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SHORT COMMUNICATION

Flavonoid-Induced Ability of Minimally Modified Low-Density Lipoproteins to Support Lymphocyte Proliferation

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Abstract. Low-density lipoprotein (LDL) peroxidation appears to be involved in atherogenesis. We studied the ability of minimally modified LDL (MM-LDL) to be used by proliferating lymphocytes and the effects of antioxidant flavonoids on this lipoprotein. MM-LDL were obtained by storing LDL at 4° for 1 month, which resulted in a decrease in lipophilic antioxidants and an increased susceptibility to oxidation when incubated with cells. MM-LDL were not cytotoxic; however, in cells treated with lovastatin that require cholesterol for cell growth, they were much less efficient than fresh LDL in sustaining proliferation as determined by [^{3}H]thymidine incorporation into DNA. Pure quercetin and grape-derived beverages restored proliferation in the presence of MM-LDL and prevented the apoptosis otherwise induced by lovastatin. These effects of flavonoids correlated with their activity in inhibiting LDL peroxidation. The results demonstrate that potent antioxidants, such as flavonoids, protect MM-LDL from lipoperoxidation and preserve their ability to efficiently deliver cholesterol to cells. BIOCHEM PHARMACOL **55**;7:1125-1129, 1998. © 1998 Elsevier Science Inc.

Key Words. Minimally modified LDL; lipoperoxidation; cell proliferation; flavonoids; wine; lymphocyte

On the basis of certain properties exhibited *in vitro*, oxidized LDL† have been involved in the pathogenesis of atherosclerosis [1]. On the other hand, oxidized LDL contain lipoperoxidation products, which are highly toxic for cells [2]. The abundance of soluble antioxidants in plasma and those lipophilic antioxidants carried in lipoproteins protect LDL from lipoperoxidation and, accordingly, only minute LDL-associated oxidation products are found in plasma [3]. In the arterial wall, LDL are retained [4] and become more susceptible to peroxidation; indeed, oxidized LDL have been detected in this tissue [5]. Interestingly, CD4+ T lymphocytes have been isolated from human atherosclerotic plaques that respond to oxidized LDL by proliferating and cytokine secretion [6]. Thus, the effects of both oxidized LDL and the more likely present *in vivo* MM-LDL [7] on lymphocytes as well as the preventive action of antioxidants merit some attention. In the present work, we studied the effects of flavonoids, which are potent antioxidants present in edible fruits and vegetables [8, 9], on the ability of MM-LDL to sustain lymphocyte proliferation and to prevent LDL cytotoxicity.

MATERIALS AND METHODS

LDL was isolated by vertical spin ultracentrifugation [10] from a patient with homozygous familial hypercholesterolemia who was being treated with LDL-apheresis [11]. The LDL used had a similar lipid composition and lipoperoxide level as did LDL from normolipidemic sera (data not shown), and also behaved similarly on LDL-receptor binding assays [12]. To obtain MM-LDL, LDL was stored in the dark at 4° for one month [7]. MOLT-4 (ATCC, CRL 1582) were cultured in sterile 96-well plates (Multiscreen-HV, Millipore), each well containing 36×10^3 cells in 225 μL of cholesterol-free medium PH-SFCM (PAA). Where indicated, the medium was supplemented with LDL, lovastatin, a specific inhibitor of HMG-CoA reductase (generously provided by Sagrario Mochales, Merck Sharp and Dohme), and different antioxidants, i.e. pure quercetin, α-tocopherol (Sigma), unfermented grape juices or wines (EVENA, Olite). After 72 hr, cell proliferation was assessed by measuring the incorporation of [^{3}H-methyl]-thymidine (5 Ci/mmol; Amersham) into DNA [13]. In some experiments, cells were seeded on cover slips, fixed and stained

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† *Abbreviations:* LDL, low-density lipoproteins (d = 1.019-1063 g/mL); MM-LDL, minimally modified LDL; TBARS, thiobarbituric acid-reactive substances.

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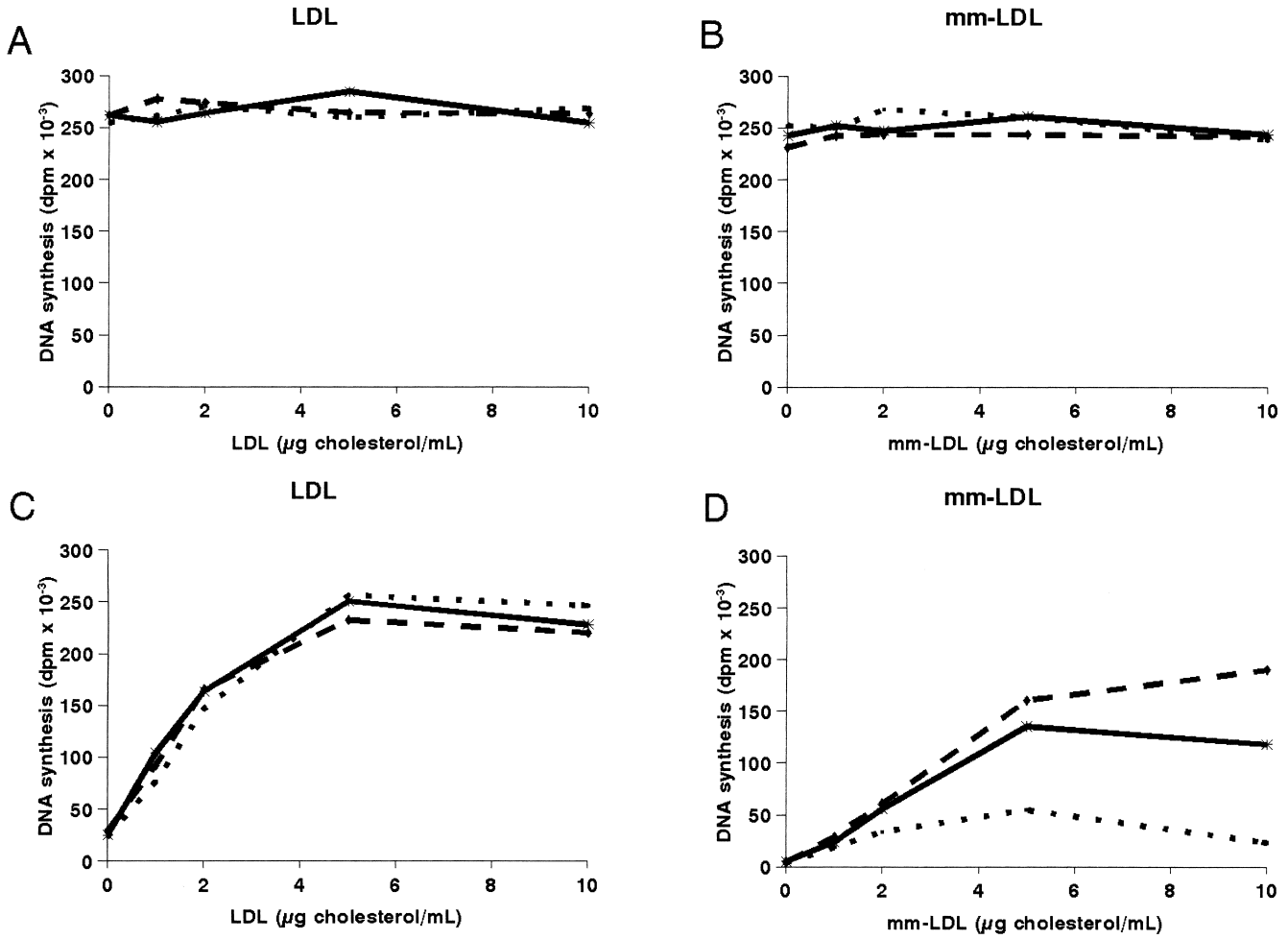

FIG. 1. Effect of quercetin on DNA synthesis in cells treated with lovastatin in the presence of minimally modified LDL (MM-LDL). MOLT-4 cells were incubated for 72 hr in cholesterol-free medium with 0 (A and B) or 10 μM of lovastatin (C and D) in the presence of either fresh LDL (A and C) or MM-LDL (B and D) as source of cholesterol, simultaneously with 0 (.....) , 10 (———), or 25 μM (– – – –) of quercetin. DNA synthesis was evaluated by [^{3}H]thymidine incorporation into DNA.

with bisbenzimide Hoechst 33258 (2.5 μg/mL) (Calbiochem). Standard methods were used for lipid peroxides [14], TBARS [15], polyphenols [16], cholesterol and apolipoprotein (apo) B [11]. Lipophilic antioxidants in LDL were measured by HPLC [17].

RESULTS AND DISCUSSION

As compared with fresh LDL, MM-LDL had similar lipid composition and electrophoretic mobility (data not shown), slightly higher lipid peroxides (49.8 ± 7.6 vs 16.4 ± 4.9 nmol/mg LDL-protein, $N = 3$) and diminished lipophilic antioxidant content (α-tocopherol: 1368 ± 18 vs 1541 ± 11; lycopene: 11.7 ± 1.0 vs 16.8 ± 0.25; β-carotene: 12.8 ± 0.2 vs 15.5 ± 1.0 μg/g of LDL-apo B, respectively), which likely determines a greater susceptibility to oxidation [18]. To evaluate the biologic properties of MM-LDL, we studied their ability to sustain cell growth in cells cultured in a cholesterol-free medium containing

TABLE 1. Effect of different wines, unfermented grape juices and ethanol on DNA synthesis in cells exposed to minimally modified LDL

	DNA synthesis (DPM × 10^{-3})	
	Control	Lovastatin
Basal	238.6 ± 7.5	23.5 ± 1.3
Quercetin (10 μM)	243.4 ± 6.8	118.1 ± 4.1
Vitamin E (50 μM)	255.1 ± 10.3	85.4 ± 5.1
Red wine (1:100)	246.3 ± 11.1	149.6 ± 6.2
Rosé wine (1:100)	240.1 ± 2.9	97.3 ± 7.3
White wine (1:100)	241.1 ± 6.3	63.7 ± 5.0
Red must (1:100)	249.2 ± 4.8	108.5 ± 4.3
White must (1:100)	243.8 ± 10.0	31.3 ± 1.1
12% ethanol (1:100)	242.7 ± 4.7	12.2 ± 2.7

MOLT-4 cells were cultured with MM-LDL (10 μg of cholesterol/mL) in the absence or in the presence of 10 μM of lovastatin. DNA synthesis was assayed after 72 hr by measuring the incorporation of [^{3}H]thymidine. In the absence of both lovastatin and LDL, DNA synthesis was 252.2 ± 20.8 × 10^3 dpm. Data are means ± SEM. of quintuplicate determinations. Total polyphenol (mg/L): red wine, 1659; rosé wine, 785; white wine, 732; red must, 1519; white must, 406.

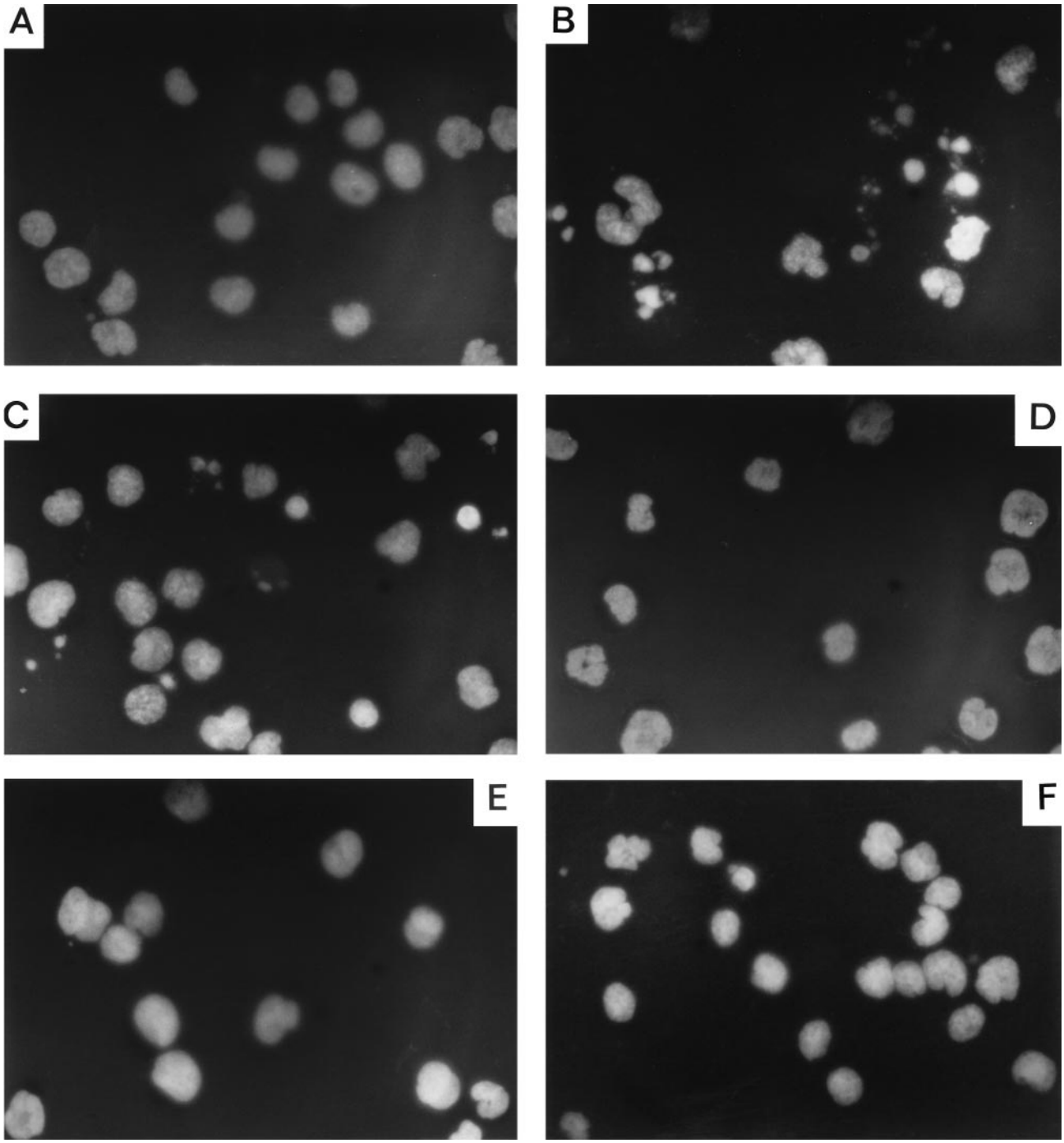

FIG. 2. Fluorescence microscopic appearance of Hoechst 33258-stained nuclei of cells treated with quercetin, red wine or unfermented grape juice. MOLT-4 cells were incubated in cholesterol-free medium for 72 hr in the absence (A) or in the presence of 10 μM of lovastatin (B—F) and 30 μg of LDL-cholesterol/mL (C—F) plus 10 μM of quercetin (D), red wine (final concentration 1:100, v/v) (E), or red unfermented grape juice (final concentration 1:100, v/v) (F). At the end of the incubation, the cells were fixed and stained with Hoechst 33258 for fluorescence microscope analysis of the nuclei.

lovastatin; in this way cells were made dependent on lipoprotein cholesterol for proliferation [13]. In the absence of lovastatin, neither fresh LDL (Fig. 1A) nor MM-LDL (Fig. 1B) affected DNA synthesis, demonstrating that MM-LDL were not cytotoxic. Lovastatin markedly inhibited [^{3}H]thymidine incorporation into DNA, and this inhibition was fully reversed by fresh LDL (Fig. 1C), which validates this technique for evaluating the activity of lipoproteins to be used by cells [13]. In contrast, MM-LDL could not restore cell proliferation in cells treated with lovastatin (Fig. 1D). Interestingly, supplementing the media with quercetin greatly increased the proliferative response to MM-LDL (Fig. 1D). It is worth mentioning that at the doses used, quercetin was not toxic (Fig. 1, A—C); at higher doses ([mt]50 μM), however, it was cytotoxic (data not shown), corroborating previous results by others [19].

In studies conducted in parallel, TBARS in media were determined at the end of the incubation. Oxidation product formation was higher from MM-LDL than fresh LDL (2.32 ± 0.19 vs 0.39 ± 0.11 nmol MDA/mg LDL-cholesterol, $P < 0.001$), and quercetin (10 μM) inhibited this process (0.42 ± 0.30 and 0.26 ± 0.12 with MM-LDL and fresh LDL, respectively). It has been reported that, in contrast to fully oxidized LDL, MM-LDL interact normally with the LDL-receptor [7]. Thus, it is conceivable that in our *in vitro* system, MM-LDL, as a result of their ready oxidation, were no longer used by cells or even became cytotoxic, whereas in the presence of quercetin, which prevented peroxidation, MM-LDL still sustained cell growth in cholesterol-deprived cells.

To study the action of flavonoid-rich products, cells incubated with MM-LDL were supplemented with wines or unfermented grape juices. As shown in Table 1, the stimulatory effect of wines on DNA synthesis was proportional to their flavonoid content (red > rosé > white), and the unfermented grape juices behaved in a similar manner to their wine counterparts. Red wine diluted 1:100 was more effective than 10 μM of quercetin, which in turn was more potent than vitamin E (50 μM), results that parallel their relative antioxidant activity [9]. By using several red wines made with different grapes, we found in separate experiments that TBARS production from MM-LDL inversely correlated to the polyphenol content in wines (multiplicative regression: $y = 2.43x^{-0.3634}$, $r = -0.8968$, $N = 10$, $P < 0.00044$; units: y, nmol MDA/mg LDL-cholesterol; *x*, mg polyphenol/L) and in turn, DNA synthesis inversely correlated to TBARS in medium (lineal regression: $y = 232.2\text{-}50.65x$, $r = -0.9124$, $N = 11$, $P < 0.0001$; units: y, ^{3}H-DNA dpm/well; *x*, nmol MDA/mg LDL-cholesterol). On the other hand, pure ethanol did not affect cell proliferation. All these results point to flavonoids as the active compounds in grape-derived beverages.

The inability of MM-LDL to sustain DNA synthesis in lovastatin-treated lymphocytes was accompanied by the induction of apoptosis, as assessed by fluorescence microscopy (Fig. 2). In comparison with the control (Fig. 2A), lovastatin produced both a loss of cells and the appearance of condensed and fragmented nuclei (Fig. 2B), which is in accordance with the DNA fragmentation observed by others [20]. On supplementing with MM-LDL, the number of viable cells increased although some apoptotic bodies were still present (Fig. 2C). Finally, when either quercetin, red wine, or red must (final concentration 1:100, v/v) was added simultaneously with MM-LDL (Fig. 2, D—F, respectively), the apoptotic bodies were no longer observed. Without discarding direct actions on cells [21], both the morphological observations and the proliferation studies commented on above indicate that flavonoids, by inhibiting MM-LDL peroxidation, permit the cholesterol provision to cells to occur, allowing them to proliferate and to escape from apoptosis. Since quercetin is present in human plasma [22], our results support the notion that a moderate intake of dietary flavonoids may have relevant physiological significance by reducing lipoprotein oxidation and preventing LDL-cytotoxicity.

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