

Age-related increased susceptibility of high-density lipoproteins (HDL) to in vitro oxidation induced by γ -radiolysis of water

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Abstract In the present study, we investigated the age-related susceptibility of high-density lipoproteins (HDL) to oxidation. HDL were obtained from healthy, normolipidemic young, middle-aged and elderly subjects. Oxidation of HDL was induced in vitro by oxygen free radicals generated by water γ -radiolysis, and followed by the decrease of endogenous vitamin E and the formation of conjugated dienes and thiobarbituric acid-reactive substances, as well as the alterations of apolipoproteins A-I/A-II. The resistance of HDL to oxidation, evaluated by the length of the lag phase, decreased with aging. This increased oxidizability of HDL with aging could have a dramatic impact on the development of atherosclerosis in the elderly population.

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Key words: High density lipoprotein; γ -Radiolysis; Oxygen free radical; Aging; Vitamin E; Conjugated diene; Thiobarbituric acid-reactive substance; Apolipoprotein A-I/A-II

1. Introduction

There is increasing evidence that oxidatively modified lipoproteins, particularly low-density lipoproteins (LDL), play an important role in the pathogenesis of atherosclerosis [1,2] and that a relationship exists between the susceptibility of LDL to in vitro oxidation and the atherosclerotic risk [3,4]. However, compared to the large body of data related to oxidized LDL, less is known about the peroxidation of high-density lipoproteins (HDL) and their susceptibility to oxidation in different pathologies [5–7].

In population studies, the risk for an atherosclerotic event is strongly and inversely related to HDL levels [8]. In particular, HDL have been shown to exert a protective effect against coronary heart disease [5,9]. Several lines of evidence have suggested that HDL, which are present in the interstitial space of the artery wall at a much higher concentration than LDL [10], may achieve such a protection role by inhibiting LDL oxidation [2] and cell toxicity, as well as by effluxing free cholesterol from peripheral cells to the liver ('reverse cholesterol transport') [11,12]. It has also been proposed that HDL act as a sink for preformed lipid hydroperoxides either from the plasma or from circulating LDL [9].

Oxidative modifications can occur in HDL as in LDL. Although there is only some scarce indirect evidence available yet for the existence of oxidized HDL in vivo [13], oxidation of HDL has been demonstrated to occur in vitro under different conditions: incubation with human polymorphonuclear

leukocytes [13], UV irradiation [14], interaction with transition metal ions Fe^{2+} , Mn^{2+} , Cu^{2+} [15,16] or exposure to oxygen free radicals generated in solution by water γ -radiolysis [17,18]. In in vitro studies, Nagano et al. [19] also reported that Cu^{2+} -induced oxidation of HDL resulted in denaturation of apolipoprotein A-I (apoA-I), the major apolipoprotein of HDL, and increased their net negative surface charge. Other common oxidative modifications of HDL were observed such as an increase in peroxidized lipids and the appearance of apoA-I oligomers [20].

A strong variation in the length of the oxidation 'lag phase' and therefore in the resistance of LDL and HDL against oxidative modification has been demonstrated. A shorter lag phase was found for HDL, indicating a higher susceptibility of HDL to oxidation when compared to LDL [21–23]. This higher susceptibility was explained by the lower content of endogenous vitamin E in HDL [24,25]. However, this phenomenon has not been clearly elucidated and it was suggested that vitamin E-independent variables may also contribute to this increased susceptibility of HDL to oxidation [21,26].

The oxidative modifications of HDL can reduce their ability to function in reverse cholesterol transport [15,17,19,27], one of the mechanisms through which HDL are believed to protect against the development of atherosclerosis by preventing the accumulation of unesterified cholesterol in macrophages [28,29]. Moreover, recent work has shown that oxidized HDL can lose their capacity to protect LDL against in vitro oxidation and that this loss of protective effect may be due, in part, to a concomitant decrease in the activity of certain HDL-associated enzymes such as paraoxonase or platelet-activating factor acetylhydrolase [9,30]. HDL oxidation, if occurring in vivo, would thus generate modified lipoproteins that could contribute to the progress of atherosclerosis [5,20,31]. Within the oxidative theory of atherosclerosis, HDL might thus be considered Janus-faced, with either antiatherogenic (in their native form) or proatherogenic (when oxidatively modified) properties, depending on their oxidative status [30,31].

It is now established that the incidence of cardiovascular diseases due to atherosclerosis increases with aging [32]. In our previous studies [26], we have demonstrated that LDL from elderly subjects are more prone to oxidation when exposed to oxygen free radicals (OH/O_2^-) generated in solution by γ -irradiation. This radiolytic oxidation of LDL induced lipid peroxidation and led to the consumption of vitamin E and carotenoids, the major classes of endogenous antioxidants found in these lipoproteins [26]. The aim of the present study was to establish, as for LDL and under identical in vitro conditions, whether the susceptibility of HDL to oxidation is increased with aging. The use of water γ -radiolysis allowed an accurate estimation of the nature and quantity of free radicals able to react with the HDL, unlike the commonly

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used techniques of incubation with cells or in presence of transition metal ions [17,18,21].

2. Materials and methods

2.1. Reagents

Acetic acid, sulfuric acid, *n*-butanol, sodium phosphate, thiobarbituric acid, methanol and hexane were purchased from Fisher (Montreal, Qué., Canada), 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. (Houston, TX, USA).

2.2. Subjects

Sera were obtained from 18 healthy normolipidemic male subjects of various ages after an overnight fast. In each age group, six subjects were analyzed independently: six young subjects, age 20–25 years; six middle-aged subjects, age 30–48 years; and six aged subjects, age 68–85 years. They were all in general good health, without symptoms and signs of any arterial diseases established by a complete and negative clinical examination and a normal 12-lead ECG according to the World Health Organization (WHO) criteria [33]. No study subjects had kidney, liver or thyroid diseases. 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 HDL

Isolation of HDL ($1.063 < d < 1.210$ g/ml) was performed according to the method of Sattler et al. [34], using the Beckman Optima TLX ultracentrifuge equipped with a TLA-100.4 rotor, in the presence of ethylenediaminetetraacetic acid (EDTA) (0.4 mg/ml) as already described [22]. After separation, HDL were dialyzed overnight at 4°C against a 10^{-2} M sodium phosphate buffer (pH 7). HDL concentrations are expressed in terms of total protein concentrations. Prior to irradiation, the dialyzed HDL solutions were adjusted to a concentration of 100 μ g protein/ml by dilution in the same buffer. Proteins were measured by commercial assay (Pierce method, Rockford, IL, USA).

2.4. HDL oxidation by gamma radiolysis of water

Oxygen free radical species were generated by irradiation of aqueous solutions of HDL using a ^{60}Co Gamma cell 220 (Atomic Energy of Canada Ltd.) at a dose rate of 0.18 Gy/s as determined with the Fricke (ferrous sulfate) dosimeter [35,36]. Irradiations were performed at room temperature as previously described [18]. In brief, samples (2 ml) of HDL solutions containing 10^{-2} M sodium phosphate buffer (pH 7), saturated with oxygen, were exposed to γ -radiation. Under these conditions, the main free radical species produced selectively and simultaneously were hydroxyl ($\cdot\text{OH}$) and superoxide anion ($\text{O}_2^{\cdot-}$) radicals with yields of 2.8×10^{-7} and 3.4×10^{-7} mol/J, respectively [36]. Hydrogen peroxide (H_2O_2), a species also produced in water γ -radiolysis with a yield of about 0.7×10^{-7} mol/J [36], was not considered in this study, in view of previous results showing that this product had no effect on the oxidation of LDL under similar experimental conditions [37]. The total radiation doses were varied from 0 to 200 Gy.

2.5. Measurement of conjugated dienes, thiobarbituric acid-reactive substances, differential fluorescence and vitamin E

Different parameters were used to monitor the progress of HDL oxidation *in vitro*, including the formation of conjugated dienes (CD) and thiobarbituric acid-reactive substances (TBARS), the increase of differential fluorescence emission associated to HDL-bound protein alterations, and the disappearance of endogenous vitamin E (major antioxidant in HDL). The CD (early products of lipid peroxidation) produced were measured by recording differential absorbance spectra of irradiated and control HDL between 200 and 700 nm using a Hitachi U-300 spectrophotometer as described [26]. An increase in the differential absorbance at 234 nm constituted evidence for CD formation [38]. TBARS were determined as end-products of lipid peroxidation by the spectrofluorimetric method of Yagi [39], but without precipitation by phosphotungstic acid, using 1,1,3,3-tetraethoxypropane as a standard. The concentration of TBARS produced was calculated as the difference between the concentration of TBARS in

the irradiated samples and that in the non-irradiated controls [26]. Differential fluorescence emission spectra of oxidized and control HDL were recorded between 380 and 520 nm (excitation at 360 nm), using a Hitachi U-4500 spectrofluorimeter as previously described [26]. Endogenous vitamin E was assayed as α -tocopherol before and after irradiation by reverse-phase HPLC, with spectrophotometric detection at 292 nm as already described [17,40,41].

Radiolytic yields for the disappearance of vitamin E [$G(-\text{vit. E})$], calculated from the initial slopes of the curves representing the concentration of vitamin E as a function of the radiation dose] and for the formation of TBARS [$G(\text{TBARS})$], determined from the maximal slopes of the curves representing the concentration of TBARS as a function of the radiation dose] were expressed in mol of vitamin E lost and of TBARS formed per unit of energy (J) absorbed, respectively [36]. These yields reflected the rates of disappearance of vitamin E and of TBARS formation, as a result of the action of oxygen free radicals generated at steady-state concentrations and hence at a constant rate by γ -radiolysis of aqueous HDL solutions [18].

2.6. Statistical analysis

Results are presented, for each age group, as pooled data from six independent experiments performed in duplicate (mean \pm S.D.). Mean values were compared using the Student's *t*-test, for detection of significant differences. A *P*-value less than 0.05 was considered significant.

3. Results

Fig. 1 shows the formation of CD (differential absorbance at 234 nm) after exposure of HDL solutions to radiolytically induced $\cdot\text{OH}/\text{O}_2^{\cdot-}$ free radicals as a function of radiation dose and age. For young and middle-aged subjects, the CD curves obtained showed early resistance against oxidation, judging from the initial inhibited phase (or 'lag phase', expressed as the radiation dose below which little or no CD formation was detectable) preceding the phase of rapid CD formation ('propagation phase'). Although the CD curve for elderly subjects displayed essentially the same characteristic oxidation profile,

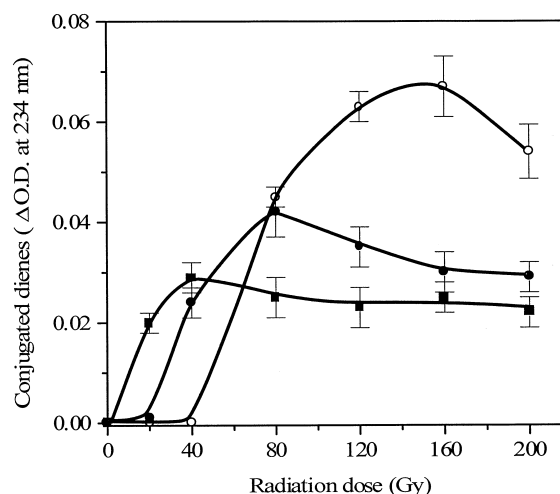


Fig. 1. Conjugated diene formation, followed by differential absorbance at 234 nm ($\epsilon_{234\text{nm}} = 27000 \text{ M}^{-1} \text{ cm}^{-1}$), in HDL exposed to $\cdot\text{OH}/\text{O}_2^{\cdot-}$ free radicals generated by water γ -radiolysis (dose rate = 0.18 Gy/s) as a function of radiation dose and age. The concentration of HDL was 100 μ g protein/ml in O_2 -saturated aqueous solutions containing 10^{-2} M sodium phosphate buffer at pH 7. The radiation doses absorbed by the solutions were obtained by multiplying the dose rate by the irradiation times. Symbols are as follows: \circ , young subjects, age 20–25 years; \bullet , middle-aged subjects, age 30–48 years; \blacksquare , aged subjects, 68–85 years. Each value is expressed as mean \pm S.D. (see text).

Table 1

Comparative results of endogenous vitamin E consumption and TBARS formation during in vitro oxidation of HDL at a concentration of 100 µg protein/ml (equal to ~ 0.8 µM HDL) with radiolytically generated $\cdot\text{OH}/\text{O}_2\cdot$ free radicals, for the three age groups considered

Age (years)	$G(-\text{vit. E})^a$ ($\times 10^{-7}$ mol/J)	$G(\text{TBARS})^a$ ($\times 10^{-7}$ mol/J)	[vit. E]/[HDL particle] ^b	[TBARS]/[HDL particle] ^c	$G(\text{TBARS})/G(-\text{vit. E})^d$
20–25	0.35	0.45	1.40	2.0	1.29
30–48	0.24	0.25	1.29	1.01	1.04
68–85	0.023	0.10	0.44	0.89	4.35

^aRadiolytic yields of disappearance of HDL endogenous vitamin E, $G(-\text{vit. E})$, and of TBARS formation, $G(\text{TBARS})$, expressed in mol of vitamin E lost and TBARS formed per unit of energy (J) absorbed in the solutions, respectively (see text).

^bVitamin E content of freshly isolated (unirradiated) HDL, expressed as the number of endogenous vitamin E molecules per HDL particle.

^cMaximum amount of TBARS produced per HDL particle, representing the number of oxidation target sites per HDL particle.

^dIndex of HDL oxidation efficiency.

no such early resistance could be observed in HDL isolated from this age group, where the lag phase was approximately 0 Gy (Fig. 1). For each age group, the CD produced increased to a maximum as a function of radiation dose, and then decreased at higher doses of radiation ('decomposition phase'). At the highest radiation doses supplied, a plateau was reached for middle-aged and elderly subjects, while it was still not observed at 200 Gy for young donors (Fig. 1).

The production of TBARS (mainly malondialdehyde) from $\cdot\text{OH}/\text{O}_2\cdot$ free radical-exposed HDL as a function of radiation dose and age is shown in Fig. 2. Similar to CD formation, the TBARS curves also displayed a characteristic triphasic oxidation profile. However, the initial lag phase could only be obtained for HDL from young subjects. In the case of HDL isolated from middle-aged and elderly donors, the formation of TBARS started at very low radiation doses, practically without lag phase (Fig. 2). When compared to our previous results on LDL oxidation under similar experimental conditions [26], HDL seemed to be more easily oxidized by $\cdot\text{OH}/\text{O}_2\cdot$ free radicals or, in other words, less resistant to oxidation. Our findings are in agreement with the data reported by Babiy et al. [21].

As seen in Figs. 1 and 2, the value of the plateau observed at high radiation doses in the formation of CD and TBARS in HDL from the three age groups decreased with aging. Similarly, the maximum value of the CD and TBARS curves was also found to decrease when the age of the subjects increased.

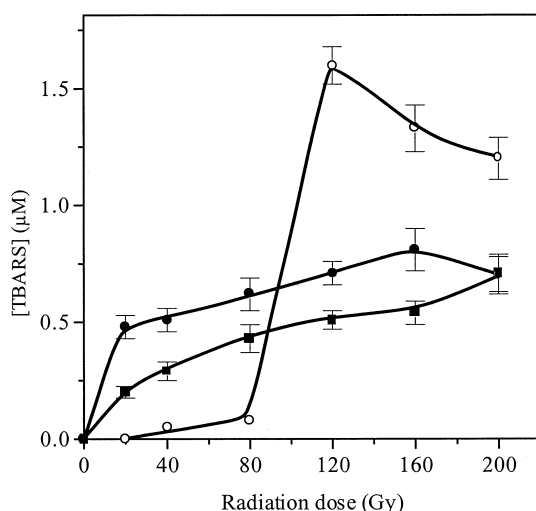


Fig. 2. TBARS formation in $\cdot\text{OH}/\text{O}_2\cdot$ free radical-exposed HDL as a function of radiation dose and age. Conditions were as described in Fig. 1.

Comparing with our previous age-related LDL oxidation studies [26], it is worth noting that the CD and TBARS maxima observed in HDL from elderly subjects were more than three-fold smaller than those found in LDL from the same age group under similar oxidation conditions. Such a decrease, compared to LDL [26], was also observed for the other age groups.

Results of measurements of the endogenous vitamin E content of $\cdot\text{OH}/\text{O}_2\cdot$ free radical-exposed HDL as a function of radiation dose and age are given in Fig. 3. The amount of vitamin E present in freshly isolated (unirradiated) HDL was significantly higher for young (1.12 ± 0.11 µM) and middle-aged (1.03 ± 0.10 µM) subjects than for elderly (0.35 ± 0.04 µM) donors. Adopting a mean HDL molecular weight of 250 kDa [42,43] and assuming the HDL-bound apoproteins to represent $\sim 50\%$ of the weight of HDL [22,42,44], the number of endogenous vitamin E molecules per HDL particle can be calculated to be 1.40, 1.29 and 0.44 for young, middle-aged and elderly individuals, respectively (Table 1). These values compare well to those recently reported by Suzukawa et al. (1.92 ± 0.38 , for donors age 28.8 ± 6.6 years) [45] and by Laureaux et al. (0.58, average for pooled HDL) [18,43]. As seen in Fig. 3, there was a rapid loss of vitamin E content in HDL from young and middle-aged subjects with increasing radiation dose. The amount of vitamin E initially present decreased by more than 70% after exposure to a dose of 40 Gy, and completely disappeared in HDL from young do-

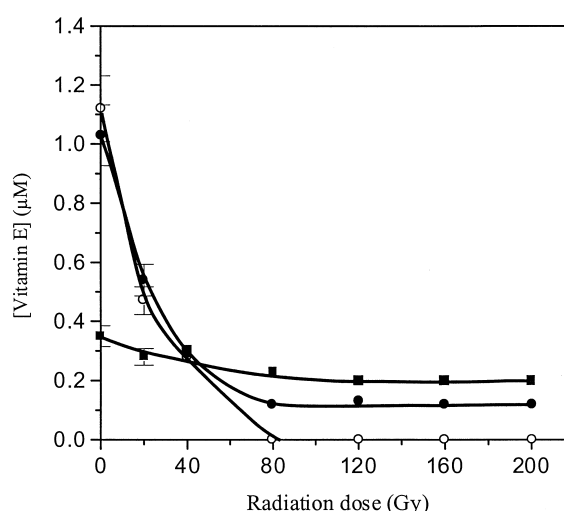


Fig. 3. Evolution of the amount of endogenous vitamin E in HDL during oxidation by $\cdot\text{OH}/\text{O}_2\cdot$ free radicals as a function of radiation dose and age. Conditions were as described in Fig. 1.

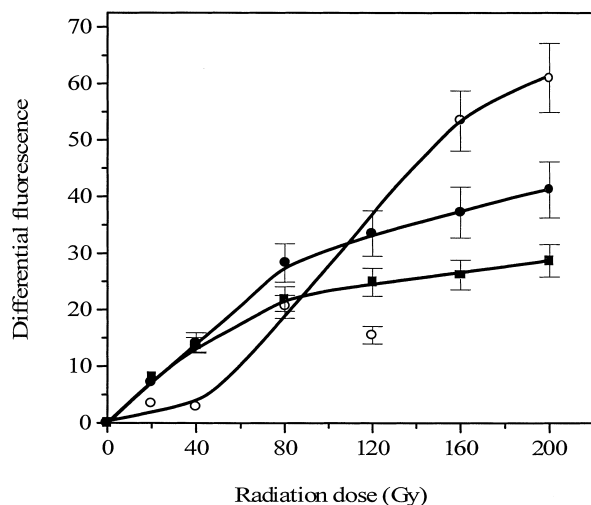


Fig. 4. Protein alterations in HDL exposed to $^{\bullet}\text{OH}/\text{O}_2^{\bullet-}$ free radicals, measured by the differential fluorescence emission at 440 nm (with excitation wavelength at 360 nm) as a function of radiation dose and age. Conditions were as described in Fig. 1.

nors above about 80 Gy. In contrast to these two age groups, only a very slow decrease of vitamin E content was observed in HDL from elderly subjects with increasing radiation dose.

As shown in Figs. 1 and 2, the lag-phase lengths for CD and TBARS formations decreased with increasing age of the donors. For irradiated HDL from young and middle-aged subjects, it is relevant to note that the lag phases occurred following the depletion of substantial amounts of the initial HDL vitamin E contents (Fig. 3). For these two age groups, such a depletion of endogenous vitamin E correlated significantly with CD and TBARS productions (Figs. 1 and 2). In HDL obtained from elderly subjects, however, the formation of CD and TBARS did not correlate with the disappearance of vitamin E.

Alterations induced to the HDL protein moiety during oxidation were measured by differential fluorescence emission at 440 nm ($\lambda_{\text{exc}} = 360$ nm) as a function of radiation dose and age (Fig. 4). The results show that the appearance of fluorescence in $^{\bullet}\text{OH}/\text{O}_2^{\bullet-}$ free radical-exposed HDL occurred with essentially no initial lag phase, for the three age groups studied. These HDL protein alterations were, however, markedly reduced at radiation doses below 100 Gy for HDL isolated from young subjects, in correlation with the vitamin E consumption observed for this age group in the same dose range. As for the CD and TBARS formations, at the highest radiation doses supplied, the fluorescence of irradiated HDL seemed to reach a plateau, whose value was inversely correlated with the age of the subjects (Fig. 4).

4. Discussion

Human HDL are well known for their protective effect against the development of the atherosclerotic process, because of their ability to promote cholesterol efflux from cells [11,12,46] and to inhibit oxidative modification of LDL [2,38,47,48]. However, compared to LDL, less is known about the peroxidation of HDL and their susceptibility to oxidation in different pathologies.

We have recently demonstrated that the susceptibility of

LDL to oxidation was increased with aging [26] and that the decreased levels of endogenous vitamin E in LDL from elderly subjects were largely responsible for this greater oxidation susceptibility of LDL. The present study was the first, to our knowledge, to investigate the *in vitro* oxidizability of human HDL as a function of age. Our findings demonstrate that HDL from elderly donors are more susceptible to oxidation, while less oxidizable, than HDL from young and middle-aged subjects.

Under our experimental conditions (O_2 -saturated solutions), it can be implied from previous studies [18] that all radiolytically generated $^{\bullet}\text{OH}$ and $\text{O}_2^{\bullet-}$ free radicals simultaneously react with the different molecular HDL components, thereby leading to oxidation of their lipid (CD and TBARS formations) and protein (fluorescence appearance) moieties, and to the disappearance of endogenous vitamin E. As seen in Fig. 1, CD formation curves showed a lag phase only for HDL from young and middle-aged subjects. This lag phase can be partly explained by the protection provided by the HDL endogenous antioxidant (vitamin E) content, which concomitantly declined in the process (Fig. 3) [49]. For elderly donors, endogenous vitamin E levels in native HDL were about three-fold lower than those in native HDL from young and middle-aged subjects. Nevertheless, at radiation doses above about 80 Gy, the amount of vitamin E initially present completely disappeared in HDL from young subjects, while on the contrary, $\sim 65\%$ still remained in HDL from elderly subjects. As shown in Fig. 3, the rate of vitamin E loss in HDL dropped when its level decreased. This is in agreement with the observations of Babiy et al. [21] showing that the higher the level of vitamin E the higher its rate of consumption, as well as with the work of Laureaux et al. [43] using α -tocopherol-enriched HDL.

The maximum value of CD and TBARS formed was found to be the highest in HDL from young subjects, when compared to the other two age groups (Figs. 1 and 2). These high amounts of CD and TBARS may explain the complete depletion of endogenous vitamin E in HDL from young donors above about 80 Gy (Fig. 3). In this respect, vitamin E seems to be more used, as the first line of antioxidant defense, to break the free radical-induced lipid peroxidation chain in HDL from young subjects than in HDL from elderly subjects. According to Sato et al. [50], the length (or the efficiency) of the peroxidation chain could be evaluated by the ratio $G(\text{TBARS})/G(-\text{vit. E})$. In the case of HDL from elderly subjects, this ratio is indeed higher (4.35) than those in HDL from young (1.29) or middle-aged (1.04) subjects (Table 1). A longer peroxidation chain length thus consistently characterizes the kinetic mechanism for the oxidation of HDL from elderly subjects as compared to that of HDL from the young or middle-aged donor groups.

However, at identical endogenous vitamin E concentrations, corresponding to the irradiation dose of ~ 40 Gy (Fig. 3), the formation of CD in HDL from young subjects was still inhibited (lag phase), whereas it was about 50% of its maximum, or at its maximum level, for middle-aged and elderly subjects, respectively (Fig. 1). Such variations suggest that other, vitamin E-independent variables may also contribute to explain the $^{\bullet}\text{OH}/\text{O}_2^{\bullet-}$ free radical-induced oxidative modifications of HDL [21,26,51]. Factors conferring enhanced susceptibility of HDL to oxidation with aging may be complex, including the fatty acid composition, antioxidant (other

than vitamin E) content, hydroperoxide level, apoA-I/A-II protein content, HDL particle size and the HDL₂/HDL₃ sub-fraction ratio. A study by Frey et al. [52] demonstrated, in this respect, significant changes in the composition of HDL₂ sub-fractions in men with aging, especially a reduction in the phospholipid content, an increase in protein and a decrease in the apoA-I/A-II ratio. These observations might contribute to explain the differences in the susceptibility of HDL to oxidation with aging shown in our conditions.

As shown in Figs. 1 and 2, the CD and TBARS maxima decreased when the age of the subjects increased. HDL is a macromolecular complex with many 'potential targets' for free-radical attacks and when a plateau and/or a maximum formation of lipid peroxidation products is reached, this would signify that all targets have been oxidized [53]. The maximum of targets can be estimated as the number of TBARS molecules per HDL particle. These values are 2, 1.01 and 0.89 for young, middle-aged and elderly subjects, respectively (Table 1), with a mean value for all three age groups of 1.3, as has been already demonstrated for pooled HDL [53]. Hence, these results strongly suggest that the number of targets on HDL decreases with aging. In the oxidative theory of atherosclerosis based on the reciprocal protection of LDL and HDL [47], reduction in the number of potentially oxidizable targets on the HDL may direct radical attacks onto LDL, thus contributing indirectly to LDL oxidation and in turn to the enhancement of the incidence of cardiovascular diseases with aging.

In our experimental conditions, radiolytically generated $\cdot\text{OH}/\text{O}_2\cdot$ free radicals also induced alterations in the protein moiety of HDL (Fig. 4). The extent of such alterations at high radiation doses decreased with the age of the donors to reach a plateau and the curves bear a similarity to CD and TBARS curves (Figs. 1 and 2). In fact, oxidative modifications affecting the HDL protein moiety resulted from the derivatization of lipid peroxidation products with amino groups of apolipoprotein A.

Our results showed that there is an increased susceptibility of HDL to oxidation as well as a decrease in their oxidizability with aging. Vitamin E seems to act as a lipid peroxidation chain-breaking molecule in HDL, but due to its reduced level in HDL compared to LDL, vitamin E may be, at least in part, responsible for the reduction of the resistance of HDL to oxidation, even more in HDL from elderly subjects. However, other factors might contribute to these observed alterations in HDL oxidation with aging, including the frequency of radical collision due to the fluidity of the lipid environment depending on the fatty acid profile, the levels of peroxidizable substrates, the concentration of antioxidants, the amounts of hydroperoxides and the apoA-I/apoA-II density ratio. We are currently investigating the role of these various factors in the alterations of HDL oxidation susceptibility and oxidizability with aging. These modifications could be important in the increased development of atherosclerosis with aging as these HDL might be unable to perform their protective role as the reverse cholesterol transport and the protection of LDL from oxidation.

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