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Red Wine administration to Apolipoprotein E-deficient Mice reduces their Macrophage-derived Extracellular Matrix Atherogenic Properties

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ABSTRACT

Proteoglycans (PGs) from the arterial extracellular matrix (ECM) contribute to the trapping of LDL and oxidized LDL (Ox-LDL) in the arterial wall, a phenomenon called "lipoprotein retention". Moreover, we have shown that subsequent to their binding to the matrix, LDL and Ox-LDL are taken up by macrophages. Oxidative stress significantly increases macrophage secretion of ECM-PGs, lipoprotein binding to the ECM and the uptake of ECM-retained lipoproteins by macrophages. The aim of the present study was to determine whether red wine administration to atherosclerotic mice would affect their peritoneal macrophage-derived extracellular matrix properties, such as the glycosaminoglycan content and the ability to bind LDL. In addition, we questioned the ability of LDL bound to the mice peritoneal macrophages-derived ECM to be taken up by macrophages. Red wine administration to atherosclerotic mice did not affect the mice peritoneal macrophages-derived ECM glycosaminoglycan content but it significantly reduced the mice peritoneal macrophages-derived ECM ability to bind LDL and the subsequent uptake of ECM-retained LDL by the macrophages. The present study thus clearly demonstrated the inhibitory effect of red wine consumption by E^0 mice on their peritoneal macrophage-derived extracellular matrix atherogenic properties.

Key words: Antioxidants, atherosclerosis, extracellular matrix, macrophages, polyphenols, proteoglycans, red wine.

Abbreviations: CE: cholesterol ester; DMMB: 1,9-dimethylmethylene blue; ECM: extracellular matrix; E^0: apolipoprotein E deficient; FCS: fetal calf serum; GAG: glycosaminoglycan; LDL: low density lipoprotein; LDL-R: LDL receptor; LPL: lipoprotein lipase; MPM: mouse peritoneal macrophages; Ox-LDL: oxidized LDL; PBS: phosphate buffered saline; PGs: proteoglycans.

INTRODUCTION

The development of atherosclerotic lesion is initiated with the accumulation of cholesterol in monocyte-derived macrophages (2,3) as well as with the retention of lipoproteins in the extracellular matrix of the subendothelial wall (22,30). Extracellular matrix (ECM) contains collagen and elastic fibers embedded in a viscoelastic gel consisting of proteoglycans (PGs), hyaluronan and glycoproteins (29). Arterial ECM contributes to the trapping of LDL and oxidized LDL (Ox-LDL) in the arterial wall, a phenomenon called "lipoprotein retention" (7, 18, 31). Specifically, the PGs from the ECM were shown to be responsible for the entrapment of LDL and Ox-LDL in the arterial wall (19, 27). Moreover, we have shown that subsequent to their binding to the matrix, LDL and Ox-LDL are taken up by macrophages (20). ECM can be produced in vitro by arterial cells, including endothelial cells, smooth muscle cells and also by macrophages (19). The amount and composition of ECM produced by all major cells of the arterial wall, determine the extent of lipoprotein cellular uptake. We have demonstrated that under oxidative

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stress, the macrophage ECM-PG secretion, the binding of lipoproteins to the ECM and the uptake of the ECM-retained lipoproteins by macrophages, are all significantly increased (20).

The intake of flavonoids, which constitute one of the largest groups of antioxidant phytochemicals, was shown to be inversely related to morbidity and mortality from coronary heart disease (16) and this phenomenon could be associated with polyphenol-mediated fibrinolytic effects (24), vasodilator effects (8) and most important, antioxidant effects (5,12). The bioavailability and metabolic modifications of flavonoids determine the antioxidative capacity of these potent antioxidants *in vivo*. Flavonoids can reduce LDL lipid peroxidation by acting as free-radical scavengers, as metal ion chelators, or by sparing LDL-associated antioxidants (10, 12). Flavonoids can also reduce macrophage oxidative stress by inhibition of cellular oxygenases (such as NADPH oxidase) and/or by activating cellular antioxidants such as the glutathione system (25). The effect of flavonoids on LDL oxidation is determined, by their accumulation in the lipoprotein on one hand, and in arterial cells (such as macrophages) on the other hand (10, 11).

Red wine is a dietary source of polyphenols, such as flavonols, quercetin and myricetin, and 3-flavanols, catechin and epi(gallo)catechin. Following wine ingestion, some wine-derived polyphenols bind to the LDL particle and protect the lipoprotein against oxidation (9). In the presence of wine-derived flavonoids, the activity of HDL-associated paraoxonase (an esterase that hydrolyzes oxidized lipids) is preserved and thus further reduces the lipoprotein oxidative state (14,21). Wine-polyphenols, which accumulate in arterial wall cells such as macrophages, can protect them from lipid peroxidation and thus further reduce macrophage lipid peroxidation and cell-mediated oxidation of LDL, leading to attenuation in foam cell formation (4). Furthermore, wine administration to atherosclerotic mice (apolipoprotein E deficient mice) attenuates the development of atherosclerotic lesions in this animal model (15).

The aim of the present study was to determine whether red wine administration to atherosclerotic mice would affect their peritoneal macrophage-derived ECM properties, including their glycosaminoglycan (GAG) content and their ability to bind LDL. In addition, we questioned the effect of red wine on the cellular uptake of LDL bound to mouse peritoneal macrophages-derived ECM.

EXPERIMENTAL DESIGN

Mice

The study protocol was approved by the Committee for the supervision of animal experiments of the Technion Israel Institute of Technology (approval number IL-066-10-2001/04) and was conducted in accordance with the guiding principles in the care and use of laboratory animals.

Apolipoprotein E deficient (E^0) mice were provided by Dr. Jan Breslow (Rockefeller University, NY). E^0 mice are widely used as an animal model for atherosclerosis as they develop severe hypercholesterolemia on a chow diet. E^0 atherosclerotic mice at 4 weeks of age were assigned randomly into three groups; 5 mice in each group. The mice were supplemented for 5 weeks with the following substances that were added to their drinking water: (1) Placebo (water), (2) red wine (0.125 ml red wine *Cabernet Sauvignon*/mouse/day), (3) vitamin E (50 mg/mouse/day).

Mouse peritoneal macrophages isolation

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal fluid of E^0 mice, 4 days after intraperitoneal injection into each mouse of 3 ml of thioglycolate (24g/l) in saline. The cells ($10\text{-}20*10^6$/mouse) were washed, dispensed into plastic Petri dishes and incubated in a humidified incubator (5 % CO_2, 95 % air) for 2h at 37°C.

Extracellular matrix preparation

Macrophages (MPM from E^0 mice) were cultured for 4 days at 37°C in DMEM

containing 10 % fetal calf serum (FCS). Then, cells were removed using 0.5 % Triton X-100 and 20mM NH_4OH. Dishes coated with the ECM were then washed with phosphate buffered saline (PBS) and kept at 4ºC for use within two weeks (28). The macrophage ECM remained intact and was free of cellular elements. The above treatment completely removes the cell layer leaving behind only the matrix secreted by the cells. ECM protein content was assessed by the Lowry assay.

ECM Glycosaminoglycans determination

Macrophage GAG content was analyzed using the 1,9-dimethylmethylene blue (DMMB) spectrophotometric assay for sulfated glycosaminoglycans (26).

Degradation and cell-association of ECM-retained lipoproteins by macrophages

Dishes coated with the macrophage-derived-ECM, were preincubated with 8 mg/ml of lipoprotein lipase (LPL) for 1 hour at 4°C. After 3 washes with PBS, radioiodinated-LDL (25 mg of protein/ml) was added for 4 hours at 37°C. Finally, after 3 more washes with PBS, fresh macrophages were seeded onto the ECM layer in presence of the cellular activator phorbol myristate acetate (100 nM) for 18 hours at 37°C. Cell-mediated hydrolysis of LDL protein was assayed by determination of the trichloroacetic acid soluble, chloroform-insoluble radioactivity in the incubation medium (20). Degradation of lipoproteins, that were added to control empty plates, was minimal (<10 %) and was always subtracted from the degradation rates of the ECM-retained lipoproteins.

Statistics

T test was performed for all statistical analyses. Results are given as mean±SD

RESULTS

Mouse peritoneal macrophages (MPMs) were harvested from the 3 groups (placebo, red wine-treated and vitamin E-treated) of apolipoprotein E deficient (E^0) mice and these cells were used to produce ECM. ECM derived from the E^0 harvested MPMs was analyzed for GAG content, and its ability to bind LDL. In addition, we analyzed the ability of LDL-bound to ECM to be taken up by J-774 A.1 macrophages.

The GAG content of the ECM derived from MPM was not affected either by red wine treatment, or by vitamin E treatment, in comparison to the placebo-treated mice (Fig. 1A).

ECM obtained for the 3 groups of mice (placebo, red wine-treated and vitamin E-treated) were then incubated with ^{125}I-LDL to compare their ability to bind LDL. The binding of LDL to the macrophage-derived-ECM was significantly reduced by 77 % following mice treatment with red wine but by only 18 % following mice treatment with vitamin E, in comparison to the placebo-treated mice (Fig. 1B).

Finally, LDL that was retained to ECM derived from MPM was incubated with J-774 A.1 macrophages in the presence of 100 nM PMA (cellular activator), in order to determine the ECM-retained-LDL cellular uptake by the macrophage cell-line. Macrophage degradation of ECM-retained LDL was decreased by 56 % when the ECM was prepared from MPMs harvested from red wine–treated mice in comparison to ECM prepared from MPM harvested from placebo-treated mice (Fig. 1C). In contrast, macrophage degradation of LDL that was retained to ECM from MPMs harvested from vitamin E–treated mice was similar to that of placebo-treated mice (Fig. 1C).

DISCUSSION

The present study clearly demonstrated the inhibitory effect of red wine consumption by E^0 mice on their peritoneal macrophage-derived ECM atherogenic properties, such as LDL binding to the ECM and its subsequent uptake by macrophages, leading to an inhibitory effect on foam cell formation (Fig. 2).

ECM produced by the cells of the arterial wall participated in the

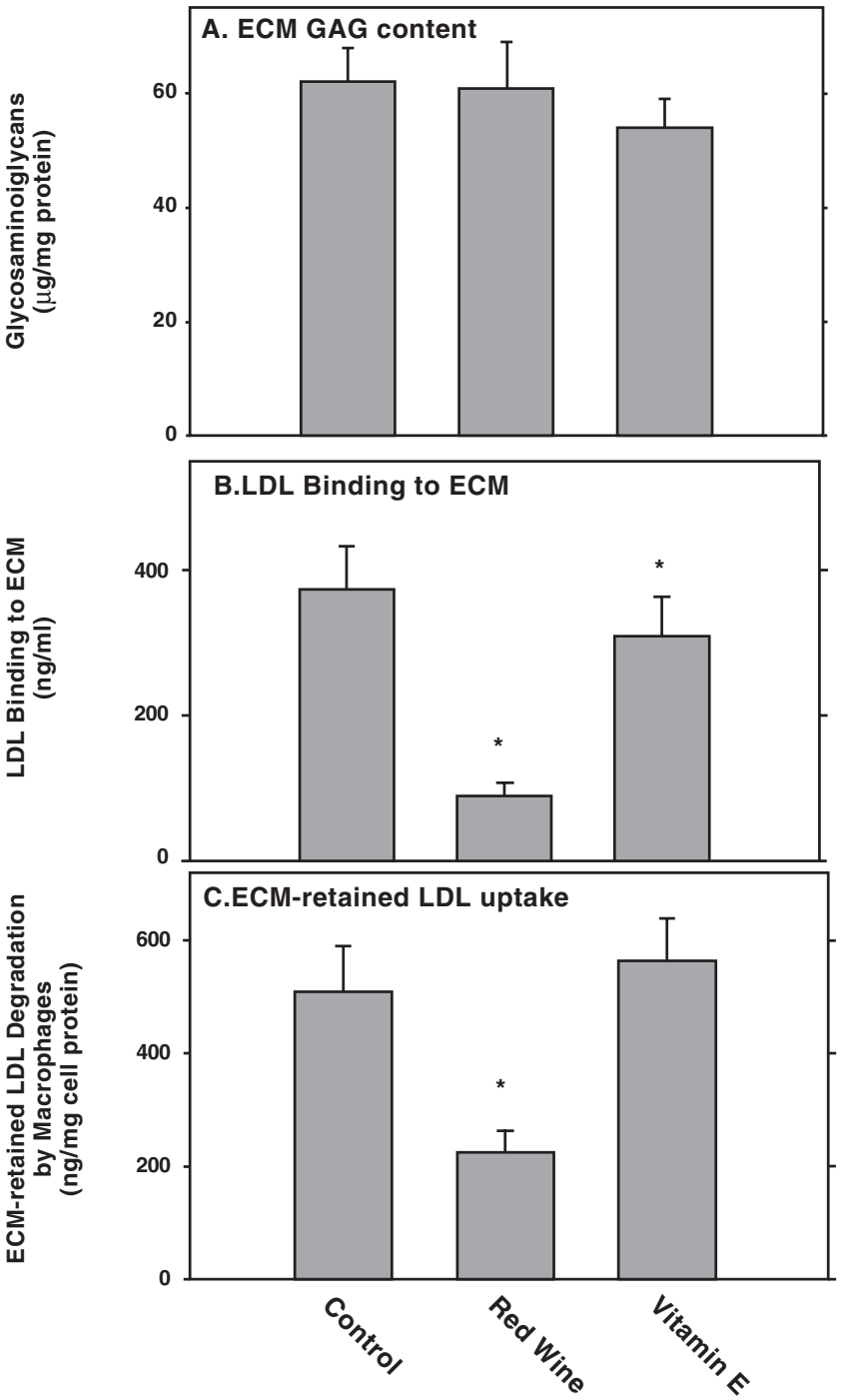

Figure 1. The effect of red wine or vitamin E supplementation to apolipoprotein E deficient (E^0) mice on their peritoneal macrophage derived extracellular matrix (ECM) atherogenicity.
ECM prepared from mouse peritoneal macrophages (MPM) that were isolated from 3 groups of apolipoprotein E deficient mice (red wine-treated, vitamin-E treated and placebo-treated) were analyzed for their glycosaminoglycan content (A), and their ability to bind LDL (B). This latter ECM-retained LDL was used to analyze the lipoprotein uptake by J-774 A.1 macrophages (C). Results are given as mean ± SD, *$p<0.01$ (n=5).

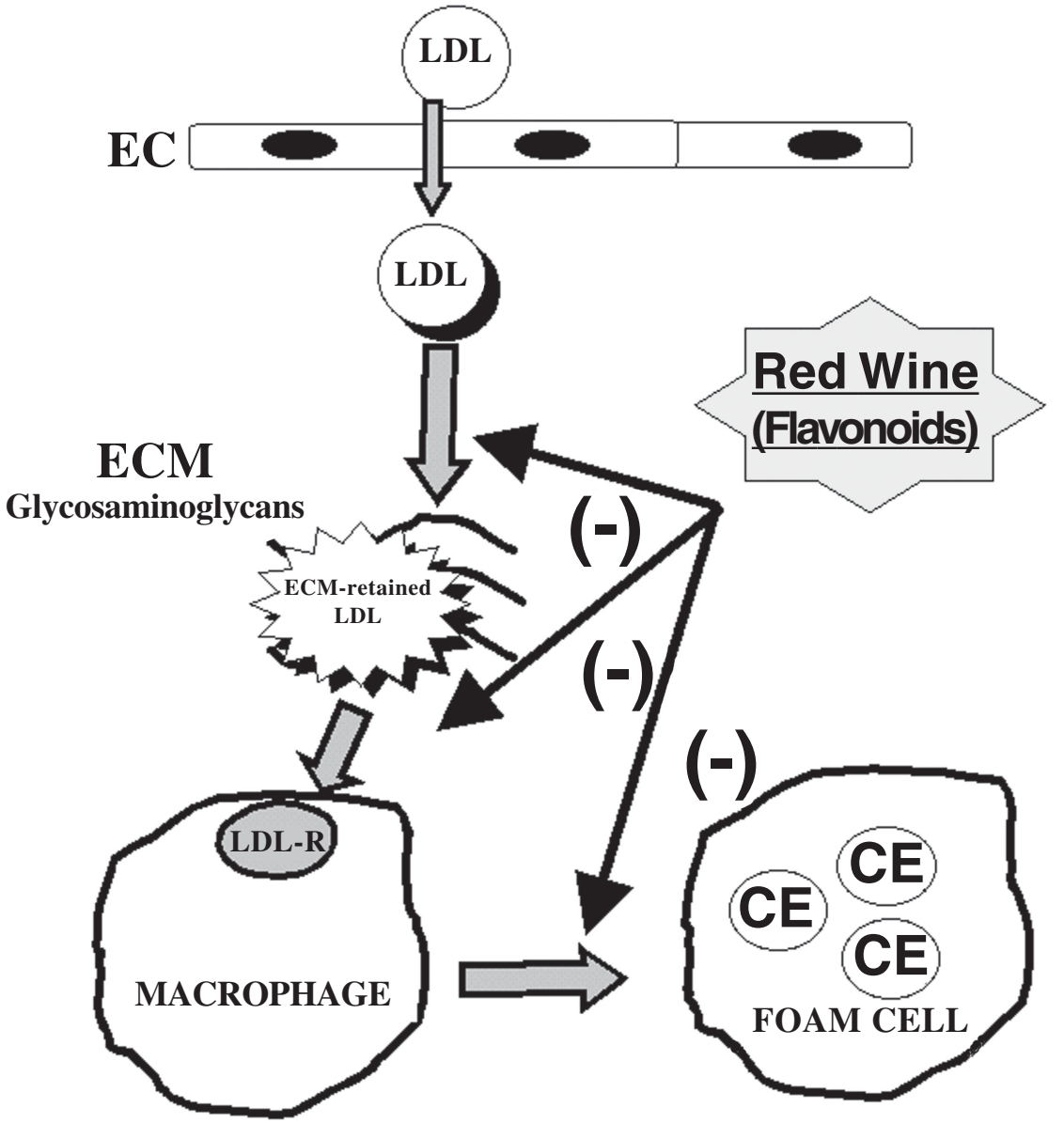

Figure 2. Red wine supplementation to apolipoprotein E deficient (E^0) mice inhibits LDL retention to the peritoneal macrophage derived-extracellular matrix (ECM).
Following red wine consumption, flavonoids present in red wine can reduce retention of LDL to the ECM, thus inhibiting the macrophage uptake of ECM-retained LDL, and therefore leading to a reduction in macrophage cholesterol accumulation and foam cell formation. EC: Endothelial cell, ECM: Extracellular matrix, LDL: Low density lipoprotein, CE: cholesterol ester, LDL-R: LDL receptor.

development of the atherosclerotic lesion (30). ECM-derived-GAGs are able to trap LDL in the arterial wall, a process called "LDL retention" (7, 18, 31). Following its retention in the arterial wall, the lipoprotein becomes more susceptible to oxidation, and it is taken up by arterial cells such as macrophages at enhanced rate (20, 27). Thus, modulation of the ECM content (especially its GAG content) could affect the ECM atherogenic properties.

In the present study we have shown for the first time that although total GAGs content of the macrophages derived ECM was not affected by red wine treatment, it significantly reduced the ability of the matrix layer to bind LDL. More important, this reduction in macrophage LDL binding was associated with a marked decrement in the subsequent uptake of the ECM-retained LDL by macrophages. Vitamin E, as opposed to red wine flavonoids caused only a slight reduction in LDL retention to the macrophages derived ECM, and did not affect the ECM-retained LDL uptake by macrophages.

Once LDL is trapped in the arterial wall and isolated from the plasma antioxidant-rich environment, the lipoprotein becomes more susceptible to oxidation. Oxidation of LDL then, results in its separation from the ECM, followed by the lipoprotein (Ox-LDL) uptake by macrophages. The inhibitory effect of red wine on LDL retention thus affects its subsequent oxidation and the cellular uptake of the oxidized lipoprotein. In addition, red wine supplementation to E^0 mice also reduced macrophage uptake of the ECM-retained LDL. The ability of red wine to affect LDL retention to ECM and its subsequent uptake by arterial wall cells, in spite of the fact that it did not affect total GAG content in the ECM, can be explained by the fact that ECM is composed of several classes of GAG. The effect of red wine consumption on the ECM-GAGs could thus be due to their composition rather than to the total GAG content.

Red wine supplementation to apolipoprotein E-deficient mice was previously shown to reduce oxidative stress, both in serum and in peritoneal macrophages (1, 9, 15). However the inhibitory effect of red wine on the retention of LDL to the ECM may not be related to additional effects of red wine besides its antioxidant effects (6), as vitamin E was ineffective.

The ability of red wine flavonoids to attenuate atherogenesis is determined by the capacity of these substances to scavenge reactive oxygen/nitrogen species (and not a single type of free radical) as shown by comparing several different varieties of red wine (17). Different types of flavonoids exert different capacities to scavenge free radicals as we have recently shown (13).

In conclusion, red wine, in addition to its known anti-oxidative characteristics, also significantly reduces macrophage-derived ECM atherogenic properties. These properties contribute to the ability of red wine consumption to attenuate the development of atherosclerosis (23).

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