EARLY PATHOLOGICAL CHANGES OF ENDOTHELIA IN A MODEL USING LDL PERFUSION AT PHYSIOLOGICAL LDL-CHOLESTEROL CONCENTRATION

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The aim of the present research was to provide further insight into the debated problem of the existence of modified LDL in vivo. For this purpose a novel model was devised for studying LDL injurious effect on endothelial cells (EC) by infusing native cholesterol rich LDL, diluted to physiological LDL cholesterol concentration. Normal rabbits were infused with LDL separated from rabbits previously fed either with standard food (I-LDL Group), 1% cholesterol (II-LDL Group) or 1% cholesterol plus probucol (IV-LDL Group). Cu++ modified II-LDL was infused as well (III-LDL Group). After dilution as above, lipid oxide (LP) significantly increased in III- and II-LDL media, as compared to I- and IV-LDL media. EC of III- and II-LDL Groups showed irregular shape and surface pattern. Further, they showed adhering clusters of monocytes, platelets and erythrocytes. Endocytic vesicles and ruthenium red-positive particles increased too. EC of IV-LDL Group were only slightly affected as compared to I-LDL Group.

These data suggest that native LDL from hypercholesterolemic rabbits contain an oxidized form which is noxious to EC even when LDL is infused at physiological LDL-cholesterol concentration. This early injury is in part LP-associated and actively involves platelets and monocytes.

Introduction

It has been shown in various experimental animal models that by feeding them with high cholesterol food, there occurs damage to endothelial cells (EC) of arterial walls (1). Recently, attention has been paid to modified LDL rather than serum cholesterol as such as an important pathogenetic mechanism inducing atherosclerotic changes. We have recently devised a new method to study EC injury by infusing rabbits with LDL obtained from animals which had been fed with different diets (2,3). The purpose of this research was to ascertain if cholesterol-rich LDL, although diluted to physiological LDL-cholesterol concentration, shares some injurious effects with oxidatively modified LDL, thus supporting the hypothesis of the existence of a modified form of LDL in vivo.

Materials

Seventy-three month old New Zealand White rabbits, weighing around 3kg, were used in the study. Serum LDL were extracted from rabbits after different five-week dietary regimens (Oriental Labochow Co.,Japan) which were free of oxidized products, as ascertained by prior assays, as well as of antioxidants: I,was given standard food (no. 40); II, 1% cholesterol food (no. 5); III, LDL extracted from II was modified by Cu++ as described below; IV was given 1% cholesterol food plus 1% Probucol (no. 5). LDL extracted after each of these diets and diluted,as described below, was infused in healthy rabbits which were named as I-, II-, III- and IV-LDL Group, respectively.

Methods

Preparation of LDL. Following overnight fasting, blood samples were drawn from the femoral artery into syringes containing sodium citrate 3.8% w/vol. LDL was extracted by sequential ultracentrifugation according to the methodof Havel (4) and it was extensively dialyzed with three changes of saline (0.15 M NaCl), pH 7.4. For oxidation, LDL was collected into Visking seamless cellulose tubes and modified by dialysis against saline containing 3.5 uM Cu++ for 24 to 120 hr at room temperature, according to Steinbrecher, et al. (5). After dialysis, the contents of the tubes were centrifuged at 400 xg for 20 min. and the supernatants were used. Lipid peroxide (LP) of LDL media was measured by El-Saadani's method (6).

Lipoprotein perfusion. LDL perfusion was done as originally devised by Chui (2,3). I-, II-, III- and IV-LDL fractions were dialyzed with saline over a 24 hr period at 4°C. LDL obtained as above was diluted with physiological saline to LDL-cholesterol concentration of 53mg/dl (within normal range in rabbits) which represented the final LDL media. Healthy rabbits, anesthetized with 0.25mg/kg Nembutal, had their external carotid artery exposed. A venular cannula was connected to a bottle with 120mm Hg pressure and inserted into the external carotid artery as close as possible to the inlet of the internal carotid artery. Through this cannula, 400ml of the above media at 37°C were infused at 2.2ml/min. flow rate and 120mm Hg pressure (rabbits systolic pressure 120±15mm Hg) over 3hr (at a time when we had found from preliminary studies that early pathological changes would occur). Care was paid so as not to obstruct the blood flow of the internal carotid artery during the study. The lipids of LDL were extracted as from Bligh and Dyer's method (7) and measured by an enzymatic method (Lipid Enzyme, Eiken Chemicals, Japan).

Pathologic examination. While rabbits were still alive and under anesthesia, 500ml of physiological saline at 37°C were infused at 120mm Hg pressure into

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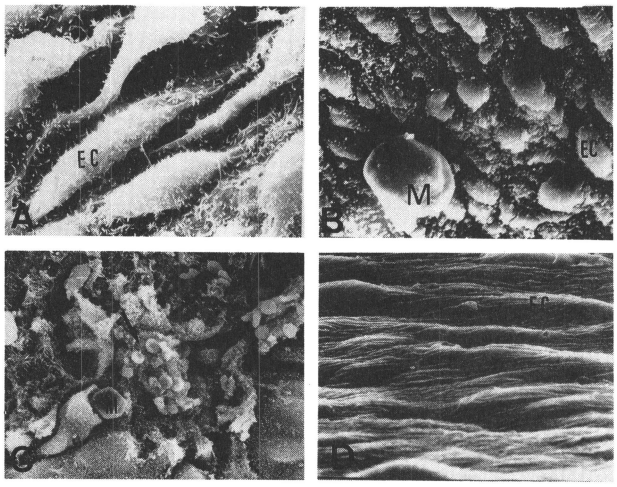

Figure 1. SEM findings of internal carotid artery in normal rabbits after perfusion with LDL from Group I (A ×3500), Group II (B ×4500), Group III (C ×2000), and Group IV (D ×3500). EC, endothelia cell; M, macrophage; arrows, red blood cells and platelets.

the left ventricle soon after ligation of the upper part of the abdominal aorta and the sectioning of the right atrium. Soon after, fixation was performed by infusing 1000ml of 0.05% tannic acid in 0.1 M 2.5% glutaraldehyde (pH 7.4). A 1.0cm segment was sectioned from the internal carotid artery starting from 0.5cm above the conjuction with the external carotid artery. This segment was split lengthwise in two halves which were examined for scanning electron microscopy (SEM) and, after staining with ruthnium red, for transmission electron microscopy (TEM). TEM was carried out only on strips adjacent to the SEM half. SEM and TEM were performed as devised by Takahashi (8).

Results

Lipoprotein analysis. LP (nmol/mg) increased significantly ($p<0.05$) in II-LDL (6.3±0.08) and in III-LDL media (177±13.1), as compared to I-LDL (4.1± 0.06). IV-LDL did not show any significant increase in LP (5.0±0.09) but rather a significant decrease as compared to II- and III-LDL ($p<0.05$).

Pathologic examination. I-LDL Group showed normal spindle-shaped EC running parallel to the blood flow and with normal villi structure and no macrophage (fig.1a). II-LDL Group EC looked round and oval shaped. Most of them were laying flat and presented several folds on their surface with adherent platelets (fig.1b). Monocytes in this group showed adhering clusters of platelets and erythrocytes (RBC) (fig.2a). EC of III-LDL Group showed a more severe degree of the previous findings with plenty of grossly abnormally shaped monocytes bearing blood elements on their surface (fig.1c). As compared to I-LDL Group, EC of IV-LDL Group showed only slight changes consisting in a tendency to look round and oval shaped and no blood element was found attached (fig.1d). The rarely observed monocytes showed a regular surface (fig.2b). EC structure in I-LDL Group appeared well preserved and their endocytic vesicles (EV) showed rare ruthenium red-positive (RRP) particles (fig.3a). EC of II-LDL Group had a very irregular surface with clusters of platelets and showed

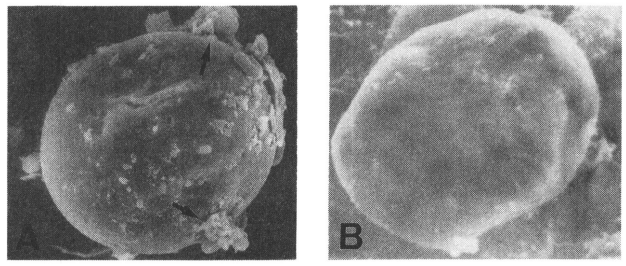

Figure 2. Monocyte from II-LDL Group (A × 12,000) and from IV-LDL Group (B ×12,000), as observed by SEM. Arrows, platelets and red blood cells.

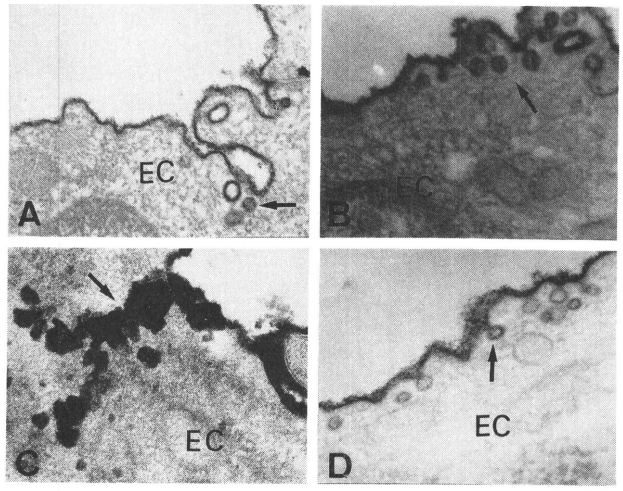

Figure 3. TEM findings of internal carotid artery in normal rabbits after perfusion of LDL from Group I (A, ×28,000), Group II (B × 28,000), Group III (C, × 28,000), and Group IV (D, × 28,000). EC, endothelial cell; arrows, RRP particles.

under their luminal side an increased number of EV with RRP granules (fig.3b). III-LDL showed a more pronounced degree of the above changes. EV were present also down to the basement membrane and were crowded with RRP granules (fig.3c). IV-LDL Group showed only a slight increase of EV which contained rare RRP bodies (fig.3d). Tight junctions were not affected in any of the groups studied.

Discussion

The evidence of the contribution of oxidative modification of LDL to the atherogenic process has been elucidated (9). Steinbrecher et al.(5) have recently reported that the LDL oxidative modification by Cu++ resembled that one induced by cultured EC. Therefore, we used such a method in our study.

Perfusion of modified LDL confirmed to cause clear pathological changes of EC with adhesion of platelets, RBC and monocytes. A recent observation has shown that hypercholesterolemia alone elicits monocytes adhesion and EC disturbance in the absence of injury and desquamation (10). Interestingly, even after dilution of native II-LDL to physiological LDL-cholesterol concentration, the EC of healthy rabbits perfused with such a mediumshowed adhesion of platelets and monocytes, the latter bearing on their surface clusters of platelets and RBC. These data are

strengthened by our recent finding of PAF acetylhydrolase activation and consequent platelet aggregation by employing the same model (11).

The EV of this group clearly increased in number and contained a large amount of RRP particles. Hollander et al.(12) have reported that LDL internalized in the intima are bound by glycosaminoglycans which have been shown to form insolvable complexes with ruthenium-red (13). Therefore, it is likely that the abundant RRP particles in the EC of II-LDL Group can be interpreted as an increased amount of LDL internalized in the EC. The modified LDL medium showed the highest LP level but it was more remarkable that also the native LDL separated from hypercholesterolemic rabbits, although diluted to physiological LDL-cholesterol concentration, still contained a significantly increased LP level as compared to I-LDL medium. Heinecke et al.(14) have shown in vitro that LDL modified by human arterial smooth muscle cells exhibit increased LP level. Conversely, IV-LDL medium contained an LP level comparable to that one of LDL separated from control rabbits and caused only slight EC damage. These findings can be explained by taking into account the recent results of Kuzuya et al. (15) who have highlighted the role of probucol in preventing oxidative injury to EC. Furthermore, by using clinical samples, Kita et al.(16) have shown in vitro that LDL separated from hypercholesterolemic patients treated withprobucol were not oxidized by Cu++ and had a lower level of LP as compared to non-treated patients.

Accordingly, Carew et al.(17) have recently provided the evidence in vivo that the anti-oxidant property of probucol, unrelated to its hypocholesterolemic effect, is capable of reducing the development of fatty streak lesions in hyperlipidemic rabbits. These findings suggest the conclusive hypothesis that LDL in hypercholesterolemic rabbits contain an oxidized form which is responsible for inducing EC disturbance leading to higher LDL internalization into EC even when infused at physiological LDL-cholesterol concentration.

One mechanism of such injury is likely to be accounted for by an LP-associated cyto-toxicity which takes place at a very early stage, together with active platelet involvement, as recently suggested (18) and with the participation of monocytes as well.

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