ELSEVIER

Atherosclerosis 121 (1996) 63–73

atherosclerosis

Study of causes underlying the low atherosclerotic response to dietary hypercholesterolemia in a selected strain of rabbits

J. Thiery[a], D. Teupser[a], A.K. Walli[a], B. Ivandic[a], K. Nebendahl[b], O. Stein[c], Y. Stein[d], D. Seidel[a]

[a]*Institute for Clinical Chemistry, University Hospital Großhadern, Marchioninistraße 15, D-81366 Munich, FRG*
[b]*Department of Experimental Animal Research, University Hospital Göttingen, Robert-Koch-Straße 40, D-37075 Göttingen, FRG*
[c]*Department of Experimental Medicine and Cancer Research, Hebrew University — Hadassah Medical School, Kiryat Hadassah, il-91 120 Jerusalem, Israel*
[d]*Lipid Research Laboratory, Division of Medicine, Hadassah University Hospital, Kiryat Hadassah, P.O. Box 12 220, il-91 120 Jerusalem, Israel*

Received 30 May 1995; accepted 23 August 1995

Abstract

We have recently characterized a strain of rabbits that shows a low atherosclerotic response (LAR) to dietary hypercholesterolemia in contrast to the usual high atherosclerotic response (HAR) of rabbits [1]. Presently, we have focused on three well established and important stages of atherogenesis, i.e., monocyte adhesion to endothelium, cell mediated peroxidative modification of lipoproteins and induction of a receptor that recognizes modified low density lipoprotein (LDL). The results obtained show that (1) β-very low density lipoprotein (β-VLDL) from LAR and HAR rabbits enhanced monocyte adhesion to endothelial cells to the same extent; (2) Cell mediated peroxidation of LDL and β-VLDL, tested by loss of α-tocopherol and formation of thiobarbituric acid reacting substances (TBARS), was compared using macrophages, fibroblasts and smooth muscle cells (SMC) of LAR and HAR rabbits and no significant differences were found; (3) Induction of scavenger receptor by phorbol ester (phorbol 12-myristate 13-acetate (PMA)) and platelet-derived growth factor-BB (PDGF-BB) was determined in SMC or fibroblasts from LAR and HAR rabbits using 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated LDL (DiL-acLDL). We found a significantly higher uptake of DiI-acLDL in SMC and fibroblasts derived from HAR rabbits as compared with cells from LAR rabbits. Similar results were also obtained with [^{125}I]-acLDL in fibroblasts from LAR and HAR rabbits with respect to cellular lipoprotein degradation after PMA pretreatment. Even though the attenuated atherosclerotic response to hypercholesterolemia of LAR rabbits may have multiple underlying causes, the most prominent so far is an apparent difference in inducibility of scavenger receptor in SMC and fibroblasts.

Abbreviations: acLDL, acetylated LDL; β-VLDL, beta very low density lipoprotein; BHT, butylated hydroxytoluene; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiI-LDL, DiI-labeled LDL; DiI-acLDL, DiI-labeled acLDL; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylene diamine tetraacetic acid; FBS, fetal bovine serum; HAR, high atherosclerotic response; HPLC, high performance liquid chromatography; LAR, low atherosclerotic response; LDL, low density lipoprotein; LPDS, lipoprotein deficient serum; LPS, lipopolysaccharide; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; SMC, smooth muscle cells; TBARS, thiobarbituric acid reactive substances.

* Corresponding author. Tel.: +49 89 70953210; Fax: +49 89 70958888; e-mail: thiery@klch.med.uni-muenchen.de.

SSDI 0021-9150(95)05700-7

Keywords: Cholesterol-fed rabbit; Atherosclerotic response; Alveolar macrophages; Smooth muscle cells; Fibroblasts; Endothelium; Oxidation; Cell adhesion; Scavenger receptor; DiI-acLDL; [^{125}I]-acLDL; PKH-2

1. Introduction

In a recent paper [1], we have characterized a strain of rabbits, that has a low atherosclerotic response (LAR) to feeding of 0.5% cholesterol for up to 16 weeks on this diet. Thus, in the LAR rabbits, the lipid-stainable surface area of aorta amounted to only 23% after 16 weeks of cholesterol feeding. On the other hand, in rabbits with high atherosclerotic response (HAR) to cholesterol feeding, aortic surface involvement reached 59% already after 12 weeks and 85% after 16 weeks. The measurements of surface area involvement were corroborated also by a significantly lower chemically determined cholesterol content of the aorta in the LAR rabbits [1]. Both strains of rabbits develop similar hypercholesterolemia and no differences were found in the lipoprotein distribution and composition in the LAR and HAR rabbits. The reasons for this remarkable aortic resistance to the circulating high cholesterol levels are presently unknown and this study is the first attempt to elucidate putative mechanisms that could be operative in explaining the attenuated expression of atherosclerosis in the LAR rabbits. The delineation of mechanisms responsible for the relative resistance of a strain of rabbits to high plasma cholesterol levels may be of importance for the understanding of human atherogenesis. It seems relevant to point out that the majority of humans are relatively resistant to elevated plasma cholesterol levels and the development of atherosclerosis may span over decades. This delayed onset of atherosclerosis is not seen in most mammalian species in which moderate hypercholesterolemia is induced [2, 3].

2. Materials and methods

2.1. Isolation, modification and labeling of lipoproteins

Blood from normal donors was drawn into tubes containing ethylene diamine tetraacetic acid (EDTA, 1 mg/ml). After separation of chylomicrons at 100 000 × *g* (density (*d*) < 1.006 g/ml) for 40 min, and very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) at $d = 1.019$ g/ml, the infranatant was brought to $d = 1.060$ g/ml and centrifuged at 150 000 × *g* for 24 h at 10°C. The floating low density lipoproteins (LDL) were obtained by tube slicing. Lipoprotein deficient serum (LPDS) was obtained after removal of all lipoproteins at $d = 1.24$ g/ml for 48 h. β-VLDL was isolated from cholesterol-fed rabbits as described elsewhere [4]. LDL was acetylated (acLDL) with acetic anhydride and saturated sodium acetate as described by Basu [5]. Lipoproteins were labeled with ^{125}I by the iodine monochloride method as modified for lipoproteins [6], kept sterile and used within 1–2 weeks. LDL was labeled with the fluorescent probe, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) according to the method of Pitas et al. [7], DiI-LDL was acetylated as described [5].

2.2. Isolation of rabbit alveolar macrophages

Rabbits were fed a diet enriched with 0.5% cholesterol for up to 10 weeks. They were killed with an overdose of pentobarbital and lungs and aorta were removed. The lungs were kept in phosphate buffered saline (PBS) until isolation of alveolar macrophages.

For bronchoalveolar lavage, the trachea was intubated with a flexible silicon tube and both lungs were filled with 50 ml PBS at room temperature. This procedure was repeated 5 to 6 times, all PBS washes were pooled and centrifuged for 10 min at 180 × *g* at 25°C. The cell pellet was washed once with PBS and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 100 U penicillin/ml and 100 μg streptomycin/ml; 25–80 × 10^6 cells could be isolated with this procedure. Cell viability was assessed by trypan blue exclusion and was 70%–90%. Cells were seeded in 12-well plates and used for LDL-oxidation experiments after 12 h.

2.3. Cell cultures

Monolayers of human endothelial cells (derived from the iliac artery) were established by a procedure adapted from Navab et al. [8]. The cells were seeded in 24-well plates (Falcon) in endothelial-SFM medium (Gibco, Grand Island, N.Y., USA) with L-glutamine, 20% human serum (AB) and gentamicin (0.1 mg/ml). The wells were precoated with human fibronectin (Inotech, Dottikon, Switzerland) at a concentration of 7 μg/ml PBS for 60 min at 25°C. Skin fibroblasts were obtained from rabbits of the HAR and LAR strains and cultured in DMEM with 10% fetal bovine serum (FBS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) as described for human skin fibroblasts [9]. The cells were between 5–12 passages and confluent cultures were used for all experiments. Cultures of both HAR and LAR rabbit smooth muscle cells (SMC) were obtained from explants prepared from animals fed a cholesterol-enriched diet for 4 months. Lesion-free segments of the distal thoracic aorta were stripped of the adventitia and intima and the remaining media was cut into small pieces which were then transferred to 6-well plates (5–8 explants per well) containing enough medium (SmBM, supplemented with 0.5 ng/ml human epidermal growth factor (hEGF), 2.0 ng/ml hFGF, 5% FBS, 5 μg/ml insulin, gentamicin and amphotericin-B: Clonetics, San Diego, USA) to wet but not float the explants. Once the explants had adhered to the dish, additional medium was added. Cells had grown sufficiently to permit subculturing after 3–4 weeks. Cells were used between passages 4 and 5. U937 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 with 10% FBS.

2.4. Labeling of cells with the fluorophor PKH2-GL

Human monocyte-like line U-937 cells were labeled with the green fluorescent cell linker PKH2-GL according to the recommendations of the supplier (Sigma, St. Louis, MO, USA). Cells were used for adhesion experiments immediately after labeling.

2.5. Adherence of U937 cells to endothelial monolayers

The assay used for measuring adherence of human monocyte-like U937 cells to endothelial monolayers derived from human iliac artery was adapted from a technique described by Kume et al. [10]. Confluent endothelial monolayers in 24-well plates were washed with serum free DMEM and were incubated in DMEM for 3 h with different agents: lipopolysaccharide (LPS) 1 μg/ml (derived from *Escherichia coli*, 055:B5, Sigma Chemical Co., St.Louis, USA), human LDL 100 μg protein/ml, β-VLDL isolated from three hypercholesterolemic LAR and HAR rabbits (100 μg protein/ml), and PBS as blank. Thereafter, the cells were washed twice with DMEM. PKH2-labeled monocytes were added to the wells at a final concentration of 5×10^5 cells/ml and were allowed to settle onto the monolayer for 30 min. Plates were sealed, inverted, and centrifuged for 5 min at $250 \times g$ to separate non-adherent monocytes. The remaining cells were lysed with 0.1 N NaOH/0.1% sodium dodecyl sulphate (SDS) and the fluorescence was measured (excitation 490 nm, emission 504 nm) with a Perkin-Elmer model LS 50 B luminescence spectrometer. Each adherence assay was performed in quadruplicates, the PBS and LPS values are from 8 replicates. Results are expressed as the ratio of fluorescence intensity per well normalized to the PBS-treated controls.

2.6. Cell-mediated modification of LDL, determination of α-tocopherol and thiobarbituric acid reactive substances (TBARS)

Freshly isolated macrophages were seeded in 12 well plates (1×10^6 cells/well) and kept in DMEM with 10% FBS for 12 h. Non adherent cells were removed and the remaining cells were incubated with HAM's F10 and LDL as described below. Rabbit skin fibroblasts or SMC were grown in 12-well plates to confluence and used for incubation with LDL in HAM's F10. Rabbit SMC were used for incubation with rabbit β-VLDL. Studies on cell-mediated lipoprotein modification were performed in the same manner for all types of cells. Cells were washed once with medium and incubated with 1.5 ml of HAM's F10, which was supplemented with 3 μM freshly

prepared $FeSO_4$ and LDL (200 μg cholesterol/ml) or β-VLDL (400 μg cholesterol/ml), respectively [11]. LDL and β-VLDL were used directly after desalting by chromatography (Bio-Rad Econo-Pac 10 desalting columns). In addition, LDL or β-VLDL were incubated in cell-free dishes in the presence and absence of $CuCl_2$ (4.2 μM) as control for maximal and minimal oxidation. All experiments were performed in duplicates. After incubation for up to 24 h, media were removed and 1 ml was immediately mixed with saline-EDTA (EDTA 1 mg/ml) and centrifuged. The supernatant was stored at −80°C for measurement of TBARS and α-tocopherol content of LDL or β-VLDL.

α-Tocopherol content of LDL or β-VLDL was determined fluorometrically by high performance liquid chromatography (HPLC) (Merck/Hitachi, Darmstadt, Germany) according to Lehmann and Martin [12] with some modifications. Briefly, 50 μl aliquots were mixed with 100 μl ethanol containing butylated hydroxytoluene (BHT) (1 g/l) and δ-tocopherol (1 μg/ml) as an internal standard. Thereafter, tocopherols were extracted with 400 μl *n*-hexane. After evaporation, the residue was dissolved in methanol and 10 μl of the solution was injected onto a Merck LiChrospher C18 reversed phase column (250 × 4 mm) using methanol as mobile phase. The excitation and emission wavelength was 295 and 330 nm, respectively, essentially as described by Burton et al. [13], except that the column was run at room temperature and the flow rate was 1 ml/min. The detection limit for α-tocopherol was 0.1 ng and a linear response was obtained up to 1500 ng.

TBARS were determined according to Wallin et al. [14] with some modifications. Briefly, to tubes containing 200 μl of sample, 50 μl of 50% (w/v) trichloroacetic acid (TCA) and 75 μl of 1.3% thiobarbituric acid in 0.3% (w/v) NaOH were added. The tubes were heated in a thermoblock for 60 min at 90°C and then cooled in ice water. Samples were centrifuged for 6 min to eliminate turbidity. Finally, 200 μl of the sample were transferred to a 96-well plate (Sarstedt, Nürmbrecht, Germany) and the absorbance at 530 nm minus the absorbance at 630 nm was read in a Dynatech MR 7000 microplate reader (Dynatech Deutschland GmbH, Denkendorf, Germany). TBARS were quantified using a standard curve of malondialdehyde (MDA) equivalents, generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) (Sigma T-9889).

2.7. Induction of scavenger receptor activity

Confluent cultures of skin fibroblasts or SMC derived from rabbits of the HAR and LAR strains were used in these experiments. Prior to the start of the experiment the culture medium was removed. The cells were washed with PBS and incubated for 48 h in culture medium containing 10% lipoprotein deficient serum (LPDS) without or with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) or 10 mg/ml platelet-derived growth-factor BB (PDGF-BB).

For measurement of degradation of [^{125}I]-acLDL, fibroblasts were washed with PBS and incubated in fresh medium containing 10% LPDS and 10 μg/ml of [^{125}I]-acLDL. The degradation of [^{125}I]-labeled lipoproteins was determined as described [15]. Specificity of lipoprotein degradation was determined in the presence of a 25-fold excess of unlabeled lipoprotein and these values have been subtracted from all data reported. All incubations were performed in triplicate for 4 h at 37°C.

To study the uptake of DiI-acLDL, cultured SMC were incubated at 37°C for 18 h with 20 μg/ml DiI-acLDL and specificity of acLDL uptake was determined in the presence of 25-fold excess of unlabeled lipoprotein [16]. Fibroblasts were incubated in DMEM with 20 μg/ml of DiI-acLDL for 15 h at 37°C and at 4°C. Thereafter, the cells were washed twice with 0.4% bovine serum albumin (BSA) (Behring, Marburg, Germany) in PBS and 3 × with PBS and were lysed with 0.1 N NaOH/0.1% SDS. Fluorescence intensity was determined in a Perkin Elmer model LS 50 B luminescence spectrofluorometer with excitation and emission wavelengths set at 520 and 578 nm, respectively. Standard solutions of DiI-acLDL were prepared in 0.1 N NaOH/0.1% SDS with a linear response over a concentration range from 10 to 5000 ng protein/ml. Specific uptake of DiI-acLDL by SMC (ng/mg cell protein) was determined as the difference of cell-associated fluorescence in the presence and abscence of an

excess of unlabeled acLDL. The cellular uptake of DiI-acLDL by fibroblasts was calculated as the difference between cell-associated fluorescence at 37°C and 4°C and expressed as ng of acLDL protein/mg of cell protein [16]. Protein was determined according to Lowry et al. [17]. All incubations were done in triplicate and values agreed within 10% of the mean.

2.8. Materials

HAM's F10 with glutamine was obtained from Gibco (Grand Island, NY, USA) and Biochrom (Berlin, Germany). DMEM with 1000 mg glucose/l was obtained from Sigma (St. Louis, MO, USA). RPMI 1640 was obtained from Sigma (St. Louis, USA). FBS was obtained from Biochrom (Berlin, Germany). Sodium ^{125}iodide was obtained from Amersham (Braunschweig, Germany). DiI was obtained from Molecular Probes (Eugene, OR, USA). Recombinant human platelet derived growth factor BB homodimer was obtained from Genzyme (Cambridge, MA, USA)

2.9. Statistical analysis

P-values were calculated by Student's *t*-test.

3. Results

In this study, we attempted to unravel some of the mechanisms which could be responsible for the attenuated atherosclerotic response of LAR rabbits to cholesterol feeding, in spite of marked hypercholesterolemia. First, we inquired whether β-VLDL of LAR and HAR rabbits will affect differently the interaction between monocytes and endothelial cells, which is one of the first steps in atherogenesis. To that end, human endothelial cells in culture were pretreated for 3 h with β-VLDL (isolated from 3 LAR and 3 HAR rabbits), with LPS or with human LDL. Thereafter the medium was removed, the endothelial cell layer washed and monocytes (U 937) labeled with PKH2 were added for 30 min. Endothelial cells preincubated with PBS served as controls. The number of adhering U937 cells was determined and normalized to the PBS values. As shown in Fig. 1, pretreatment with β-VLDL resulted in a marked stimulation of monocyte adhesion which was higher than that elicited by LPS or LDL. However, the response to β-VLDL from LAR rabbits was not different from that to β-VLDL isolated from HAR rabbits. Since lysophosphatidylcholine was shown to influence monocyte adhesion to endothelium, we also determined the phospholipid composition of the HAR and LAR β-VLDL; the finding of similar concentrations of lysophosphatidylcholine (data not given) corroborated the results described above.

Peroxidation of lipoproteins is thought to play an important role in the early stages of atherogenesis by promoting foam cell formation. Most probably peroxidation of lipoproteins occurs within the aortic wall where they come in contact with macrophages and SMC. Therefore, in the next experiments we compared the effects of incubation of LDL with alveolar macrophages derived from LAR and HAR rabbits; the parameters measured were loss of α-tocopherol and formation of TBARS. The data of the experiments were normalized, taking the zero time values of α-tocopherol as 100%, and it can be seen that during

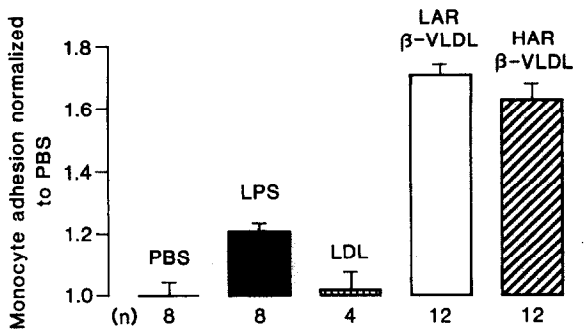
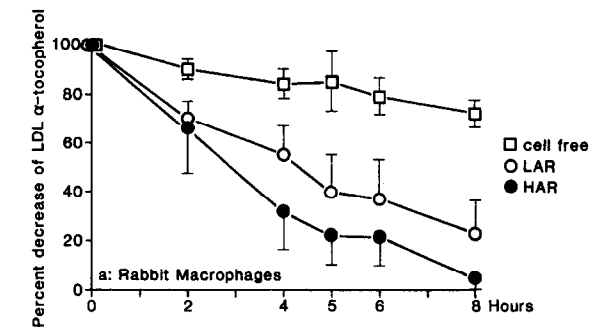
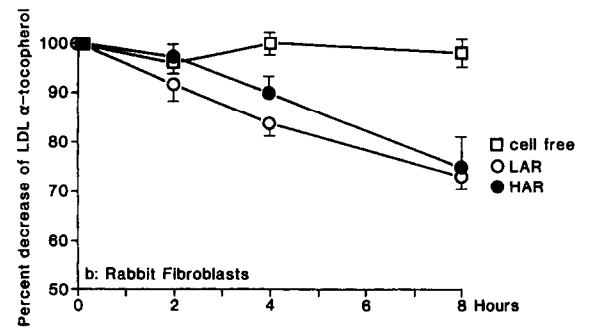


Fig. 1. Adhesion of U 937 monocytes to human endothelial cells preincubated with LPS, LDL or β-VLDL from HAR and LAR rabbits. Confluent human endothelial cells, grown in 24-well plates, were incubated for 3 h with LPS (1 μg/ml), human LDL, LAR β-VLDL and HAR β-VLDL (100 μg protein/ml) or PBS alone. After washing, cells were incubated with PKH2-labeled U 937 monocytes (0.5×10^6cells/well) for 30 min. Supernatant was removed, plates were inverted and centrifuged for 5 min at 250 $\times$ *g*. The adhering cells were lysed in 1 ml 0.1 N NaOH/0.1% SDS and fluorescence was determined and corrected for autofluorescence of endothelial cells alone. Fluorescence of each well was then normalized to fluorescence intensity of endothelial cells preincubated with PBS. Values represent mean $\pm$ S.E.M. of 4–12 samples (*n*).



(a)



(b)

Fig. 2a,b. Time course of loss of LDL-α-tocopherol in the presence of alveolar macrophages (a) and fibroblasts (b) derived from LAR-rabbits (open circles) and HAR-rabbits (closed circles). Cell-free values are indicated by open squares. All experiments were performed in HAM's F-10 medium supplemented with 3 μM $FeSO_4$. LDL (200 μg cholesterol/ml) was incubated in the presence or absence of rabbit alveolar macrophages or skin fibroblasts, in a humidified atmosphere (5% CO_2) at 37°C. LDL-α-tocopherol concentrations were measured fluorometrically by HPLC as described under methods and the zero time value was expressed as 100%. Individual experiments were carried out in duplicates. Values are means ± S.E. of all experiments in each category. The differences between LDL-α-tocopherol incubated with cells derived from HAR and LAR rabbits were not statistically significant at any time point studied. In Fig. 2a (macrophages), n = number of experiments: LAR = 5; HAR = 2; cell-free values = 5. All experimental values from 5 h onwards are significantly different from cell-free values ($P < 0.05$–0.001). In Fig. 2b (fibroblasts), n = number of experiments: LAR = 2; HAR = 3; cell-free values = 3. All experimental values from 4 h onwards are significantly different from cell-free values ($P < 0.05$–0.001).

8 h of incubation with macrophages there was a 70% decrease in α-tocopherol content of LDL exposed to cells of the HAR and LAR rabbits (Fig. 2a). The experiments performed with macrophages were repeated with rabbit skin fibroblasts (Fig. 2b). As shown in Fig. 2a,b, no significant differences between the cells of the 2 rabbit strains were seen with respect to the loss of α-tocopherol from LDL. As the α-tocopherol content of LDL was almost depleted after 24 h, we determined TBARS at this time interval. Increase of LDL TBARS formed in the presence of macrophages or fibroblasts from both rabbit strains occurred at this time interval and no significant difference was seen between the cells of HAR and LAR rabbits (Table 1).

We determined also the peroxidation of rabbit β-VLDL by aortic SMC from 3 LAR and 3 HAR rabbits. In analogy to the findings with rabbit skin fibroblasts or macrophages and LDL, no difference was seen in the formation of TBARS by SMC of the two strains. The mean TBARS values were 2.7 and 2.8 nmol MDA equivalents/mg β-VLDL cholesterol/24 h for the SMC of HAR and LAR rabbits, respectively.

Another parameter studied was the induction of the scavenger receptor activity in non-macrophage cells. This receptor and especially its induction by PMA has been well documented by Pitas for rabbit aortic SMC and fibroblasts [18]. In the present experiments, we have asked whether there might be a difference in the induction of scavenger

Table 1
TBARS formation after 24 h incubation of LDL in presence of alveolar macrophages or fibroblasts from LAR- and HAR-rabbits

	TBARS, nmol/mg LDL cholesterol
HAR-macrophages	4.1 ± 1.3 (3)
LAR-macrophages	7.0 ± 1.6 (5)
HAR-fibroblasts	2.6 ± 0.4 (3)
LAR-fibroblasts	4.0 ± 1.4 (2)
$CuCl_2$ (4.2 μM)	21.0 ± 3.2 (5)

All experiments were performed in HAM's F-10 medium supplemented with 3 μM $FeSO_4$. LDL was incubated for 24 h in a humidified atmosphere (5% CO_2) at 37°C in the presence or absence of cells. As control for maximal oxidation, LDL was incubated in the absence of cells with 4.2 μM $CuCl_2$. TBARS were measured as described under Methods. All values were corrected for TBARS formation in the absence of cells. All experiments were performed in duplicates. Values represent means ± S.E.M. of all experiments in each group. (n) = number of experiments with cells derived from different rabbits.

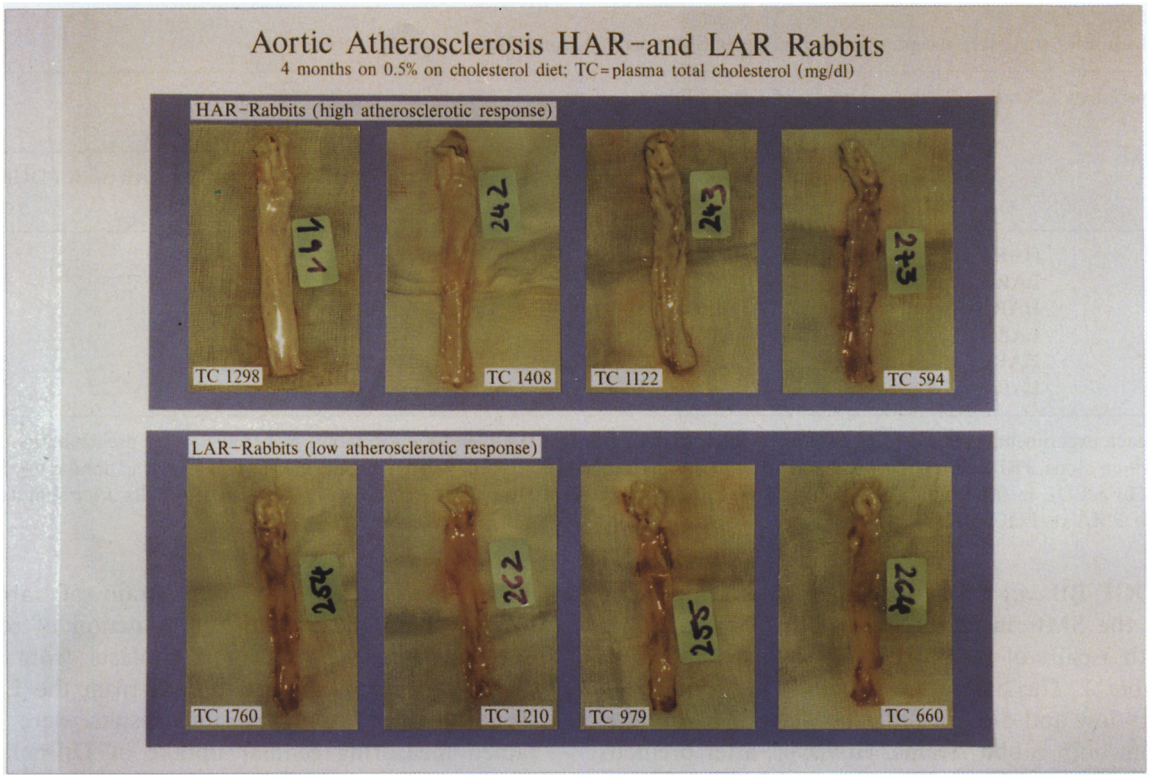


Fig. 3. Extent of atherosclerotic involvement of the aortic arch and thoracic aorta in 4 HAR (upper row) and 4 LAR rabbits (lower row) fed a 0.5% cholesterol enriched diet for 4 months. The aortae are unstained and the lesions appear white while areas without lesions appear dark. TC = plasma cholesterol concentrations at sacrifice (mg/dl).

receptor activity in cells derived from LAR and HAR rabbits. We examined cultured SMC from aortae of HAR and LAR rabbits fed cholesterol for 4 months. The aortae of these rabbits are depicted in unstained form in Fig. 3. The induction of scavenger receptor activity by PMA or

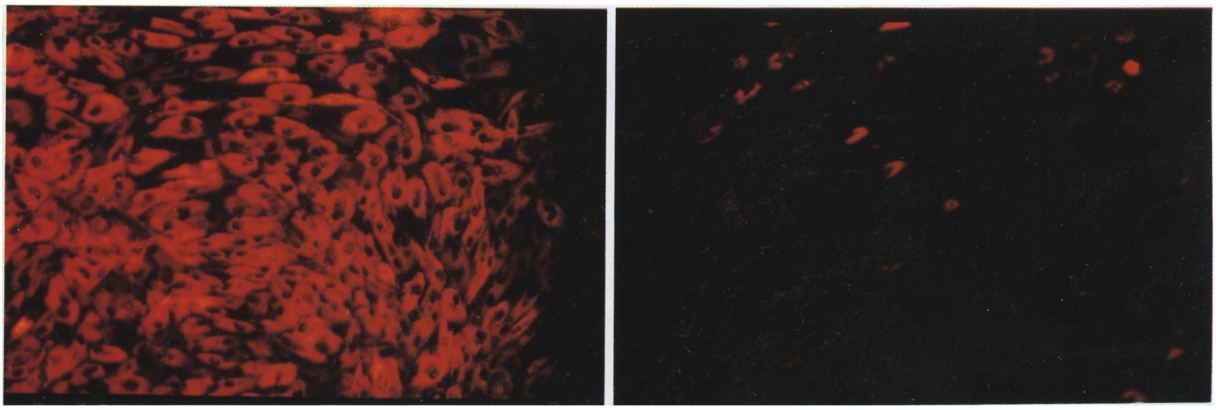


Fig. 4. Photomicrographs showing the uptake of fluorescently labeled DiI-acLDL by smooth muscle cells derived from one HAR (No. 273, left) and one LAR rabbit (No. 255, right). Cells were preincubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h, and incubated with DiI-acLDL (20 μg/ml) for 18 h.

Table 2
Induction of scavenger receptor activity in aortic SMC derived from HAR- and LAR-rabbits

Experiment	Strain	Rabbit no.	Uptake of DiI-acLDL ng/mg cell protein/18 h				
			Medium	With PMA	HAR vs. LAR with PMA *P*	With PDGF-BB	HAR vs. LAR with PDGF-BB *P*
I	HAR	273	372 ± 14	14 886 ± 448	<0.001	1616 ± 100	0.005
	LAR	255	206 ± 72	821 ± 75		538 ± 167	
II	HAR	243	445 ± 59	9399 ± 363	<0.001	3789 ± 263	<0.001
	LAR	262	647 ± 109	2033 ± 96		834 ± 49	
III	HAR	191	336 ± 12	5939 ± 194	<0.001	886 ± 44	<0.001
	LAR	254	200 ± 124	1497 ± 169		346 ± 38	

In each experiment, uptake of DiI-acLDL by SMC derived from an HAR and a LAR rabbit was studied after preincubation with medium alone, PMA (50 ng/ml), or PDGF-BB (10 μg/ml). Scavenger receptor activity is expressed as mean specific uptake (± S.E.) of DiI-acLDL in triplicate dishes. Comparisons represent uptake of DiI-acLDL by SMC of each pair of rabbits after stimulation with PMA or PDGF-BB.

PDGF-BB was measured as uptake of DiI-acLDL by the SMC in culture and data of experiments with 3 pairs of HAR and LAR cells are shown in Table 2. The baseline uptake of DiI-acLDL was very low and did not differ between SMC derived from both rabbit strains. However, after preincubation with PMA, the uptake of DiI-acLDL by SMC derived from HAR rabbits increased 18–40-fold while the uptake of DiI-acLDL by SMC from LAR rabbits was only 3–7-fold above baseline (Table 2). This finding is illustrated in the photomicrographs showing the uptake of DiI-labeled acLDL by SMC derived from both rabbit strains (Fig. 4).

The induction of scavenger receptor activity was also measured as degradation of [^{125}I]-acLDL by fibroblasts derived from both rabbit strains. The results of one single experiment with cells of each strain are given in Fig. 5. It can be seen that a 10-fold induction of degradation of [^{125}I]-acLDL occurred after PMA stimulation of the fibroblasts from the HAR rabbit; the induction was much lower in the cells of the LAR rabbit (Fig. 5). In order to validate the findings shown in Fig. 5, comparison of scavenger receptor induction in fibroblasts was repeated with cells from 6 LAR and 6 HAR rabbits. The results are summarized in Table 3 as the difference (Δ) between degradation of [^{125}I]-acLDL obtained with PMA and without PMA. The means ± S.E. of the paired comparisons for each strain of rabbits showed a significantly higher induction of scavenger receptor activity in fibroblasts from the HAR strain compared with cells from the LAR strain (Table 3). Comparable results were obtained measuring cellular uptake of DiI-acLDL (Table 4). The inducibility of scavenger receptor activity was determined also in skin fibroblasts from 3 'wild strain' rabbits by measuring the degradation of [^{125}I]-acLDL. The mean value of Δ + PMA-PMA was 91.4, similar to that observed with fibroblasts from HAR rabbits, i.e., 82.2 (Table 3).

The LDL receptor activity as measured by [^{125}I]-LDL degradation in cultured fibroblasts was similar in both strains. The ratio of degradation + PMA/-PMA was 1.6 ± 0.46 and 1.5 ± 0.5 for the HAR and LAR rabbit strains, respectively; it was 1.3 ± 0.14 for cells from control rabbits.

4. Discussion

In the present study, we have considered several possible mechanisms that could contribute to the low atherosclerotic response in our strain of rabbits in spite of prolonged exposure to high levels of plasma cholesterol. Feeding of cholesterol to rabbits results in elevation of plasma β-VLDL and the latter has been shown to enhance monocyte adhesion to endothelium [19, 20]. Among the

first questions posed was whether there might be a difference in the β-VLDL of the LAR and HAR rabbits with respect to enhancement of monocyte adhesion to endothelium, which is the first step in the cellular sequence of events which culminates in atherogenesis [21]. In a model system, using a human monocyte cell line (U937) and human endothelial cells, β-VLDL isolated from both strains of rabbits markedly enhanced monocyte adhesion, but there was no difference between LAR and HAR β-VLDL. The other parameter studied with respect to β-VLDL was the phospholipid composition in view of the chemotactic effect of lysophosphatidylcholine for monocyte recruitment [22, 23] and its role in upregulation of the expression of inducible leukocyte adhesion molecules [10, 24]. However, the lysophosphatidylcholine content in β-VLDL isolated from the plasma of both strains did not differ.

It is generally accepted that peroxidation of lipoproteins plays a prominent role in atherogenesis [25,26] and the presence of oxidized LDL in arterial lesions supports this concept [27,28]. We

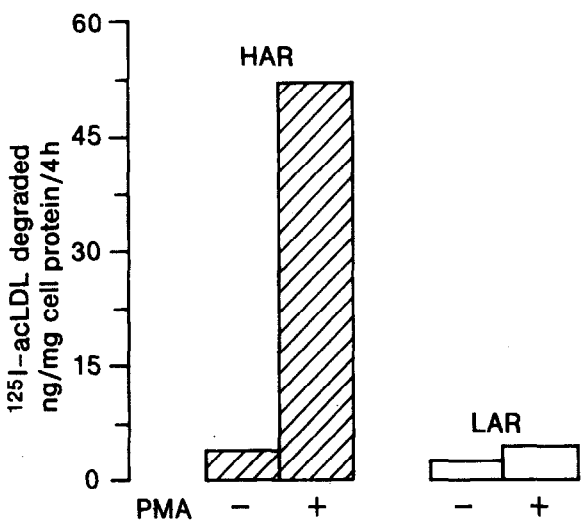


Fig. 5. Induction of scavenger receptor activity in fibroblasts from HAR and LAR rabbits. Confluent cultures of fibroblasts from one HAR and one LAR rabbit were preincubated for 48 h with or without PMA. Thereafter, cells were incubated for 4 h with [^{125}I]-acLDL in presence and absence of a 25-fold excess of unlabeled acLDL. Noniodide degradation products in medium were determined. All data presented are after subtraction of nonspecific degradation. Values are means of triplicate dishes in each experiment, which agree within 10%.

therefore investigated the peroxidative capacity of cells from LAR and HAR rabbits. So far, the two parameters of cell-mediated modification of LDL studied were loss of vitamin E and formation of TBARS. In the classical studies by Esterbauer [29], it has been established that in the presence of Cu^{++} a relatively rapid loss of α-tocopherol from LDL preceded the generation of aldehydes. Incubation of alveolar macrophages and skin fibroblasts from LAR and HAR rabbits with human LDL resulted in a linear decrease of lipoprotein α-tocopherol which was followed by the generation of TBARS. The kinetics of macrophage-mediated depletion of α-tocopherol in human [^{125}I]-LDL were determined using mouse peritoneal macrophages and our findings are comparable in general to those reported earlier [11]. However, we observed a much slower rate of α-tocopherol consumption, which may be explained by the use of native LDL in our studies, since iodination of lipoproteins has been shown to enhance peroxidation [30]. With respect to the parameters measured, fibroblasts and macrophages from HAR and LAR rabbits exhibited the same peroxidative potential to modify LDL. The same was true using aortic SMC and β-VLDL as substrate.

Modified lipoproteins have been shown to be recognized by scavenger receptors present in macrophages and their uptake does result in

Table 3
Scavenger receptor induction in skin fibroblasts of HAR and LAR rabbits determined by degradation of [^{125}I]-acLDL

	Fibroblasts derived from rabbits	
Rabbit strain	HAR	LAR
Rabbit cell donors (n)	6	6
Experiments (n)	11	12
Mean of Δ + PMA-PMA	82.2	8.1
S.E. of mean	23.6	8.6
P-value	<0.01	

In each experiment, the degradation of [^{125}I]-acLDL was studied in triplicate dishes with or without pretreatment with 50 ng/ml of phorbol myristate acetate (PMA). Scavenger receptor stimulation was calculated as the difference (Δ) of degradation of [^{125}I]-acLDL (ng/mg cell protein/4 h) determined in cells pretreated with or without PMA. P-value by t-test.

Table 4
Analysis of scavenger receptor induction in skin fibroblasts of HAR and LAR rabbits determined by uptake of DiI-acLDL

	Fibroblasts derived from rabbits	
Rabbit strain	HAR	LAR
Rabbit cell donors (n)	3	3
Experiments (n)	7	9
Mean of Δ + PMA-PMA	1118	216
S.E. of mean	250	61
P-value	<0.001	

In each experiment, the uptake of DiI-acLDL was studied in triplicate dishes with or without pretreatment with 50 ng/ml of phorbol myristate acetate (PMA). Scavenger receptor stimulation was calculated as the difference (Δ) of uptake of DiI-acLDL (ng/mg cell protein/15 h) determined in cells pretreated with or without PMA. P-value by t-test.

cholesterol accretion [31]. The presence and inducibility of scavenger receptor has been demonstrated also in rabbit aortic smooth muscle cells and rabbit skin fibroblasts [18,32–34]. Recently, scavenger receptor expression in rabbit SMC in vivo and its regulation by atherogenic diets was also reported [35]. In the present study, we compared the catabolism of acLDL by SMC and fibroblasts isolated from LAR and HAR rabbits prior to and after stimulation with phorbol ester. In analogy to the findings of Pitas [17, 33, 34], a very significant induction of the scavenger receptor activity, as evidenced by an enhanced degradation of [^{125}I]-acLDL or uptake of DiI-acLDL was demonstrated in cells from HAR rabbits. However, in SMC and fibroblasts derived from LAR rabbits, this stimulation of scavenger receptor activity was markedly attenuated.

What could be the in vivo relevance of these findings? Oxidatively modified lipoproteins have been demonstrated in atherosclerotic vessels [27,28]. The scavenger receptor which can be induced in rabbit SMC has been shown to recognize oxidized lipoproteins in addition to acLDL [34]. The induction of scavenger receptor in SMC has been shown to proceed through activation of protein kinase C [18] by platelet secretory products [33]. Moreover, rabbit aortic SMC were shown to have increased scavenger receptor activity following incubation with interferon-gamma or tumor necrosis factor-alpha (TNF-α) [35]. The activation of the scavenger receptor-mediated uptake of modified lipoproteins has been suggested to contribute to the formation of foam cells of SMC origin [33,34,35]. Thus, the impaired induction of scavenger receptors in cells of LAR rabbits could contribute to the attenuated development and delayed onset of atherosclerosis.

Acknowledgments

This paper includes part of the medical thesis of Daniel Teupser, Faculty of Medicine Ludwig-Maximilians-University Munich. Dr. Olga Stein and Dr. Yechezkiel Stein, Hadassah University Hospital Jerusalem, have been fellows of the Humboldt-Stiftung, 1993. We are indebted to Dr. Bernhard Engelmann for determining the phospholipid composition of β-VLDL. We are grateful for the expert technical assistance of Christiane Gross, Ulrike Haas and Barbara Siegele. We also thank Dr. P. Scheuber and Anni Krieg for the excellent animal care and Elke Kaufmann for the help in the preparation of the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Th 374, 2-1) to Dr. Thiery.

References

[1] Thiery J, Nebendahl K, Rapp K, Kluge R, Teupser D, Seidel D. Low atherosclerotic response of a strain of rabbits to dietary induced hypercholesterolemia. Arterioscler Thromb Vasc Biol 1995;15:1181.
[2] Goldstein JL, Brown MS. Familial hypercholesterolemia. In: Scriver CHR, Beaudet AL, Sly WS, Valle E. (eds.), The Metabolic Basis of Inherited Disease I, 6th edn., McGraw-Hill, New York, 1989, p. 1215.
[3] Wissler RW. Recent progress in studies of experimental primate atherosclerosis. Progr Biochem Pharmacol 1968;4:378.
[4] Gröne EF, Abboud HE, Höhne M, Walli AK, Gröne HJ, Stüker D, Robenek, Wieland E, Seidel D. Actions of lipoproteins in cultured human mesangial cells: modulation by mitogenic vasoconstrictors. Am J Physiol 1992; 263; Renal Fluid Electrolyte Physi., 32:F686.
[5] Basu SK, Goldstein JL, Anderson RGW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc Natl Acad Sci USA 1976;73:3178.

[6] Bilheimer DW, Eisenberg S, Levy RJ. The metabolism of very low density lipoproteins. I. Preliminary in vitro and in vivo observations. Biochim Biophys Acta 1972;260:212.
[7] Pitas RE, Innerarity TL, Weinstein JN, Mahley RW. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. Arteriosclerosis 1981;1:177.
[8] Navab M, Hough GF, Stevenson LW, Drinkwater DC, Laks H, Fogelman AM. Monocyte migration into the subendothelial space of a co-culture of adult human aortic endothelial and smooth muscle cells. J Clin Invest 1988;81:1853.
[9] Armstrong VW, Walli AK, Seidel D. Isolation, characterization and uptake in human fibroblasts of apo(a)-free lipoprotein obtained on reduction of lipoprotein(a). J Lipid Res 1985;26:1314.
[10] Kume N, Cybulsky MI, Gimbrone MA. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest 1992;90:1138.
[11] Jessup W, Rankin SM, DeWhalley CY, Hoult JRS, Scott J, Leake DS. α-Tocopherol consumption during low density lipoprotein oxidation. Biochem J 1990;265:399.
[12] Lehmann J, Martin HL. Improved direct determination of alpha- and gamma-tocopherols in plasma and platelets by liquid chromatography with fluorescence detection. Clin Chem 1982;28:1784.
[13] Burton GW, Ingold KU. Vitamin E: applications of the principles of physical organic chemistry to the exploration of its structure and function. Acc Chem Res 1986;19:194.
[14] Wallin B, Rosengren B, Shertzer HG, Camejo G. Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: it's use for evaluation of antioxidants. Anal Biochem 1993;208:10.
[15] Goldstein JL, Basu JK, Brown MS. Receptor-mediated endocytosis of low density lipoprotein in cultured cells. In: Fischer S, Fleischer B. (eds.), Methods in Enzymology, New York, Academic Press Inc. 1983;98:241.
[16] Teupser D, Gross Ch, Siegele B, Walli AK, Seidel D, Thiery J. Quantitative fluorometric assay of LDL- and scavenger-receptor activity in cultured cells. Atherosclerosis 1995;115:S38.
[17] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265.
[18] Pitas RE. Expression of the acetyl low density lipoprotein receptor by rabbit fibroblasts and smooth muscle cells: Up-regulation by phorbol esters. J Biol Chem 1990;265:12722.
[19] Endemann G, Pronzcuk A, Friedman G, Lindsey S, Alderson L, Hayes KC. Monocyte adherence to endothelial cells in vitro is increased by β-VLDL. J Pathol 1987;126:1.
[20] Gerrity RG. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 1981;103:181.
[21] Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990's. Nature 1993;362:801.
[22] Quinn MT, Parthasarathy S, Steinberg D. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. Proc Natl Acad Sci USA 1988;85:2805.
[23] Kume N, Gimbrone MA Jr. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. J Clin Invest 1994;93:907.
[24] Li H, Cybulsky MI, Gimbrone MA, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule in rabbit aortic endothelium. Arteriosclerosis Thromb 1993;13:197.
[25] Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol — Modifications of low density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915.
[26] Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. J Clin Invest 1991;88:1785.
[27] Ylä-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J Clin Invest 1989;84:1086.
[28] Palinski W, Rosenfeld ME, Ylä-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew TE, Steinberg D, Witztum JL. Low density lipoprotein undergoes oxidative modification in vivo. Proc Natl Acad Sci USA 1989;86:1372.
[29] Esterbauer H, Quehenberger O, Jürgens G. Effect of peroxidative conditions on human plasma low density lipoproteins. In: Nigam SK, McBrien DCH, Slater TF (eds.), Eicosanoids, Lipid Peroxidation and Cancer, Springer-Verlag, Berlin und Heidelberg. 1988;203.
[30] Khouw AS, Parthasarathy S, Witztum JL. Radioiodination of low density lipoprotein initiates lipid peroxidation: protection by use of antioxidants. J Lipid Res 1993;34:1483.
[31] Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 1979;76:333.
[32] Bickel PE, Freeman MW. Rabbit aortic smooth muscle cells express inducible macrophage scavenger receptor messenger RNA that is absent from endothelial cells. J Clin Invest 1992;90:1450.
[33] Pitas RE, Friera A, McGuire J, Dejager S. Further characterization of the acetyl LDL (scavenger) receptor expressed by rabbit smooth muscle cells and fibroblasts. Arterioscler Thromb 1992;12:1235.
[34] Dejager S, Mietus-Snyder M, Pitas RE. Oxidized low density lipoproteins bind to the scavenger receptor expressed by rabbit smooth muscle cells and macrophages. Arterioscler Thromb 1993;13:371.
[35] Li H, Freeman MW, Libby P. Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vivo by cytokines. J Clin Invest 1995;95:122.