*-test. [a]Statistically different ($P < 0.05$) from the result obtained with control cells.

association by 30% comparatively to control cells. The ^3H-CE association of M-OxLDL was increased by 50% in cells pre-treated with HDL_3, but lipid association of H-OxLDL was not modulated by HDL_3 (Fig. 9). CE-selective uptake values were also obtained by subtracting protein association data from those of CE. The data obtained indicated that HDL_3 pre-treatment increased the selective uptake of ^3H-CE from OxLDL (Fig. 9). Therefore, these results indicated that HDL_3 modulated OxLDL metabolism by reducing the global uptake in favor of selective uptake of lipids from OxLDL.

IMPACT OF HDL_3 ON LYSOSOMAL MEMBRANE STABILITY

To determine if this effect of HDL_3 on OxLDL uptake protects against lysosomal membrane damage, we next stained MG-63 cells with acridine orange. AO is a lysosomotropic weak base and a metachromatic fluorochrome showing red fluorescence at high and green fluorescence at low concentrations. Confocal scanning microscopy presented in Figure 10 demonstrated fewer red fluorescence spots in the cells exposed to OxLDL, indicating lysosomal membrane damage (Fig. 10B,E) comparatively to control cells incubated in absence of OxLDL (Fig. 10A,D). This reduced red staining was accompanied with increased green cytosolic fluorescence, reflecting a lower cytosolic pH due to proton redistribution and thus the trapping of protonized HAO+ in the cytosol of damaged cells. However, when cells were pre-treated with HDL_3 (200 μg/ml for 24 h), this reduction of red fluorescence spots induced by OxLDL was abolished (Fig. 10C,F). Importantly, results also indicated that HDL_3 alone have no impact on lysosomal membrane

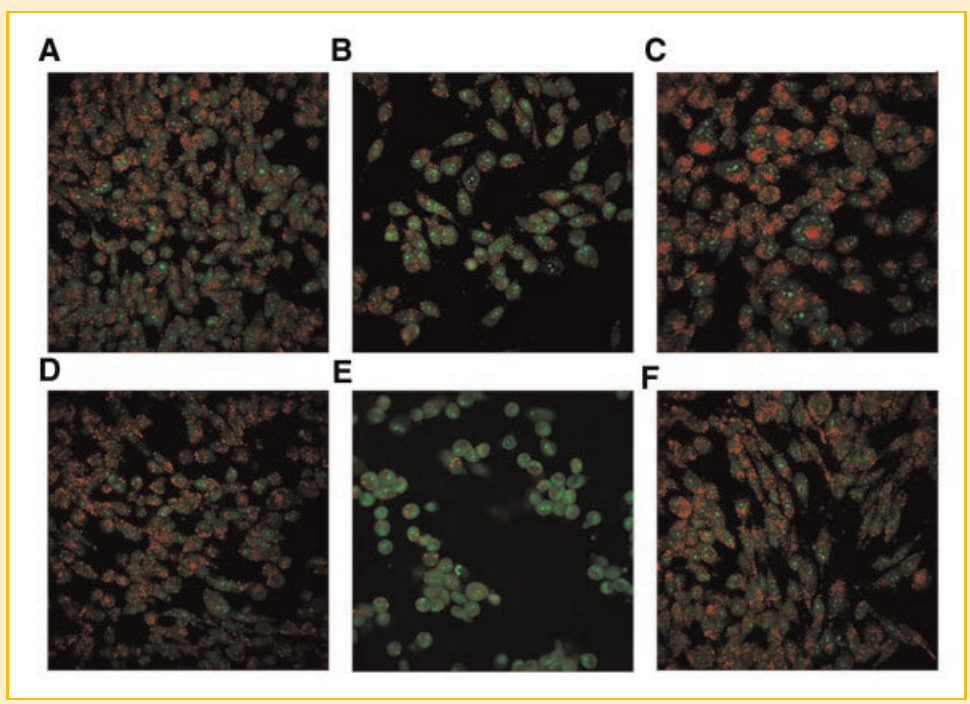

Fig. 10. Effect of HDL_3 on OxLDL-induced lysosomal membrane damage. MG-63 cells seeded in LabTek chambers were incubated in the absence (A,B,E) or presence of 200 μg/ml of HDL_3 (C,D,F) during 24 h in DMEM-F12 supplemented with 2% LPDS, and then incubated with 200 μg/ml of M-OxLDL (B,C) or H-OxLDL (E,F) during 24 h in DMEM-F12 supplemented with 2% LPDS. The cells were then labeled with acridine orange as described in Experimental Procedures Section and examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) with a Plan-Apochromatic 60× oil N.A. 1.4 objective lens. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

integrity (Fig. 10D). Therefore, these results suggest that exposition of cells to HDL_3 prior OxLDL results in the conservation of lysosomal integrity and inhibition of cells death induced by OxLDL.

DISCUSSION

Growing evidence suggests that OxLDL particles are a potential risk factor for the development of osteoporosis. The deleterious action of OxLDL on bone metabolism has been attributed to the stimulation of proliferation and the concomitant inhibition of osteoblastic differentiation [Parhami et al., 1997; Mody et al., 2001], as well as to the cytotoxic effects of OxLDL on osteoblasts [Klein et al., 2003; Hirasawa et al., 2007; Brodeur et al., 2008a]. Therefore, the prevention of osteoblast function alterations induced by OxLDL takes great importance in view to eventually prevent the development of osteoporosis associated with atherogenic conditions. Given that it was demonstrated that HDL exert a beneficial effect on cardiovascular system [Gordon and Rifkind, 1998] and bone tissue [Yamaguchi et al., 2002; Solomon et al., 2005; Dennison et al., 2007], we evaluated if HDL have the capacity to inhibit the deleterious effects of OxLDL on osteoblastic cells. Our results demonstrated that HDL_3 totally offset MG-63 cell death induced by OxLDL (Figs. 1 and 3) while being without effect per se on osteoblast viability and proliferation. Moreover, we demonstrated that HDL_3 have also the ability to reduce the loss of viability induced by OxLDL in hOB (Fig. 2). However, this protecting action of HDL_3 did not reach a statistical difference for M-OxLDL in hOB cells. The reason for this difference between MG-63 and hOB cells remains to be establish, but may be attributed to the uptake of M-OxLDL by others receptors (scavenger receptors or LDLr) that are not able to bind HDL. However, it cannot be excluded that a prolonged exposition of hOB to HDL_3 could protect against the deleterious effects of M-OxLDL as suggested by the results obtained in the pre-incubation experiments (Fig. 7).

As the cholesterol efflux by HDL was shown to inhibit macrophage apoptosis induced by OxLDL [Jiang et al., 2006], we investigated if this process also account for the HDL protection against the induction by OxLDL of cell death in osteoblasts. Although HDL_3 and β-cyclodextrin induced cholesterol efflux from MG-63 cells, β-cyclodextrin did not protect cells from death induced by OxLDL (Fig. 6B) which indicates that HDL are not protecting the cells by removing cell cholesterol. Instead, we showed that OxLDL have also the capacity to induce cholesterol efflux (Fig. 6C). Furthermore, the incubation of osteoblastic cells with OxLDL and β-cyclodextrin led to an increased loss of cell viability, suggesting that enhanced cholesterol efflux occurred upon the treatment. In accordance, we also found that higher concentrations of β-cyclodextrin, which promoted higher cholesterol efflux levels, reduced MG-63 cell viability (Fig. 6D). Therefore, OxLDL may jeopardize the survival mechanisms of osteoblast by promoting cholesterol efflux (Fig. 6C) and also may promote the induction of cell death mechanisms by loading cells in oxidized cholesterol that is not processed by the lysosomal system [Brodeur et al., 2008a], inducing a net decrease in total cell cholesterol availability.

As OxLDL cytotoxicity was shown to be dependent on the amount of OxLDL internalized by cells [Negre-Salvayre et al., 1990] and as we demonstrated that internalization of OxLDL by osteoblasts induced apoptosis, we speculated that the protective effect of HDL could result from a competition of the association of OxLDL to osteoblasts. Our data support the competition as the underlying mechanism for HDL protection against cell death induced by OxLDL, since HDL_3 efficiently reduced OxLDL association (Fig. 5). Moreover, the demonstration that the simultaneous incubation of cells with OxLDL and LDL reduced cell death induced by OxLDL and that LDL also significantly displaced OxLDL association further highlighted the importance of OxLDL association (binding and/or internalization) for the cell death action of OxLDL. Interestingly, the competition of OxLDL association by HDL_3 and LDL was around 50–60% which may indicate that only these binding sites/receptors are associated with OxLDL cytotoxicity or that the HDL protection may be related in part to the competition of OxLDL association and also to additional mechanisms that could protect the cells from the remaining association.

Although, the mechanism(s) and pathway(s) (receptor(s)) by which OxLDL act on osteoblastic cell viability remain(s) unknown, our results suggest that the level of SR-BI expression by osteoblastic cells could modify the metabolism of OxLDL and dictate their cytotoxicity. Indeed, we showed that pre-incubation of MG-63 cells with HDL_3 prior to incubation with OxLDL preserve the lysosomal integrity and reduced cell death. This pre-incubation with HDL_3 was correlated with an increase of SR-BI expression (Fig. 8). At first glance, this protection may appear to be associated with the reduction of OxLDL uptake as it was shown that the SR-BI pathway is less efficient than the CD36 pathway to internalize OxLDL [Sun et al., 2007]. Indeed, this higher level of SR-BI expression could shift the total cell surface binding of OxLDL to CD36 and SR-BI in favor of SR-BI, and thereby may protect the cells by limiting the entry of OxLDL in osteoblasts as suggested by the protection of lysosomal integrity (Fig. 10). In accordance, we have demonstrated that the association of ^{125}I-H-OxLDL with MG-63 cells was reduced. On the other hand, the protein association of M-OxLDL was not affected (Fig. 9). As our protocol for the determination of OxLDL association does not discriminate between cell surface binding and internalization, it cannot be excluded that there has been a shift of internalization in favor of binding. Despite the concomitant protection of lysosomes and the reduced uptake of OxLDL upon pre-treatment with HDL_3, cells are not protected against cell death induced by H-OxLDL, suggesting that OxLDL cause also their deleterious effect by other(s) pathway(s) which remain(s) to be identified in osteoblasts. However, given that in co-incubation of OxLDL and HDL, cells are totally protected despite an incomplete competition of the OxLDL association (Fig. 5), this lysosomal protection may be involved in the total inhibition of cell death obtained at 48 h in Figures 1 and 3.

We have recently reported that scavenger receptors of class B expressed by osteoblastic cells are implicated in the uptake of CE from LDL and HDL_3 [Brodeur et al., 2008b]. Interestingly, cells exposed to HDL_3 presented higher SR-BI levels and concomitantly became more efficient in CE selective uptake from OxLDL. Therefore, these results indicated that SR-BI is involved in the selective uptake

from OxLDL. As lipoproteins were labeled with ^{3}H-CE after oxidation, we cannot evaluate the impact of the HDL_3-induced selective uptake of oxidized lipids by cells. Nevertheless, the demonstration that cells are not more susceptible to cell death induced by OxLDL when SR-BI expression is up regulated suggests that SR-BI activity could modify the fate of lipids coming from selective uptake and reduce their cytotoxicity. Indeed, our results showed that cells exposed to HDL_3 are rather protected against cell death induced by M-OxLDL (Fig. 7) despite an increase of selective uptake, suggesting that the latter process is beneficial. This is supported by the demonstration that death induced by M-OxLDL in cells exposed to HDL_3 was reduced whereas death induced by H-OxLDL in these cells was not prevented, which correlated with the levels of augmentation of selective uptake by 2- and 1.25-fold, respectively. Thus, more investigation is required to determine the possible mechanism of protection afforded by the selective uptake against the cell death induced by OxLDL.

In conclusion this study reveals that HDL_3 have the capacity to inhibit the toxic effect of OxLDL on osteoblastic cells. This inhibition of apoptosis is related to the displacement by HDL_3 of the OxLDL association to osteoblasts and to the modulation of OxLDL metabolism related to an increase of SR-BI expression. This higher SR-BI expression reduced the global uptake of OxLDL in favor of selective uptake, resulting in the protection against lysosomal membrane damage induced by OxLDL. Therefore, the displacement of OxLDL association and the modulation of OxLDL metabolism have to be included to the numerous properties of HDL for the protection against OxLDL deleterious effects.

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REFERENCES

Akatsu T, Murakami T, Nishikawa M, Ono K, Shinomiya N, Tsuda E, Mochizuki S, Yamaguchi K, Kinosaki M, Higashio K, Yamamoto M, Motoyoshi K, Nagata N. 1998. Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. Biochem Biophys Res Commun 250:229–234.

Barengolts EI, Berman M, Kukreja SC, Kouznetsova T, Lin C, Chomka EV. 1998. Osteoporosis and coronary atherosclerosis in asymptomatic postmenopausal women. Calcif Tissue Int 62:209–213.

Brissette L, Charest MC, Falstrault L. 1996. Selective uptake of cholesteryl esters of low-density lipoproteins is mediated by the lipoprotein-binding site in HepG2 cells and is followed by the hydrolysis of cholesteryl esters. Biochem J 318:841–847.

Brodeur MR, Brissette L, Falstrault L, Ouellet P, Moreau R. 2008a. Influence of oxidized LDL on the viability of osteoblastic cells. Free Radic Biol Med 44:506–517.

Brodeur MR, Brissette L, Falstrault L, Luangrath L, Moreau R. 2008b. Scavenger receptors expressed by osteoblasts are implicated in the selective uptake of cholesteryl ester and estradiol from low and high density lipoprotein. J Bone Miner Res 23:326–337.

Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A, Zukel WJ. 1997. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation 55: 67–72.

Cui LH, Shin MH, Chung EK, Lee YH, Kweon SS, Park KS, Choi JS. 2005. Association between bone mineral densities and serum lipid profiles of pre- and post-menopausal rural women in South Korea. Osteoporosis Int 16:1975–1981.

D'Amelio P, Pescarmona GP, Gariboldi A, Isaia GC. 2001. High density lipoproteins (HDL) in women with postmenopausal osteoporosis: A preliminary study. Menopause 8:429–432.

de Nigris F, Franconi F, Maida I, Palumbo G, Anania V, Napoli C. 2000. Modulation by alpha- and gamma-tocopherol and oxidized low-density lipoprotein of apoptotic signaling in human coronary smooth muscle cells. Biochem Pharmacol 59:1477–1487.

Dennison EM, Syddall HE, Aihie Sayer A, Martin HJ, Cooper C. 2007. Lipid profile, obesity and bone mineral density: The Hertfordshire Cohort Study. Q J Med 100:297–303.

Gordon DJ, Rifkind B. 1998. High-density lipoprotein: The clinical implications of recent studies. N Engl J Med 321:1311–1316.

Hirasawa H, Tanaka S, Sakai A, Tsutsui M, Shimokawa H, Miyata H, Moriwaki S, Niida S, Ito M, Nakamura T. 2007. ApoE gene deficiency enhances the reduction of bone formation induced by a high-fat diet through the stimulation of p53-mediated apoptosis in osteoblastic cells. J Bone Miner Res 22: 1020–1030.

Jiang P, Yan PK, Chen JX, Zhu BY, Lei XY, Yin WD, Liao DF. 2006. High density lipoprotein 3 inhibits oxidized low density lipoprotein-induced apoptosis via promoting cholesterol efflux in RAW264.7 cells. Acta Pharmacol Sin 27:151–157.

Kilsdonk EP, Yancey PG, Stoudt GW, Bangerter FW, Johnson WJ, Phillips MC, Rothblat GH. 1995. Cellular cholesterol efflux mediated by cyclodextrins. J Biol Chem 270:17250–17256.

Klein BY, Rojansky N, Ben-Yehuda A, Abou-Atta I, Abedat S, Friedman G. 2003. Cell death in cultured human Saos2 osteoblasts exposed to low-density lipoprotein. J Cell Biochem 90:42–58.

Klein BY, Kerem Z, Rojansky N. 2006. LDL induces Saos2 osteoblasts death via Akt pathways responsive to a neutral sphingomyelinase inhibitor. J Cell Biochem 98:661–671.

Kodama H, Nose M, Niida S, Yamasaki A. 1991. Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. J Exp Med 173:1291–1294.

Kwiterovich PO, Jr. 1998. The antiatherogenic role of high-density lipoprotein cholesterol. Am J Cardiol 82:13Q–21Q.

Langer T, Strober W, Levy RI. 1972. The metabolism of low density lipoprotein in familial type II hyperlipoproteinemia. J Clin Invest 51:1528–1536.

Laroche M, Pouilles JM, Ribot C, Bendayan P, Bernard J, Boccalon H, Mazieres B. 1994. Comparison of the bone mineral content of the lower limbs in men with ischaemic atherosclerotic disease. Clin Rheumatol 13:61–64.

Lee CM, Chien CT, Chang PY, Hsieh MY, Jui HY, Liau CS, Hsu SM, Lee YT. 2005. High-density lipoprotein antagonizes oxidized low-density lipoprotein by suppressing oxygen free-radical formation and preserving nitric oxide bioactivity. Atherosclerosis 183:251–258.

Li W, Yuan XM, Brunk UT. 1998. OxLDL-induced macrophage cytotoxicity is mediated by lysosomal rupture and modified by intralysosomal redox-active iron. Free Radic Res 29:389–398.

Lougheed M, Steinbrecher UP. 1996. Mechanism of uptake of copper-oxidized low density lipoprotein in macrophages is dependent on its extent of oxidation. J Biol Chem 271:11798–11805.

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

Mackness MI, Durrington PN. 1995. HDL, its enzymes and its potential to influence lipid peroxidation. Atherosclerosis 115:243–253.

Majima T, Shimatsu A, Komatsu Y, Satoh N, Fukao A, Ninomiya K, Matsumura T, Nakao K. 2008. Increased bone turnover in patients with hypercholesterolemia. Endocr J 55:143–151.

Mardones P, Rigotti A. 2004. Cellular mechanisms of vitamin E uptake: Relevance in alpha-tocopherol metabolism and potential implications for disease. J Nutr Biochem 15:252–260.

Martínez-Ramírez MJ, Palma S, Martínez-González MA, Delgado-Martínez AD, de la Fuente C, Delgado-Rodríguez M. 2007. Dietary fat intake and the risk of osteoporotic fractures in the elderly. Eur J Clin Nutr 61:1114–1120.

McFarlane AS. 1948. Efficient trace-labelling of proteins with iodine. Nature 182:53–54.

Mody N, Parhami F, Sarafian TA, Demer LL. 2001. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. Free Radic Biol Med 31:509–519.

Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, Vahabzadeh K, Hama S, Hough G, Kamranpour N, Berliner JA, Lusis AJ, Fogelman AM. 2004. The oxidation hypothesis of atherogenesis: The role of oxidized phospholipids and HDL. J Lipid Res 45:993–1007.

Negre-Salvayre A, Lopez M, Levade T, Pieraggi MT, Dousset N, Douste-Blazy L, Salvayre R. 1990. Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. II. Uptake and cytotoxicity of ultraviolet-treated LDL on lymphoid cell lines. Biochim Biophys Acta 1045:224–232.

Nofer JR, Levkau B, Wolinska I, Junker R, Fobker M, von Eckardstein A, Seedorf U, Assmann G. 2001. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. J Biol Chem 276:34480–34485.

Ozaki H, Hla T, Lee MJ. 2003. Sphingosine-1-phosphate signaling in endothelial activation. J Atheroscler Thromb 10:125–131.

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, Tintut Y, Beamer WG, Gharavi N, Goodman W, Demer LL. 2001. Atherogenic high-fat diet reduces bone mineralization in mice. J Bone Miner Res 16:182–188.

Poli A, Bruschi F, Cesana B, Rossi M, Paoletti R, Crosignani PG. 2003. Plasma low-density lipoprotein cholesterol and bone mass densitometry in postmenopausal women. Obstet Gynecol 102:922–926.

Rämet ME, Rämet M, Lu Q, Nickerson M, Savolainen MJ, Malzone A, Karas RH. 2003. High-density lipoprotein increases the abundance of eNOS protein in human vascular endothelial cells by increasing its half-life. J Am Coll Cardiol 41:2288–2297.

Robbesyn F, Garcia V, Auge N, Vieira O, Frisach MF, Salvayre R, Negre-Salvayre A. 2003. HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-kappaB activation in smooth muscle cells. FASEB J 17:743–745.

Rodan GA, Martin TJ. 2000. Therapeutic approaches to bone diseases. Science 289:1508–1514.

Schmidt A, Geigenmüller S, Völker W, Buddecke E. 2006. The antiatherogenic and antiinflammatory effect of HDL-associated lysosphingolipids operates via Akt –>NF-kappaB signalling pathways in human vascular endothelial cells. Basic Res Cardiol 101:109–116.

Solomon DH, Avorn J, Canning CF, Wang PS. 2005. Lipid levels and bone mineral density. Am J Med 118:1414.

Suc I, Escargueil-Blanc I, Troly M, Salvayre R, Nègre-Salvayre A. 1997. HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL. Arterioscler Thromb Vasc Biol 17:2158–2166.

Sun B, Boyanovsky BB, Connelly MA, Shridas P, van der Westhuyzen DR, Webb NR. 2007. Distinct mechanisms for OxLDL uptake and cellular trafficking by class B scavenger receptors CD36 and SR-BI. J Lipid Res 48:2560–2570.

Tintut Y, Morony S, Demer LL. 2004. Hyperlipidemia promotes osteoclastic potential of bone marrow cells ex vivo. Arterioscler Thromb Vasc Biol 24:E6–E10.

Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, Shinki T, Gillespie MT, Martin TJ, Higashio K, Suda T. 2000. Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology 141:3478–3484.

Uyama O, Yoshimoto Y, Yamamoto Y, Kawai A. 1997. Bone changes and carotid atherosclerosis in postmenopausal women. Stroke 28:1730–1732.

Vieira O, Escargueil-Blanc I, Meilhac O, Basile JP, Laranjinha J, Almeida L, Salvayre R, Negre-Salvayre A. 1998. Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. Br J Pharmacol 123:565–573.

von Eckardstein A, Hersberger M, Rohrer L. 2005. Current understanding of the metabolism and biological actions of HDL. Curr Opin Clin Nutr Metab Care 8:147–152.

Yamaguchi T, Sugimoto T, Yano S, Yamauchi M, Sowa H, Chen Q, Chihara K. 2002. Plasma lipids and osteoporosis in postmenopausal women. Endocr J 49:211–217.