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ENHANCEMENT OF CHOLESTEROL ESTERIFICATION IN AORTIC SMOOTH MUSCLE CELLS BY MEDIUM OF MACROPHAGES CONDITIONED WITH ACETYLATED LDL

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1. Introduction

Deposition of cholesteryl ester in the arterial wall is a well-established hallmark of atheroma. The accumulating lipid originates from plasma lipoproteins and from in situ synthesis. Cultured aortic smooth muscle cells (SMC) served as a model system for the in vitro study of atheroma [1] and modulation of cellular cholesteryl ester deposition in and mobilization from the lysosomal [2,3] and cytoplasmic [4] compartments has been described. Recent findings of massive depositions of cholesteryl ester in cultured macrophages, exposed to modified low density lipoproteins [5], have again directed attention to the possible role of these cells in atheroma. This research was designed to learn whether an interaction between modified low density lipoproteins and macrophages will affect not only cholesteryl ester metabolism in these cells, but will induce also cholesterol esterification in SMC. Such a finding would have some implications on the formation of atheroma.

2. Materials and methods

Peritoneal macrophages were obtained from male albino mice following intraperitoneal injection of phosphate-buffered saline (PBS) [6]. The cells were pelleted and washed by resuspension in Dulbecco-Vogt medium containing 10% new-born calf serum. The cell suspension was plated to give 4×10^6 cells/60 mm petri dish, assuming that the level of macrophages in the peritoneal fluid was ~30%. After 2 h pre-plating, the medium and non-attached cells were removed, the cell layer was washed 3 times with phosphate-buffered saline and the cells were incubated for 24–48 h in medium containing 10% new-born calf serum. 100% of attached cells phagocytosed latex particles.

Rat and bovine aortic SMC were cultured as in [2]. Aortae were removed from 3-month-old male rats under ether anaesthesia; bovine aortae were obtained from the abattoir. The aortae were dissected and medial explants were prepared [7]. The cells were cultured in modified Dulbecco-Vogt medium containing 5% fetal calf serum and 5% new-born calf serum. Prior to each experiment, the aortic SMC were trypsinized and seeded in 30 mm petri dishes at 7×10^4 cells/dish. The medium used consisted of Dulbecco-Vogt medium supplemented with 10% FCS to which 7α-N-[^{3}H]cholesterol was added [8] to give 1 μCi/ml medium. After 48 h incubation in the presence of the labeled medium, the medium was removed. The cell layer was washed 3 times with PBS and the cells were post-incubated for 24 h or 48 h with the various media.

Bovine endothelial cells were obtained from the aortae used to prepare medial SMC and during the first passages were cultured in the presence of fibroblast growth factor [9]. Human low density lipoproteins (LDL) were isolated by centrifugation as in [10] at d = 1.019–1.063 from serum containing 1 mg EDTA/ml. Acetylation of LDL was carried out with acetic anhydride [11]. [1-^{14}C]Oleic acid was converted to sodium salt and complexed to bovine serum albumin [12].

The peritoneal macrophages were incubated with acetylated LDL or native LDL 25 μg protein/ml added to the complete culture medium on day 1 or 2 after plating. Following 48–72 h the medium was collected under sterile conditions, centrifuged at 2000 rev./min for 10 min and used for incubation

with the pre-labeled SMC. As controls, served media collected from macrophages incubated in the absence of lipoproteins, to which LDL or acetylated LDL were added at the onset of post-incubation to give 25 μg protein/ml. Another control was non-preincubated medium, supplemented with LDL or acetylated LDL. To terminate the experiment, the medium was collected, the cell layer was washed 3 times with 0.2% albumin and 3 times with PBS and the cells were scraped with a Teflon policeman into methanol [3]. Following addition of chloroform and campesterol, the lipids were extracted and purified according to [13] and aliquots of the chloroform phase were used for the determination of radioactivity. The % of labeled cholesterol or oleic acid recovered as cholesteryl ester was determined by thin-layer chromatography [14]. Protein was determined on the delipidated cell pellet according to [15]. All radioactive materials were obtained from the Radiochemical Centre, Amersham.

3. Results

The data presented in table 1 are from a representative experiment in which the effect of post-incubation of ^{3}H-labeled SMC in macrophage medium, conditioned in presence of acetylated LDL, is compared to post-incubation with various other media. It can be seen that after 48 h post-incubation the labeled cholesterol which had been present in the cells prior to post-incubation (A) had become distributed between the cells and the post-incubation medium (B–H). While labeled cholesterol in the medium was only in unesterified form, the labeled cellular cholesterol was partly esterified. The amount of labeled esterified cholesterol increased markedly above that present at zero time (A) when the medium used had been derived from incubation of macrophages with acetylated LDL (B). The amount of labeled cholesteryl ester obtained in (B) was ~3-fold that shown in (C) in which the acetylated LDL had been added to macrophage conditioned medium only during post-incubation with the labeled SMC. Addition of acetylated LDL to non-preincubated medium (F) resulted in a lower amount of labeled cholesteryl ester, which was similar to that observed when post-incubation was carried out without acetylated LDL (H). Preincubation of macrophages with LDL (D) or addition of LDL to macrophage conditioned medium (E) or to non-preincubated medium (G) resulted in a higher amount of labeled cholesteryl ester than that

Table 1
Effect of medium preincubated with macrophages and acetylated LDL on [^{3}H]cholesterol ester content of bovine smooth muscle cells

Postincubation of SMC in medium preincubated in presence of:	Added to medium after preincubation	^{3}H Label after 48 h incubation in:		
		Medium	Cells	
		Total ($\times 10^{-3}$)	Total ($\times 10^{-3}$)	CE ($\times 10^{-2}$)
(A) Zero time	–	–	260 ± 6	120 ± 4
(B) Macrophages + AcLDL	–	186 ± 3	91 ± 1	295 ± 8
(C) Macrophages	AcLDL	174 ± 7	83 ± 2	115 ± 3
(D) Macrophages + LDL	–	179 ± 5	80 ± 1	148 ± 6
(E) Macrophages	LDL	176 ± 4	79 ± 7	133 ± 16
(F) Non-preincubated medium	AcLDL	192 ± 3	70 ± 2	83 ± 3
(G) Non-preincubated medium	LDL	189 ± 2	71 ± 4	104 ± 8
(H) Non-preincubated medium	–	186 ± 3	74 ± 2	77 ± 6

Conditions: Mouse peritoneal macrophages were cultured in medium containing 10% new-born calf serum. 24 h after plating acetylated LDL (AcLDL) or LDL (25 μg protein/ml) were added to fresh medium and the incubation was carried out for 72 h. The medium was collected and centrifuged for 10 min at 2000 rev./min. Media derived from incubation of macrophages without lipoproteins were supplemented with AcLDL or LDL to give 25 μg protein/ml final conc. These media, as well as non-preincubated medium, were used for post-incubation with smooth muscle cells (SMC). SMC were plated on day 0 at 7×10^{4}/30 mm petri dish in medium containing 10% serum and [^{3}H] cholesterol. After 48 h labeling, the medium was removed, the cell layer washed thrice with PBS and post-incubated in the presence of the indicated media. After 48 h the medium was collected, the cell layer washed and extracted as in section 2. Following lipid extraction the amount of label recovered in cholesteryl ester (CE) was determined by thin-layer chromatography. Zero time = petri dishes terminated prior to post-incubation. Values are ±SE of triplicate dishes

encountered with non-preincubated medium (H). In 5 out of 6 expt, the values of (D), (E) and (G) were lower than the value for labeled cholesteryl ester seen in (B). This difference between the effect of macrophage conditioned medium with acetylated LDL and with LDL was even more pronounced when rat SMC were used. In cells post-incubated in medium conditioned with macrophages in the presence of acetylated LDL the label in cholesteryl ester amounted to 28 000 dpm. This value was 6200 and 4400 dpm with control media similar to those under (D) and (G) in table 1.

To determine whether the increased content of labeled cholesteryl ester observed in SMC, post-incubated in medium of macrophages, conditioned in presence of acetylated LDL, was due to stimulation of cholesterol esterification, [1-^{14}C]oleic acid was added during post-incubation. The data presented in table 2 show a 4-fold increase in cholesteryl [^{14}C]-oleate in cells post-incubated in medium conditioned with macrophages in the presence of acetylated LDL, when compared to those exposed to non-preincubated medium supplemented with acetylated LDL during post-incubation. Some increase in content of labeled esterified cholesterol can be obtained also, with medium derived from endothelium which had been incubated with acetylated LDL, but not with medium derived from smooth muscle cells (table 2).

Table 2
Labeled cholesteryl ester content of bovine smooth muscle cells incubated in media conditioned in presence of acetylated LDL and various cell types

Postincubation of SMC in medium preincubated in presence of:	dpm in cellular CE	
	[^{3}H]Cholesteryl ester	Cholesteryl [^{14}C]oleate
Zero time	16 300 ± 807	–
Macrophages + AcLDL	48 425 ± 2650	4172 ± 563
Endothelium + AcLDL	24 191 ± 1826	3207 ± 142
SMC + AcLDL	11 364 ± 1762	1085 ± 158
Non-preincubated medium + AcLDL	14 205 ± 635	1362 ± 80
Non-preincubated medium	13 653 ± 1700	1125 ± 30

Conditions: Incubation of macrophages with AcLDL as in table 1. Confluent cultures of bovine aortic smooth muscle cells (SMC) and endothelial cells were used for preincubation with AcLDL, 25 μg protein/ml medium. All cell types had been exposed to AcLDL for 72 h. Bovine SMC were plated and labeled with [^{3}H]cholesterol as in table 1. After 48 h the medium was removed, the cell layer was washed 3 times with PBS and post-incubation with the various media was started. [^{14}C]Oleic acid which had been complexed to bovine serum albumin (see section 2) was added at the onset of post-incubation to give 0.05 mM final conc. Post-incubation was carried out for 24 h and the experiment was terminated as in table 1. (CE) cholesteryl ester. Values are means ± SE of triplicate dishes

4. Discussion

These results are a first example of a system in which interaction between a lipoprotein and a cell of one type result in stimulation of esterification in another cell type. Acetylated LDL interacts with cultured macrophages through a receptor-mediated pathway and these receptors are not present in either fibroblasts [5] or smooth muscles, but have been observed in aortic endothelial cells [9]. In smooth muscle cells the esterification of cholesterol can be modulated by native LDL [4], which is recognized by a specific receptor, but not by acetylated LDL, which is not recognized by these cells [9]. These findings indicate that following interaction between the modified LDL and cells which are able to recognize the particle, i.e., macrophages or endothelial cells, a change occurs in the conditioned medium, so that it promotes cholesterol esterification in cultured smooth muscle cells. The nature of this change, and whether it can be achieved only with acetylated or also with otherwise modified LDL is under current investigation.

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