Increased susceptibility of low-density lipoprotein (LDL) to oxidation by γ-radiolysis with age

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Abstract The susceptibility to oxidation of freshly isolated LDL from healthy normolipidemic individuals in three age groups was estimated by exposure of LDL to ionizing radiation followed by analyses of vitamin E, TBARS, conjugated dienes, and fluorescent products. The results clearly showed that LDL from elderly subjects was the most susceptible to oxidative damage in vitro. In particular, the greater susceptibility of LDL from elderly subjects in comparison to that from young subjects may be attributed to the much lower (4-fold) concentration of LDL vitamin E in the elderly subjects. The present study reinforces the notion that the susceptibility of LDL to oxidation increases with age.

Key words: LDL; γ-Radiolysis; Aging; Thiobarbituric acid reactive substances; Vitamin E

1. Introduction

There is increasing evidence that free radical-mediated oxidation of LDL is an important underlying cause of atherogenesis leading to occlusive arterial lesions which are responsible for myocardial and cerebral infarctions and other serious problems of the circulation [1]. LDL oxidation can be induced in vitro by various cells present in atherosclerotic lesions including endothelial cells [2], smooth muscle cells [3], and monocyte/macrophages [4]. Oxidized LDL (oxLDL) thus formed may promote the formation of initial atherosclerotic lesions or cholesterol-laden foam cells by way of a chemotactic effect on monocytes, an inhibitory effect on macrophage motility, immunogenic responses, changes in the production of growth factors and cytokines, and also by unregulated uptake by the scavenger receptor [5–7]. Since large amounts of oxLDL are present in atherosclerotic plaques rather than in the circulation of both rabbits and humans, it is possible that the arterial wall is a major site of LDL oxidation [8].

The propensity of LDL toward lipid peroxidation appears to be a risk factor for atherogenesis [9]. In fact, it has been shown that LDL obtained from patients with coronary heart disease [10,11], hypercholesterolemia [12,13], diabetes mellitus [14], and hypertension [15], and from smokers [16,17] has increased susceptibility to oxidation in vitro. Thus, the susceptibility of individuals' LDL to oxidation might be responsible in a large part for the increased incidence of premature atherosclerosis in such patients. The aim of the present work is to examine the effect of age on the susceptibility to free radical oxidation of LDL. To this end, we compared the susceptibility of LDL isolated from healthy, normolipidemic individuals toward radiation-induced oxidation in an attempt to find factors responsible for the association of atherosclerosis with aging.

2. Materials and methods

2.1. Reagents

Acetic acid, sulfuric acid *n*-butanol, sodium phosphate, thiobarbituric acid, methanol and hexane were purchased from Fisher (Montréal, Que.), and 1,1,3,3-tetraethoxypropane, D-α-tocopherol, and DL-α-tocopherol were obtained from Sigma (St. Louis, MO, USA). Dialysis bags were purchased from Spectrum Medical Industries Inc. (TX, USA).

2.2. Patients

Sera were obtained from 18 healthy normolipidemic male subjects of various ages after overnight fasting. In each group, 6 subjects were analyzed independently: 6 young subjects, age 20–25 years; 6 middle-aged subjects, age 30–49 years; and 6 aged subjects, age 68–85 years. They were all apparently healthy, without symptoms and signs of any arterial diseases established by a complete and negative clinical examination and a normal 12-lead ECG according to WHO criteria [18]. No study subject had kidney, liver or thyroid disease. Blood pressure profile was in the normal range and they were all non-smokers. Glycemia, fibrinogen level, lipid profile and coagulation profile were within the normal ranges.

2.3. Isolation of LDL

Isolation of LDL was performed according to the method of Sattler et al. [19], using the Beckman Optima TLX ultracentrifuge equipped with TLA 100.4 rotor, in the presence of EDTA (0.4 g/l). After separation, LDL was dialyzed overnight at 4°C with 10^{-2} M sodium phosphate buffer (pH 7). For radiolysis experiments, the dialyzed solutions of LDL were adjusted to a concentration of 100 μg/ml, expressed as total protein concentration, by dilution in the same buffer. Proteins were measured by the Pierce method.

2.4. LDL oxidation by γ-radiolysis of water

Gamma-irradiation was carried out on a ^{60}Co Gamma cell 220 (Atomic Energy of Canada Ltd.). The dose rate was 0.18 Gy/s as determined by Fricke dosimetry [20]. Irradiation was performed as previously described [21]. Briefly, LDL was irradiated in oxygenated aqueous solutions containing 10^{-2} M sodium phosphate buffer at pH 7.0. Under these conditions, the main radical species formed are hydroxyl radicals and superoxide anion radicals with respective yields of 2.8×10^{-7} mol J^{-1} and 3.4×10^{-7} mol J^{-1} [22]. The total radiation doses were 0–200 Gy.

2.5. Measurement of conjugated dienes, TBARS, differential fluorescence and vitamin E

Different parameters were used to monitor the progress of lipid peroxidation of LDL, including TBARS and conjugated diene formation, fluorescence differential spectra and the disappearance of vitamin

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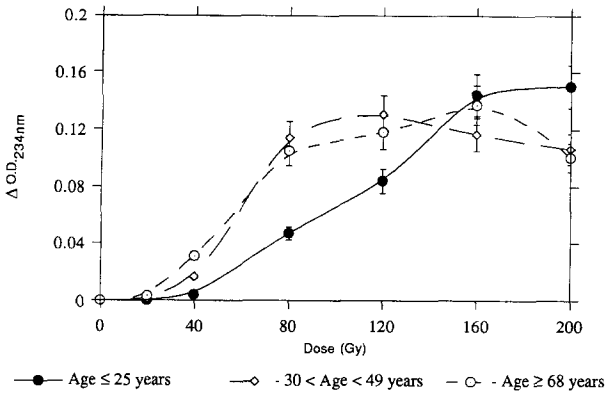

Fig. 1. Conjugated diene formation (differential absorbance at 234 nm) in LDL as a function of radiation dose and age. Dose rate=0.18 Gy/s. [LDL] 0.100 g proteins/l. Action of $OH^{\cdot}/O_2^{\cdot-}$ free radicals at pH 7 (10^{-2} M sodium phosphate, O_2-saturated solutions).

E. The conjugated dienes were measured by differential absorbance spectra of irradiated and non-irradiated LDL between 200 and 700 nm on a Hitachi U 300 spectrophotometer as described [23]. An increase in the differential absorbance at 234 nm may be explained by the formation of conjugated dienes [24,25], $\varepsilon_{234} = 27\,000$ mol^{-1} liter cm^{-1}. Thiobarbituric acid reactive substances (TBARS) were determined as end-products of lipid peroxidation by the spectrofluorometric method [26], but without precipitation by phosphotungstic acid. 1,1,3,3,-Tetraethoxypropane was used as a standard. The concentration of TBARS produced by LDL oxidation was calculated as the difference between the concentration of TBARS in irradiated samples and non-irradiated controls [23]. Differential fluorescence for control and oxidized LDL was measured between 380 and 520 nm using a Hitachi U4500 fluorescence meter as previously described [23]. Endogenous vitamin E was assayed as α-tocopherol before and after irradiation, by reverse phase HPLC, with spectrophotometric detection at 292 nm [27] in LDL as already described [23].

The rate of disappearance of vitamin E was calculated from the initial slope of −vitamin E vs. Dose (Gy) and was expressed in mol J^{-1}. Similarly, the rate of formation of TBARS was calculated from the linear part of TBARS vs. Dose. Student's unpaired *t*-test was used to determine the significance between mean values.

3. Results

The subjects for our study were normolipidemic with differences of total and LDL cholesterol of no more than 15%. The concentration of vitamin E in sera was 17.30 ± 4.10, 29.00 ± 3.42 and 28.70 ± 5.23, for young, middle-aged and elderly subjects, respectively. Thus, we assume that the ratio of vitamin E to polyunsaturated fatty acids (PUFA) was comparable for all study subjects with the exception perhaps of the young group which was low in vitamin E. In addition, TBARS were measured in both sera and native LDL from all subjects giving a range of concentrations of 3.06–4.92 μM in sera and 0.5–1.66 μM in native LDL with no significant differences in each group. The values were comparable to those reported in the literature [28].

The formation of conjugated dienes (as a measure of LDL oxidation) was first studied. Susceptibility of LDL to oxidation as a function of the age of the subject was measured by exposure to $OH^{\cdot}/O_2^{\cdot-}$ induced in vitro by the radiolysis of water. In these studies, we observed that the onset of conjugated dienes formation for the elderly takes place at a lower dose than that for the middle-aged and young subjects (Fig. 1; the onset or lag phase, before appreciable peroxidation, was measured from the intercept of the initiation and the propagation phases of peroxidation). Thereby, the lag phase for conjugated diene formation of irradiated LDL was significantly higher in young compared to elderly subjects (Table 1). At high radiation doses, the formation of conjugated dienes reached a plateau at comparable levels in each age group.

Similar to conjugated dienes, the formation of TBARS (mainly malondialdehyde) displayed a lag-phase followed by a rapid increase upon exposure of LDL to radiation-induced $OH^{\cdot}$ and $O_2^{\cdot-}$ free radicals. The lag-phase was longer for LDL isolated from young subjects compared to that from middle-aged or elderly subjects (Fig. 2 and Table 1). In addition, the lag phase for TBARS, as well as that for conjugated dienes, occurred following the depletion of endogenous vitamin E (Fig. 4). The radiolytic yield (G value) of TBARS formation was 0.015 ± 0.002 μmol J^{-1} for LDL isolated from young subjects. In contrast, the G value of TBARS formation was 0.022 ± 0.002 μmol J^{-1} and 0.038 ± 0.003 μmol J^{-1} for LDL from middle-aged and elderly donors, respectively. These values represent about 5 and 14% of the total yield of $OH^{\cdot}$ radical (0.27 μmol J^{-1}), the most damaging species generated by the radiolysis of water.

The oxidation of LDL-bound proteins was measured by differential fluorescence. These results showed that fluorescence increased after exposure of LDL to radiation doses in excess of 40 Gy ($P < 0.01$) and that the rise of fluorescence started at a lower dose and achieved a higher level in going from older to younger donors. However, the fluorescence of irradiated LDL from middle-aged donors was greater than that from elderly donors (Fig. 3).

The principal antioxidant in LDL, vitamin E, was measured in patients of various ages. Fig. 4 shows the content of vitamin E in LDL from young, middle-aged and elderly subjects

Table 1
Resistance of LDL from different subjects (young, middle-aged and elderly subjects) to oxidation promoted by $OH^{\cdot}/O_2^{\cdot-}$ free radicals induced by water radiolysis

Age (years)	Lag-phase for conjugate dienes	Lag-phase for TBARS	Lag-phase for fluorescence appearance	G(TBARS) $\times 10^{-7}$ mol J^{-1}
< 25 years	40 Gy	80 Gy	120 Gy	0.154 ± 0.020
30–48 years	20 Gy	40 Gy	40 Gy	0.222 ± 0.025*
65–68 years	< 20 Gy	20 Gy	20 Gy	0.380 ± 0.030**

The lag-phase is the radiation dose for which no appreciable oxidation takes place and is calculated at the intercept between the initiation and the tangent to the propagation phase.
G(TBARS) is expressed in mol of TBARS formed per unit of energy absorbed (Joule). The yield reflects the rate of TBARS formation as a result of the action of the oxygenated free radicals produced selectively at steady-state concentrations and hence at a constant rate by γ-radiolysis of aqueous LDL solutions.
Significance calculated as compared to young subjects: *$P < 0.05$, **$P < 0.01$.

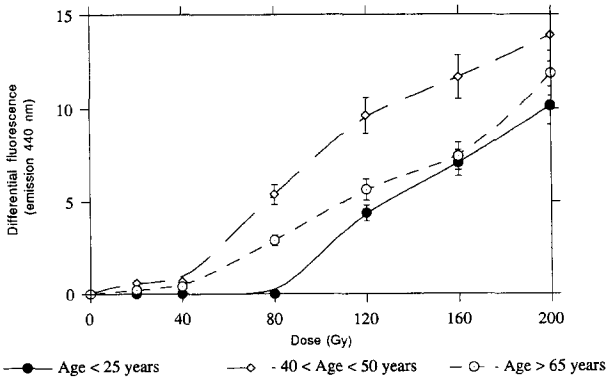

Fig. 2. TBARS formation in LDL as a function of the radiation dose and age. See notes in Fig. 1.

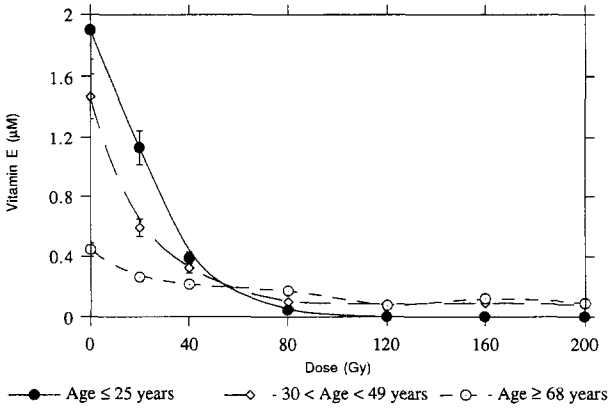

Fig. 4. Evolution of the amount of vitamin E in LDL as a function of radiation dose and age. See notes in Fig. 1.

vs. radiation doses. The amount of vitamin E in freshly isolated LDL was significantly higher in LDL for young subjects (1.90 ± 0.85) and middle-aged (1.46 ± 0.63) compared to elderly (0.44 ± 0.69) donors ($P < 0.05$). Assuming a molecular weight of LDL of approximately 2200 kDa [29], the number of molecules of vitamin E per LDL may be calculated to be 8.77 ± 3.14, 6.75 ± 2.32 and 1.62 ± 1.07 for young, middle-aged and elderly individuals, respectively. This compares to an average of 6 molecules of vitamin E per LDL particle reported for healthy young individuals [30]. The amount of vitamin E in LDL was depleted by greater than 80% after exposure to 40 Gy of ionizing radiation (Fig. 4). The rate of disappearance of vitamin E was 0.042 ± 0.026 μmol J^{-1} for young and middle-aged subjects and 0.01 ± 0.015 μmol J^{-1} for elderly donors.

At radiation doses greater than 80 Gy, it is interesting to note that the level of vitamin E was undetectably low in LDL from young donors, whereas about 0.1μM remained in LDL from middle-aged and elderly donors. The depletion of vitamin E correlates well with the formation of conjugated dienes and TBARS, as well as with differential fluorescence, observed when LDL is exposed to ionizing radiation (Figs. 1–3). This observation demonstrates the importance of vitamin E as an antioxidant against the free radicals induced peroxidation of lipoproteins.

4. Discussion

An initial step in the mechanism of atherosclerotic damage to blood vessels involves free radical processes leading to LDL oxidation. Oxidation of LDL results in its unregulated uptake by scavenger receptors of macrophages leading to foam cell formation [31]. In addition, oxidatively modified LDL is likely to play an important role in the development and progression of atherosclerosis by various biological actions, such as cytotoxicity, chemotactism for monocytes [5–7,32]. It is well known that the risk and incidence of coronary heart disease increases with age [33]. The aim of the present study was to determine whether there are changes in the level of oxidative damage in serum and LDL as a function of age (young, middle-aged, elderly); thereby, we have assessed the capacity of LDL from these donors to resist free radical induced oxidative damage.

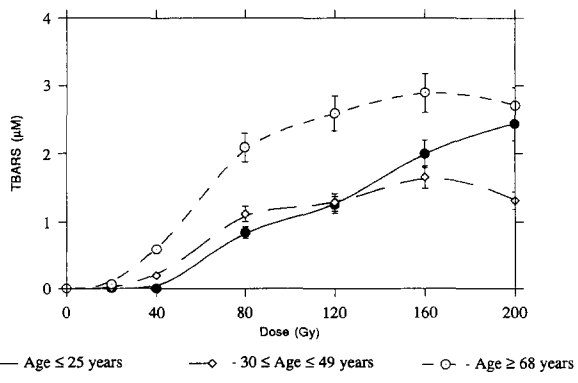

Fig. 3. Differential fluorescence ($\lambda_{emission}$ 440 nm, $\lambda_{excitation}$ 360 nm) for LDL as a function of the radiation dose and age. See notes in Fig. 1.

Our results showed that there was no significant change in the level of oxidative damage (TBARS) in the sera and native LDL of young, middle-aged and elderly donors. However, the level of vitamin E was found to be much lower (4-fold) in LDL from elderly compared to young donors, whereas the level of vitamin E in the sera was higher for the former. These results suggest that the distribution of vitamin E in sera and/or that the ability of LDL to retain vitamin E changes with age. The lower vitamin E in LDL from elderly subjects may be attributed in part to the longer residence time of LDL in circulation [34,35]. On the other hand, the low initial level of vitamin E in LDL of elderly subjects might be related to the greater susceptibility of LDL from elderly subjects to undergo autoxidation during its purification compared to that of young or middle-aged subjects.

To investigate the susceptibility of LDL toward oxidation, LDL from donors of each age group was exposed to ionizing radiation and the formation of oxidative damage was monitored. These results clearly showed that LDL from elderly donors was the most susceptible to oxidative damage, whereas that from the young donors was the least. This is consistent with previous results showing that LDL from patients with hypertension, with diabetes mellitus, with CHD and with hypercholesterolemia have an increased susceptibility to free radicals oxidation [11–17]. Therefore, our results indicate that the enhanced susceptibility of serum LDL to oxidation with age may be an important risk factor of age-associated of atherogenesis. Vitamin E is the most abundant lipid-soluble antioxidant present in LDL (approximately 6 molecules per LDL molecule) [11,36,37]. Since this antioxidant plays a ma-

jor role in protecting LDL against oxidation, the low level of endogenous vitamin E in LDL is probably responsible for the greater susceptibility of LDL to oxidation in elderly donors compared to that in young donors. However the susceptibility of LDL to oxidation from middle-aged subjects was in between that for young and elderly subjects. The initial levels of vitamin E were comparable in these LDL samples while the lag phase of oxidative damage was about twice as long for young subjects as compared to middle-aged subjects (Figs. 1–3). Thus, the observed differences in susceptibility to radiation induced oxidation are likely attributable to subtle changes in structure or composition of LDL [11–13,17].

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