Immunohistochemical Localization of Lipoprotein Lipase and Apolipoprotein E in Human Atherosclerotic Lesions

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Lipoprotein lipase (LPL) and apolipoprotein E (apo E) are both involved in the metabolism of triglyceride-rich lipoproteins. In the arterial wall, these two proteins may be associated with foam cell formation during atherogenesis, yet their localization and cellular sources in the arterial intima are not fully defined. To evaluate the potential roles of these two proteins in various stages of human atherosclerosis, we studied the distribution of LPL and apo E in diffuse intimal thickening, fatty streaks and fibrous plaques of aorta and coronary artery using specific antibodies against human LPL and apo E. In aorta with diffuse intimal thickening, LPL was found in both intimal and medial smooth muscle cells whereas apo E was present only in the intima and was mainly associated with the surrounding extracellular matrix. In fatty streaks and fibrous plaques, LPL and apo E were widely distributed, and were either cell-associated or bound to the extracellular matrix. Double immunohistochemical stain-

ing using LPL/apo E and macrophage-specific antibodies revealed that LPL or apo E was colocalized with macrophage-derived foamy and nonfoamy cells. Furthermore, LPL and apo E were variably associated with the extracellular matrix in these areas. As the lesions advanced, LPL was diffusely stained in macrophages and smooth muscle cells in the proximity of the lipid core and fibrous cap, whereas apo E was associated with macrophages around the lipid core. These immunohistochemical findings suggest that LPL can be produced by a variety of cellular sources in the arterial wall, including smooth muscle cells and macrophages, while apo E is mainly derived from macrophages. We speculate that LPL secretion and apo E secretion from macrophages in atherosclerotic lesions are regulated differently and their respective functions in foam cell formation during atherogenesis merit further investigation.

Key words: Macrophages, Foam cells, Apolipoprotein E, Lipoprotein lipase, Atherosclerosis

I. Introduction

Early atherosclerotic lesions are characterized by intimal accumulation of monocyte-derived macrophages. These intimal macrophages may engulf atherogenic lipoproteins such as modified LDL via scavenger receptors, which accumulate substantial amounts of lipids in their cytoplasm, and transform into foam cells [25]. Macrophages may also contribute to lesion development by secreting a number of biological factors, some of which modulate lipoprotein metabolism [22, 23]. In this respect, apo E and lipoprotein lipase (LPL) derived from macrophages may contribute to both receptor- and non-recep-

tor-mediated uptake of triglyceride-rich lipoproteins and modulate the foam cell formation [2, 3, 6, 9]. LPL is an enzyme ($M_r = 60$ kD) that hydrolyzes the core of triglycerides of chylomicrons and very low density lipoproteins, thereby playing a pivotal role in the lipoprotein metabolism [2, 3]. Apo E is, however, a component of all classes of lipoproteins except for LDL and its major function is to mediate the binding and uptake of remnant lipoproteins through the apo B/E receptor and LDL receptor-related protein (LRP) [9]. Apo E appears to be associated with atherosclerosis. Evidence supporting this possibility comes from the demonstration of apo E in atherosclerotic lesions [18]. Several observations have suggested that apo E is a protective factor against atherosclerosis. Apo E knock-out mice show susceptibility to atherosclerosis even in the absence of a high-fat diet [12, 15, 28], and this susceptibility can be prevented by macro-

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phage-derived apo E [1, 8]. On the other hand, Zilversmit proposed that LPL may play an important role in proatherosclerosis because increased LPL activity can result in elevation of atherogenic remnant lipoproteins thereby increasing the cholesterol and triglyceride deposition in the arterial wall [29]. Lindqvist *et al.* [7] showed that hydrolysis of triglyceride-rich lipoproteins by LPL is essential for the uptake of the lipoproteins by macrophages. Moreover, LPL may increase cellular uptake of lipoprotein particles through association with cell-surface heparan sulfate proteoglycans [24]. These studies suggest that LPL may be involved in receptor- or non-receptor-mediated uptake of lipoprotein particles; therefore, local expression of LPL in atherosclerotic lesions might modify the effects of apo E on cell cholesterol content through different mechanisms. However, the precise role of the local LPL produced by arterial cells in atherosclerosis has not been clarified. There is little information regarding the colocalization of these two proteins in atherosclerotic lesions. Several studies have demonstrated that LPL mRNA and proteins were present in atherosclerotic lesions, but their cellular sources were not fully determined

[5, 10, 13, 27]. Because of the potential importance of LPL and apo E in atherosclerosis, the current study was performed to determine the distribution of LPL and apo E in human atherosclerosis. Particular emphasis was placed on studying colocalization of these components with macrophages, which was analyzed using specific antibodies against human LPL and apo E.

II. Materials and Methods

Aortas and coronary arteries were from 12 autopsies that were performed within 4 hr of death. In addition, carotid arteries, external iliac arteries and inferior mesenteric arteries were obtained from 6 cases of endoatherectomy and 5 cases of aneurysmoplasty. The details of the clinical data are shown in Table 1. All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were cut in 5 μ m slices and routinely stained with hematoxylin and eosin (HE) and elastic van Gieson's [16]. All arterial specimens were then categorized as diffuse intimal thickening, fatty streak, fibrous plaque, or complicated plaque, based on gross and

Table 1. Summary of clinical data

Pathological finding	Autopsy/Surgery	Age, Sex	HL	HT	DM	Smoking	Clinical Diagnosis
Diffuse intimal thickening	Aneurysmoplasty	37, M	—	+	+	unknown	Aneurysm
	Autopsy	20, F	—	—	—	—	Gastric cancer
	Autopsy	40, M	—	—	—	—	Lymphoma
	Autopsy	34 wk*, F	—	—	—	—	18 trisomy
	Autopsy	58, M	—	—	—	+	Pancreatic cancer
Fatty streak	Aneurysmoplasty	72, F	—	+	—	+	Aneurysm
	Autopsy	60, M	—	—	+	+	Gastric cancer
	Autopsy	34, F	—	—	—	+	Lung cancer
	Autopsy	76, F	—	—	—	unknown	Pancreatic cancer
	Autopsy	66, M	—	+	—	—	Amyotrophic lateral sclerosis
Fibrous plaque	Aneurysmoplasty	68, M	unknown	—	+	unknown	Aneurysm
	Aneurysmoplasty	62, F	—	—	—	+	Aneurysm
	Aneurysmoplasty	80, M	—	—	—	+	Aneurysm
	Endoatherectomy	66, F	+	—	—	—	Carotid atherosclerosis
	Endoatherectomy	67, M	+	+	—	+	Carotid atherosclerosis
	Endoatherectomy	55, M	+	+	+	+	Carotid atherosclerosis
	Endoatherectomy	66, M	—	+	—	+	Carotid atherosclerosis
	Endoatherectomy	74, M	+	+	—	+	Carotid atherosclerosis
	Endoatherectomy	45, M	—	+	+	+	Carotid atherosclerosis
	Autopsy	58, M	—	+	+	—	Gastric cancer
	Autopsy	47, M	+	+	—	+	Crow-Fukase syndrome
	Autopsy	46, F	—	+	—	—	Pemphigoid
	Autopsy	60, F	+	—	—	+	Rectal cancer

Clinical details of all cases for the current study are summarized. Risk factors for atherosclerosis (hyperlipidemia, hypertension, diabetes mellitus and smoking) are included when applicable. Arterial specimens are classified into diffuse intimal thickening, fatty streak and fibrous plaque based on gross and microscopical findings.

HL, hyperlipidemia (plasma cholesterol $\geq$ 220 mg/dl and/or plasma triglycerides $\geq$ 150 mg/dl); HT, hypertension (systolic blood pressure $\geq$ 160 mmHg and/or diastolic blood pressure $\geq$ 95 mmHg); DM, diabetes mellitus (fasting blood glucose $\geq$ 140 mg/dl); +, present; —, absent;

*, gestational.

microscopical features.

Immunohistochemical staining

Immunohistochemical staining was performed using a labeled streptavidin biotin kit (Nichirei Co, Tokyo) according to the manufacturer's instructions. Paraffin sections were treated with a graded concentration series of xylene and ethanol and immersed in 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. Specimens were then rinsed with phosphate buffered saline (PBS, pH 7.4) and incubated with 10% normal goat serum for 1 hr at room temperature. The primary antibodies (Abs) used in this study included mouse monoclonal Abs Kp-1 or HAM56 against human macrophage CD68, anti- α smooth muscle actin, UCHL-1 for human helper and cytotoxic/suppressor T lymphocytes. Rabbit polyclonal Ab against human apolipoprotein E (1 : 500) was also used. All primary Abs except for HAM56 were purchased from Dako (Glostrup, Denmark). Mouse monoclonal Ab HAM56 was purchased from Enzo Diagnostics (Farmingdale, NY). Mouse mAb 5D2 (1 : 200) (a kind gift from Dr. John Brunzell, University of Washington) has been described previously [14], and recognizes denatured LPL; therefore, this Ab is suitable for the detection of LPL by immunohistochemical staining. The specimens were incubated overnight at 4°C with each primary Ab and then rinsed 3 times with PBS, and incubated with biotinylated rabbit-anti-mouse or goat-anti-rabbit IgG (Nichirei) for 30 min at room temperature. The specimens were then rinsed 3 times with PBS and incubated with peroxidase-conjugated streptavidin for 30 min and the peroxidase reaction was visualized by using a 3-amino-9-ethylcarbazole (AEC) substrate (Dako). For a negative control study, primary antibodies were replaced by normal goat serum.

Double immunohistochemical staining

Simultaneous detection of two cellular epitopes in atherosclerotic lesions was achieved by double staining, and was used to confirm the macrophage specificity of LPL or apo E expression. This method employed a combination of Kp-1 and anti-apo E or anti-LPL primary Abs. The first primary Abs were detected by peroxidase-conjugated streptavidin developed with AEC substrate as red and second primary Abs were detected by alkaline phosphatase-conjugated streptavidin developed by fast blue substrate kit (Nichirei) as blue staining. After the first immunostaining, the slides were washed with 0.1 M glycine buffer (pH 2.2) three times for 1 hr at room temperature and then, the second immunostaining was performed. Co-expression of both antigens in the same cells was detected by the observation of overlapping blue/brown staining, which usually appeared purple.

III. Results

Diffuse intimal thickening

In aortas with diffuse intimal thickening, the lesions were characterized by proliferation of smooth muscle cells (SMCs) (Fig. 1A, B). Diffuse LPL staining was observed in all layers of the aortic wall, including the intima, media and adventitia (Fig. 1C). Basically, the lining endothelial cells were stained less for LPL than were the medial and intimal smooth muscle cells. Smooth muscle cells in both the intima and media were consistently positive for LPL. In the adventitia, small vessels, i.e., vasa vasorum, and adipose tissue were strongly positive for LPL (Fig. 1D). There was little, if any, extracellular-associated LPL in the aorta with diffuse intimal thickening. Apo E distribution was different from LPL distribution in the diffuse intimal thickening of the aorta. In this regard, apo E was observed mainly in the thickened intima, whereas the media and adventitia were negative. Furthermore, apo E in the intima was predominantly associated with the extracellular matrix (Fig. 1E). These results suggest that in fibrous, thickened intima, LPL is mainly produced by smooth muscle cells, whereas apo E may originate from plasma influx rather than being produced *de novo* by vascular cells.

Fatty streaks

A typical fatty streak lesion is shown in Fig. 2A and B. Fatty streaks were composed of accumulated macrophage-derived foamy and non-foamy cells (Kp-1 positive, A) and small numbers of T lymphocytes (UCHL-1 positive, data not shown). Some SMCs were dispersed beneath the fatty streak (Fig. 2B). Macrophages, either foamy or non-foamy cells, were almost invariably stained by anti-LPL Ab (Fig. 2C). In addition, intimal SMCs located beneath foam cells and medial SMCs were also positive for LPL staining. There was little, if any, LPL associated with the extracellular matrix.

To examine whether LPL was colocalized with macrophages, double immunohistochemical staining using 5D2 and Kp-1 Abs was performed. As shown in Fig. 2E, LPL was detected within macrophages, as visualized by the overlapping staining purple pattern.

Apo E was widely stained in fatty streaks, especially in the areas of macrophages (Fig. 2D). These macrophages were variously stained; some contained apo E-positive granules in the cytoplasm and others showed apo E staining on the cellular membrane. There was a tendency for staining intensity of apo E on macrophages to be stronger for foamy cells than for non-foamy cells. In these areas, there was a lot of apo E-immunoreactive proteins associated with the extracellular matrix (Fig. 2D). We performed double immunohistochemical staining using anti-apo E and Kp-1 Abs to quantitate these apo E-positive macrophages. Apo E was stained brown by AEC and macrophages were stained blue by fast blue, and double staining of both appeared purple. As shown

Fig. 1

Fig. 2. Immunohistochemical staining of fatty streak. Serial sections of aorta with fatty streaks were immunohistochemically stained with (A) anti-macrophage CD68, (B) anti- α smooth muscle actin, (C) anti-LPL, and (D) anti-apo E. (E) and (F) were double stained using either anti-LPL or anti-apo E and anti-macrophage Abs, as described in Materials and Methods (E; anti-LPL (blown) and anti-macrophage (blue), F; anti-apo E (blown) and anti-macrophage (blue)). The typical fatty streak is mainly composed of accumulated macrophage-derived foam cells (CD68 positive), beneath which stellate or spindle-shaped SMCs are present (B). LPL is detected in both macrophages and smooth muscle cells. LPL is colocalized with macrophages, as shown by double staining. Apo E is found in macrophages and the extracellular matrix, and some macrophages are associated with apo E deposition, as shown by double staining. Magnification $\times 250$.

Fig. 1. Micrograph of aorta with diffuse intimal thickening. (A) Hematoxylin eosin staining, and (B) immunohistochemical staining with mAb against α smooth muscle actin, (C, D) against human LPL and (E) against apo E. The aorta with diffuse intimal thickening is characterized by proliferation of intimal SMCs. In these areas, LPL is detected in the SMCs in both the intima and media. Endothelial cells are weakly stained with LPL Ab. Small vessels (vasa vasorum) in the adventitia (D) are positive for LPL staining. Apo E (E) is found mainly in the intima and is associated with the extracellular matrix. The arrow indicates the internal elastic lamina. Magnification $\times 250$.

in Fig. 2F, most macrophages were stained purple. Extracellularly-associated apo E, stained brown, was dispersed around macrophages.

Fibrous plaques

Fibrous plaques were characterized by the presence of varying amounts of atheromatous substances and fibrous

Fig. 3. Micrograph of fibrous plaque. Serial sections of aorta with fibrous plaque were stained with (A) hematoxylin eosin staining, (B) anti-macrophage, (C) anti-LPL, and (D) anti-apo E Abs. (E) and (F) were double stained for either LPL or apo E and macrophage. The lipid core of the lesion is on the right and the shoulder is on the left. The shoulder is composed of an accumulation of foamy macrophages stained by HAM56 Ab (B), and some spindle-shaped SMCs positively stained by anti- α smooth muscle actin Ab (data not shown). In the shoulder region, LPL staining is almost invariably positive for both macrophages and SMCs (C). Apo E is, however, mainly present in macrophages, and only rarely found in spindle-shaped SMCs (D). Also, both LPL and apo E are noted in the lipid core in the proximity of the shoulder (C, D). To confirm the *in situ* specificity of either LPL or apo E produced by macrophages in the shoulder, double immunostaining was performed as described in Materials and Methods. LPL and apo E are associated with macrophages (E, F) as shown by the purple staining. Magnification $\times 250$ (A–D), $\times 330$ (E, F).

caps and shoulders as shown in Fig. 3A. The center of the lesion was composed of cholesterol crystals, cellular debris and amorphous materials. Together with SMCs, macrophage-derived foam cells were often markedly accumulated in the shoulder region (Fig. 3A, B). LPL was invariably observed in macrophage-derived foam cells and smooth muscle cells (Fig. 3C). In the fibrous cap and shoulder in the proximity of the lipid core, macrophages were invariably stained by anti-apo E Ab but spindle-shaped SMCs were basically negative (Fig. 3D). In addition, the lipid core region in close proximity to the shoulder was weakly stained for LPL and apo E, suggesting that these immunoreactive products are either from macrophage break-down or infusion. To confirm the *in situ* specificity of the LPL and apo E produced by macrophages in the shoulder, double immunostaining was performed using two different Abs, either anti-LPL or -apo E and anti-macrophage, as for fatty streak, and the results are shown in Fig. 3E and F. Double immunohistochemical staining revealed that both LPL and apo E staining (brown) were superimposed with macrophage staining (blue), such that the staining was visualized as purple in color. These macrophages were found to contain substantial amounts of LPL and apo E in their cytoplasm (Fig. 3E, F).

IV. Discussion

This study demonstrated that LPL is produced by a variety of vascular cells in various stages of atherosclerosis. In the arterial wall with diffuse intimal thickening, it is likely that the major source of LPL is from smooth muscle cells. LPL is synthesized in smooth muscle cells, released to the endothelial surface and anchored to the heparan sulfate proteoglycan. LPL catalyzes hydrolysis of triglycerides of triglyceride-rich lipoproteins and provides energy and nutrition for the arterial wall [11, 20].

In atherosclerotic lesions, fatty streaks and fibrous plaques, both foamy and non-foamy macrophages, along with smooth muscle cells, can produce LPL. In fatty streaks, macrophages and the extracellular matrix around them showed strong staining for LPL. In fibrous plaques, in addition to macrophages, the lipid core was also stained by anti-LPL Ab. This extracellularly associated LPL may be secreted by macrophages or smooth muscle cells, or may be due to the break-down of foam cells.

Elevated levels of macrophage-derived LPL may facilitate the production of free fatty acids through hydrolysis of triglycerides, and provide enough energy for macrophages themselves in areas where there are limited amounts of ambient glucose [26]. Furthermore, LPL may enhance the cellular uptake of lipoproteins, thus enabling macrophages to transform into foam cells [4, 7, 21]. It has been reported that LPL can increase the retention of apo B- and apo E-containing lipoproteins through binding to cells or to matrix components, especially heparan sulfate proteoglycans [11, 20]. Also, treatment of very low

density lipoproteins from hypertriglyceridemia by LPL increases the uptake of these particles by macrophages [4, 21]. Therefore, enhanced production or release of LPL in the atherosclerotic intima would facilitate the development of lesions. To test this hypothesis, it will be necessary to create transgenic animals with macrophage-specific expression of LPL. This work is currently being performed in our laboratory.

Several *in vitro* studies have shown that secretion of LPL and apo E is regulated coordinately by macrophage differentiation and various cytokines [6, 17, 19, 21]. In this study, we were particularly interested in investigating the colocalization of LPL and apo E in atherosclerotic lesions, especially in macrophage-derived foam cells. To our surprise, LPL was shown to be produced by smooth muscle cells as well as macrophages, but apo E was found only in macrophages. There was a tendency for apo E staining in macrophages to be increased in foamy macrophages compared to nonfoamy macrophages. This result indicates that in atherosclerotic lesions, LPL production and apo E production from macrophages are regulated differently. It seems likely that there is up-regulation of production of apo E in macrophages when these cells are filled with lipids, i.e., in the state of foam cells.

In conclusion, the results of this study suggest that LPL and apo E are both present in atherosclerotic lesions. LPL is produced by a variety of cellular sources, whereas apo E is produced by macrophages. Although both proteins are associated with foam cells in atherosclerotic lesions, it still remains unknown how these proteins contribute to foam cell formation during atherosclerosis.

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VI. References

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