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Localization of T lymphocytes and macrophages expressing IL-1, IL-2 receptor, IL-6 and TNF in human aortic intima. Role of cell-mediated immunity in human atherogenesis

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Abstract. Recent observations have demonstrated the presence of activated T lymphocytes and macrophages in human atherosclerotic lesions. Cells found within these lesions produce cytokines that alter vascular homeostasis in a manner that promotes atherogenesis. To elucidate the role of these immunocompetent cells in human atherosclerosis, the localization of various cytokines with an analysis of immunophenotypic features of the cellular infiltrates was studied in normal aortas from children; and in later phases of the disease (including fatty streaks and fibrous or atheromatous plaques). Semi-quantitative analysis of cytokine-expressing cells was also investigated with serial sectioning. In 4 of 9 young subjects, the grossly normal aorta contained relatively cell-rich areas which were located preferentially around the ostia of intercostal arteries and were composed of isolated or layered T lymphocytes and macrophages. In these prelesional areas, interleukin-1 (IL-1), IL-2 receptor (IL-2R) and tumour necrosis factor (TNF) were detected in the cytoplasm of the infiltrating cells, whereas no detectable reactivity was noted for IL-2, IL-6, interferon-γ (IFN-γ) or lymphotoxin (LT). In fatty streaks and full-grown atheromas including "cap" and "shoulder" regions, various numbers of T lymphocytes, macrophages and macrophage foam cells were present. In these lesion areas, especially where the cellular infiltrates were numerous, macrophage foam cells and smooth muscle cells expressed not only IL-1 and TNF but also IL-6. The ratio of IL-2R positive cells showed a tendency to decrease with advance of the disease process. Electron-microscopic examination of lesion areas demonstrated ultrastructural aspects of the cognate cell-to-cell interaction, as shown by the direct apposition of lymphocytes to macrophages or macrophage foam cells. These results suggest that a specific in situ, cell mediated hypersensitivity plays a pivotal role in the nascent as well as the progression stages of human atherosclerosis.

Key words: Cytokine – Immunocompetent cells – Immunohistochemistry – Human atherosclerosis

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Introduction

Human atherosclerosis is a progressive disease, which generally begins in childhood and does not become manifest until middle-age or later. Its aetiology and pathogenesis are multifactorial and have been difficult to elucidate. Recently, the inflammatory nature of atherosclerosis has been gaining interest (Joris and Majno 1978; Ross 1986; Schwartz et al. 1986; Munro and Cotran 1988). Histological and ultrastructural observations of human arteries indicate the presence of mononuclear phagocytes in the atherosclerotic lesion (Stary 1987; Watanabe et al. 1989). Furthermore, in experimental animals fed atherogenic diets, nascent intimal changes at the prelesional stages have been detected, such as a preferential adherence and intimal penetration of blood-borne monocytes in lesion prone areas (Gerrity 1981; Faggiotto et al. 1984; Watanabe et al. 1985; Rosenfeld et al. 1987).

Thus far, several immunocytochemical investigations have shown unequivocally that there are, in addition to macrophages, comparatively numerous T lymphocytes in atherosclerotic lesions (Jonasson et al. 1986; Munro et al. 1987; Emeson and Robertson 1988; Hansson et al. 1989a; van der Wal et al. 1989). Moreover, we demonstrated in a previous study that T lymphocytes and macrophages with little cytoplasmic lipid are present beneath the endothelium on grossly normal, prelesional areas of the aorta of young subjects (Shimokama et al. 1991). The findings obtained from such human prelesional areas were almost identical to those of the early events observed in experimental animals (Joris et al.

1983; Faggiotto et al. 1984; Masuda and Ross 1990). This selective accumulation of T lymphocytes and monocytes/macrophages may imply a possible participation of cell-mediated immune reactions in human atherosclerosis (Davies 1986; Hansson et al. 1989b; Libby and Hansson 1991). Further, their interaction and subsequent cytokine production are known to be involved in the pathogenesis of a variety of human diseases (Husby and Williams 1985; Hancock et al. 1986; Arnoldi et al. 1990; Nickoloff et al. 1991).

The purpose of the present study is to elucidate specific interaction between T lymphocytes and macrophages in the aortic intima, and to determine the in situ localization of several cytokines (IL-1, IL-2, IL-6, TNF, LT and IFN-γ), all of which are putatively implicated in vascular immune control in vivo (Hansson et al. 1989b; Libby and Hansson 1991). Particular effort was made to study the presence of cytokines in the evolutional changes of atherosclerosis, including the examination of grossly normal aortas from children. Ultrastructural studies concerning the direct in situ interactions between the immunocompetent cells are also attempted in the different evolving phases of atherogenesis.

Materials and methods

During autopsies, performed within 4 h after death, aortic specimens were collected from 79 autopsied patients with an age range from 4 to 88 years (37 males and 42 females). Septic and immunosuppressed patients were excluded from the study. After close inspection of the aorta, appropriate regions were chosen and excised. Three kinds of regions were selected: normal on gross inspection, fatty streak represented by grossly visible, slightly raised, yellow areas, and fibrous plaque or atheroma usually having grossly visible areas of central necrosis. In particular, specimens of normal tissue on gross examination were obtained from areas near the ostia of intercostal arteries of the descending thoracic aorta.

Pieces of the lesion, typically 3×15 mm in size, were bisected. One-half was embedded in OCT compound (Miles, Elkhart, Inn., USA), dropped into a bath of hexane precooled in a cryocool immersion cooler, and used for immunohistochemistry. The other half was fixed in 3% glutaraldehyde for electron-microscopic study. Frozen sections were cut at 5 µm, placed on glass slides coated with ovalbumin adhesive, and air-dried at room temperature for 1 h. Each section was fixed by cooled acetone (−20° C) for 10 min and washed vigorously in phosphate-buffered saline (PBS), pH 7.2.

The sources and specificities of the antibodies used in this study are summarized in Table 1.

The sections were processed and stained as previously described (Shimokama et al. 1991). Briefly, application of murine-derived monoclonal antibodies or rabbit-derived polyclonal antibodies was followed by a second stage of a biotin-conjugated rabbit anti-mouse antibody or biotin-conjugated goat anti-rabbit antibody (Bio Genex, San Ramon, Calif., USA), which, in turn, was followed by a third stage of peroxidase-conjugated streptoavidin (Bio Genex). Peroxidase activity was detected by incubation with diaminobenzide (Sigma, St. Louis, Mo USA; 0.6 mg/ml in 0.1 M TRIS-HCl buffer, pH 7.6, plus 0.03% hydrogen peroxide) containing 0.3% sodium azide to block endogeneous peroxidase activity. A control study was performed by omitting the primary antibody, which resulted in no labelling in all instances. The specificity of antibodies was further confirmed by pre-incubation with corresponding recombinant cytokines (Genzyme, Boston, Mass., USA). This procedure invariably abolished the immunoreaction.

Double immunostaining was carried out to elucidate the type of immunocompetent cells secreting cytokines. In the absence of normal non-immune serum, the slide was incubated with first primary antibody against IL-1, IL-6, TNF, or IL-2R overnight at 4° C. After washing, the slide was treated with the corresponding biotin-conjugated antibody and peroxidase-conjugated streptoavidin successively. The specific deposits were developed using 3-amino-9 ethylcarbazole (Sigma). After the slide was carefully washed, second primary antibody against T lymphocytes (Leu 4) or macrophages (Leu M3 or OKM1) was applied. Thereafter, the slide was incubated successively with the corresponding biotin-conjugated antibody and alkaline phosphatase-conjugated streptoavidin. The positive immunoreaction for T lymphocytes or macrophages was visualized by incubating the specimen in fast blue (Sigma) for 10 min.

Table 1. Antibodies used in this study

Antigen	Antibody	Type	Source
CD14	LeuM3	MoAb	Becton-Dickinson[a]
CD11b	OKM1	MoAb	Ortho[b]
CD3	Leu4	MoAb	Becton-Dickinson
CD4	OKT4	MoAb	Ortho
CD8	Leu2a	MoAb	Becton-Dickinson
CD21	OKB7	MoAb	Ortho
HLA-DR	OKDR	MoAb	Ortho
von-Willebrand factor	Anti-FVIII	Polyclonal	M.B.L.[c]
β chain of $\alpha\beta$ T cell receptor	βF1	MoAb	T Cell Sciences[d]
γ chain of $\gamma\delta$ T cell receptor	CγM1	MoAb	T Cell Sciences
IL1	Anti-IL1	Polyclonal	Genzyme[e]
IL1β	Anti-IL1β	Polyclonal	Endogen[f]
IL2	Anti-IL2	Polyclonal	Genzyme
IL2R	Anti-IL2R	MoAb	Becton-Dickinson
IL6	Anti-IL6	Polyclonal	Genzyme
TNF	Anti-TNF	Polyclonal	Endogen
LT	Anti-LT	Polyclonal	Genzyme
IFNγ	Anti-IFNγ	Polyclonal	Genzyme

[a] Becton-Dickinson, Mountain View, Calif., USA
[b] Ortho, Ortho Pharmaceutical Corporation, Paritan, N.J., USA
[c] M.B.L., Medical Biological Laboratories, Nagoya, Japan;
[d] T Cell Sciences, Inc., Cambridge, Mass., USA
[e] Genzyme, Genzyme Corporation, Boston, Mass., USA
[f] Endogen, Boston, Mass., USA

Semi-quantitative analysis was performed in the three different types of aortic lesions in which a series of cytokines were sequentially immunostained in parallel sections, using 6 samples of grossly normal intima from 4 young subjects in which a number of macrophages and T lymphocytes was demonstrated in the intima, 11 cases of fatty streaks, and 10 cases of atheromatous shoulder regions which contained cell-populated lesion areas. The positive cells were counted and expressed as a percent of 100 counted cells in randomly selected 4 fields of each lesion at a magnification of ×400.

The specimen for electron-microscopic study was fixed in cacodylate-buffered glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in a graded series of alcohols, and embedded in Epon 812. Silver-gray ultra-thin sections were cut on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a JEOL-100 CX transmission electron microscope.

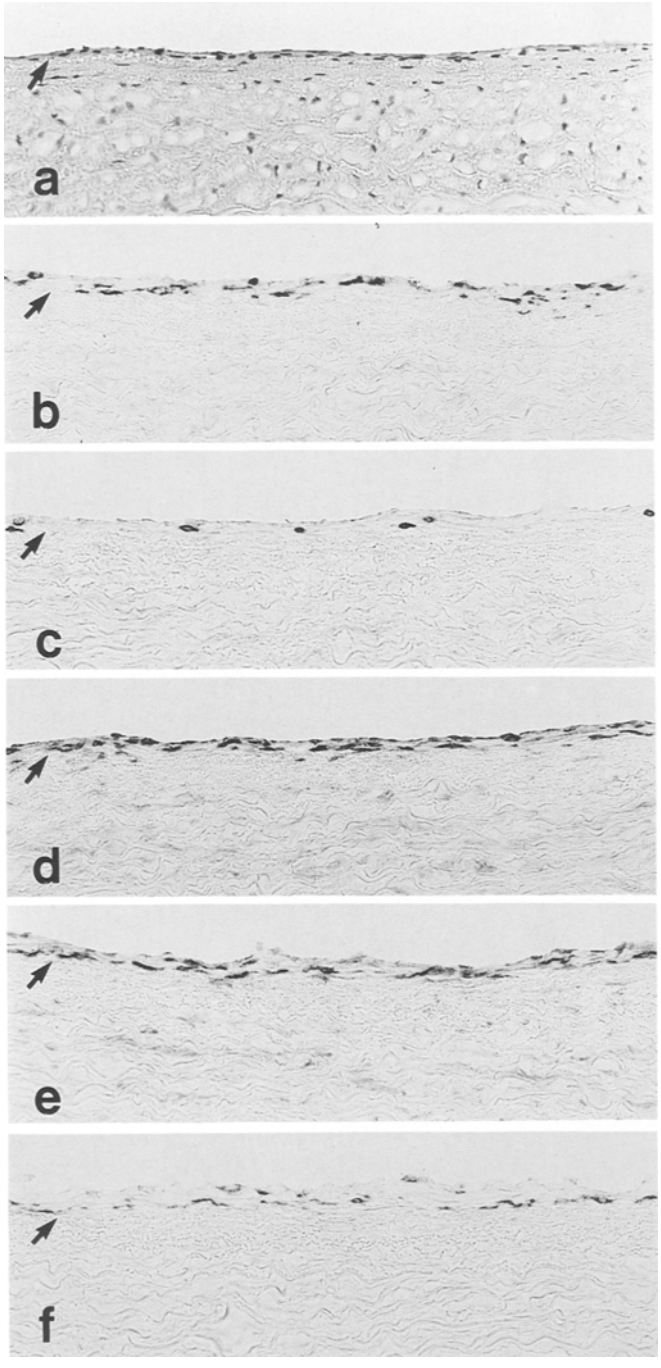

Fig. 1a–f. Grossly normal aorta of an 8-year-old female. **a** Intimal thickening is almost absent; *arrow* indicates the internal elastic lamina. Haematoxylin-eosin stain. **b** OKM1 positive monocytes/macrophages are present in rows beneath the endothelium. **c** The intima also contains scattered Leu 4 positive T lymphocytes. **d, e** Positive reaction against anti-IL-1 (**d**) or -TNF (**e**) antibody is detected in the cytoplasm of infiltrated cells. **f** Infiltrated cells in the intima express IL-2R on the cell surface. Original magnification ×80

Results

All the sections obtained from human aortas were studied under standard light microscopy, and then the sections with cell-rich areas were selected for investigation by immunohistochemistry and electron microscopy. The cell-rich areas were generally composed of mixed populations of T lymphocytes, macrophages and smooth muscle cells. Further, variable numbers of IL-1, IL-6, TNF and IL-2R expressing cells were detected in these areas. The details concerning the three different evolving phases of atherosclerosis were described separately.

Grossly normal tissue of the thoracic aorta was obtained from 9 cases of children from 4 to 14 years of age. In 5 of the cases, light microscopy revealed no thickening of the intima, and further immunohistochemical studies confirmed that there were no cells that reacted with anti-monocyte/macrophage or anti-lymphocyte antibodies in the intima. In the other 4 cases (4-, 7- and 14-year-old males and an 8-year-old female), in which

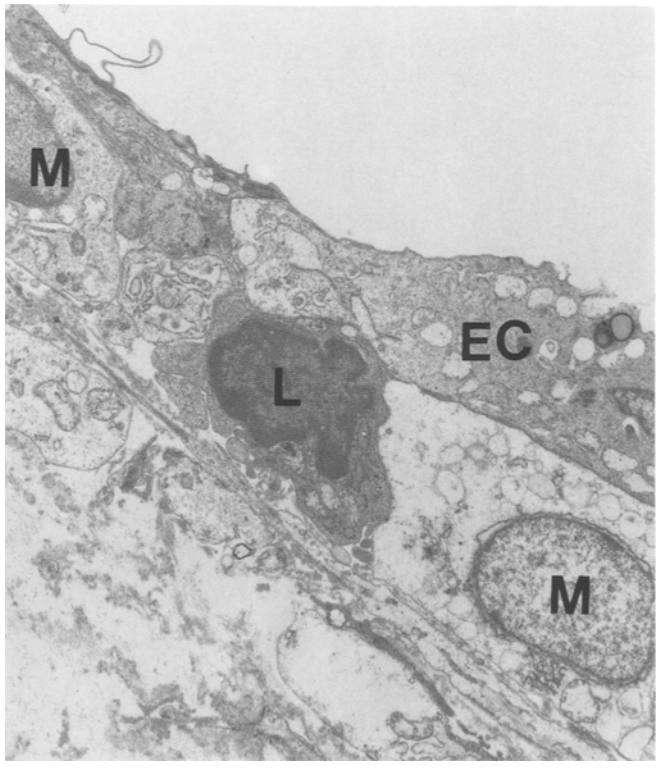

Fig. 2. Electron micrograph of grossly normal aorta of a 14-year-old male. Macrophages without lipid droplets (*M*) and a lymphocyte (*L*) are lying beneath the endothelial cells (*EC*). They are in direct contact with each other. Original magnification ×6,900

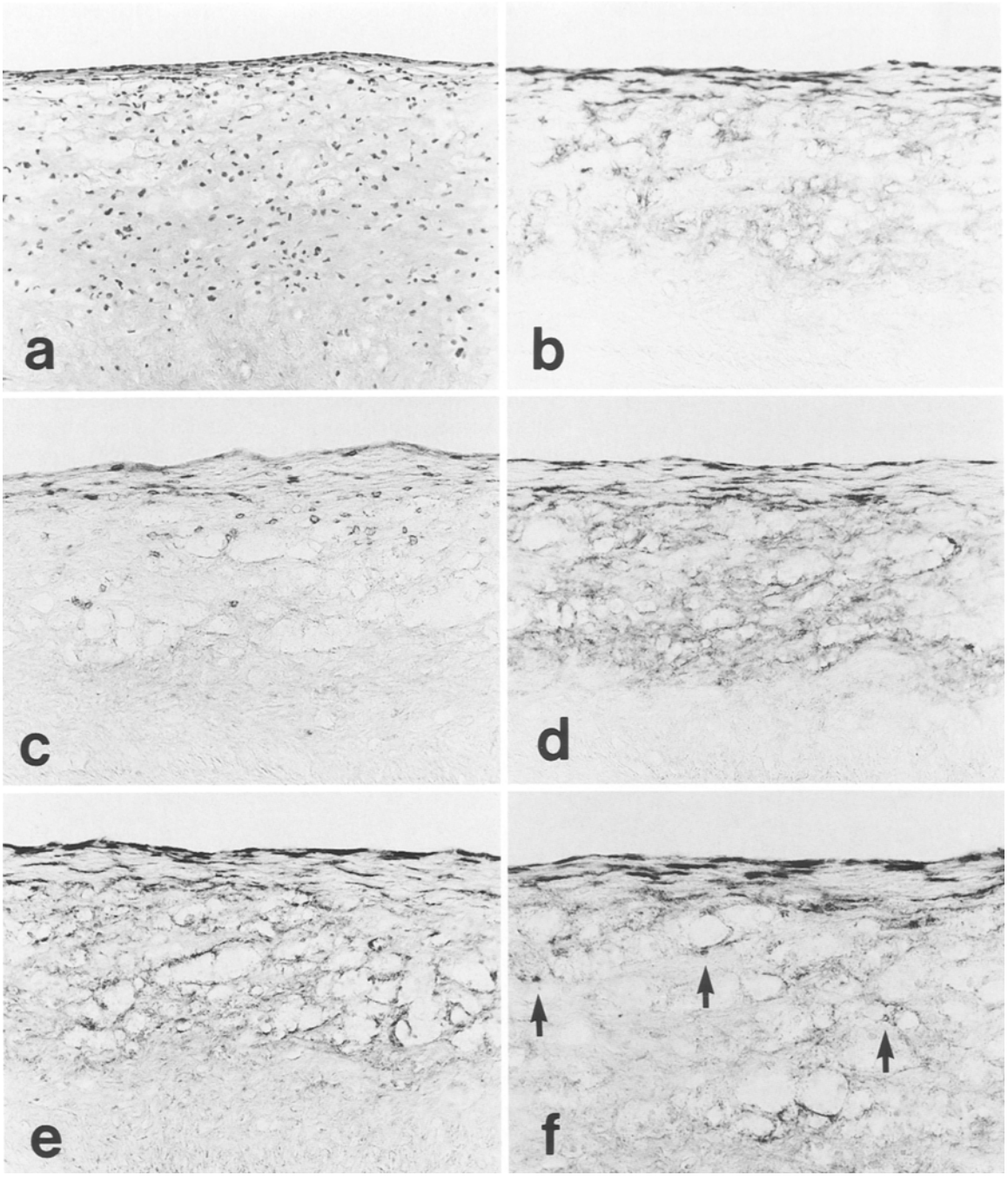

Fig. 3a–f. Fatty streak lesion from the thoracic aorta of a 32-year-old male. **a** The intima is moderately thickened due to aggregates of foam cells intermixed with mononuclear and spindle-shaped cells. Hematoxylin-eosin stain. **b** Foam cells in the deeper portion show distinct positive reaction against anti-monocyte/macrophage antibody (OKM1) along the cell membrane. Spindle-shaped cells in the upper portion are also positively stained. **c** Leu 4 positive T lymphocytes scattered throughout the lesion. **d, e** Not only macrophage foam cells but also mononuclear cells without lipid droplets show positive reaction against anti-IL-1 (**d**) and Il-6 (**e**) antibodies. **f** IL-2R positive cells are mostly spindle shaped and predominate in the superficial portion. *Arrows* show small, round cells expressing IL-2R in the deeper portion. Original magnification ×80

light microscopy showed almost normal or slightly thickened intima, immunostaining uncovered isolated or layered cells that reacted with anti-monocyte/macrophage (Fig. 1b) and anti-T lymphocyte (Fig. 1c) antibodies beneath the endothelial cells in the sections obtained from the area between the ostia of the intercostal arteries, a favorite site of fatty streak or atheromatous lesions. In contrast, these blood-derived cells were not found in the sections taken from the contralateral side of the aorta. Additionally, these macrophages were small in size and not necessarily loaded with lipid, as evidenced by oil red-o staining (data not shown). No B lympho-

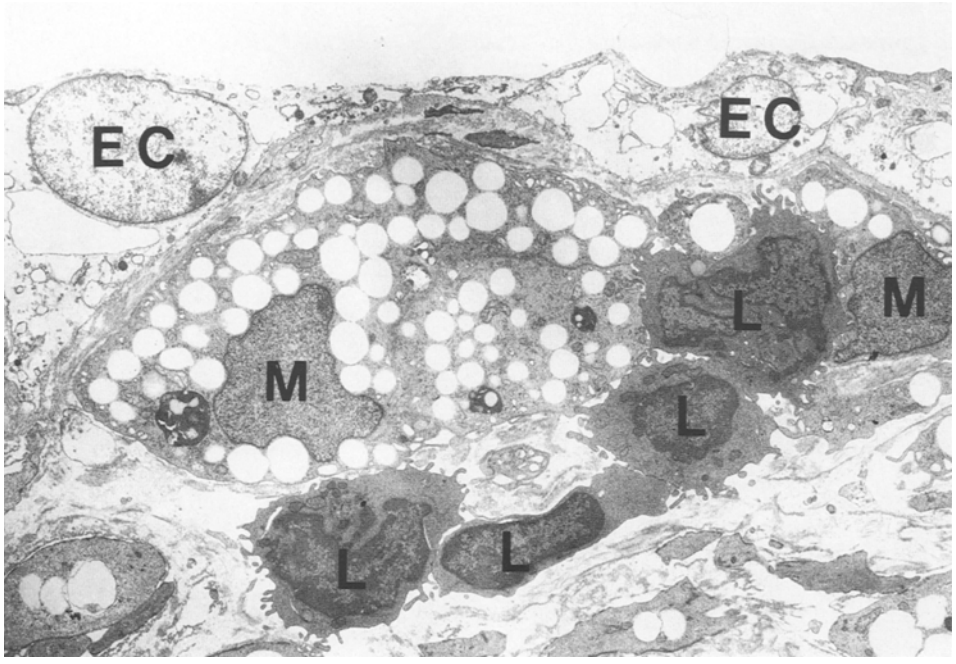

Fig. 4. Electron micrograph of a fatty streak lesion from a 32-year-old female. Macrophages laden with many lipid droplets (*M*) and smaller lymphocytes (*L*) lie beneath the endothelial cells (*EC*). The lymphocytes are in direct contact with not only macrophages but also other lymphocytes. Original magnification ×2,000

cytes were detected. Of particular note is that variable numbers of the infiltrated cells in the intima showed cytoplasmic positive reactions with the antibodies against IL-1 (Fig. 1d) and TNF (Fig. 1e). Further, a considerable number of cells expressed IL-2R on their cell surface or in the cytoplasm (Fig. 1f). No cells expressing IL-2, IL-6, LT or IFN-γ, however, were detected in any of the sections examined. No cytokine-producing cells were seen in the subjacent media.

Electron microscopy confirmed the infiltration of cells apparently derived from a monocyte/macrophage lineage and lymphocyte in the grossly normal intima of children. As was implied by light-microscopic and immunohistochemical observations, lipid inclusions were inconspicuous in the macrophages. These macrophages and lymphocytes were arranged in a row in the subendothelial space and often showed direct apposition to each other (Fig. 2).

Grossly fatty streaks were identified in 35 patients ranging in age from 32 to 56 years. Foam cells were located immediately underneath the endothelium, sometimes forming a continuous row of cells, or capped by thin layers of fibrous tissue admixed with elongated or small round mononuclear cells (Fig. 3a). These foam cells as well as a number of elongated cells in the superficial portion were labelled by antibodies directed against monocytes/macrophages (Fig. 3b). In addition, the lesion often contained T lymphocytes (Fig. 3c), among which CD8+ cells predominated over CD4+ cells in a ratio varying from 2:1 to 4:1. These T lymphocytes reacted almost exclusively with anti-T cell receptor antibody βF1 (data not shown). In contrast, B lymphocytes were very sparse. The ratio between macrophage foam cells and T cells varied from lesion to lesion. Interestingly, there were approximately twice as many T cells as macrophages in cell-populated lesion area. In addition, T cells appeared to be located closely in association with foam cells and other mononuclear cells, rather than interspersed at random. Electron microscopy demonstrated the intimate relation between macrophages and T lymphocytes. As shown in Fig. 4, lipid-laden foam cells were often surrounded by smaller lymphocytes and those two types of cells were tightly apposed to each. In addition, reciprocal cohesion of macrophage-macrophage and lymphocyte-lymphocyte was also noted.

Fatty streaks contained varying numbers of cells expressing not only IL-1 (Fig. 3d) and TNF but also IL-6 (Fig. 3e) in all the samples studied. Foamy macrophages and a number of mononuclear cells in the superficial portion showed a positive cytoplasmic reaction to these anti-cytokine antibodies. The staining property of foam cells in the deep area appeared less intense than that of mononuclear cells present in the cap region, and closer examination exhibited extracellular cytokine deposits resembling thin granules or diffuse areas surrounding the foam cells (Fig. 3d, e). The intensity of a cytoplasmic positive reaction is seen to be weakened, presumably due to accumulation of fat droplets. A number of IL-2R positive cells were interspersed in the lesion, particularly in the superficial portion (Fig. 3f), but no cells positive for IL-2, LT, IFN-γ were detected.

To identify the cell type of intimal cells producing cytokines, a double immunostaining technique was carried out. As exemplified in Fig. 5a, IL-1 producing cells were red-coloured and macrophages were blue-coloured by positive reactions with the respective antibodies. The cells elicited a reddish-blue mixed colour, and thus the results indicated that most of IL-1 positive cells were identified as macrophages. A small number of spindle-shaped IL-1 positive cells which had not reacted with

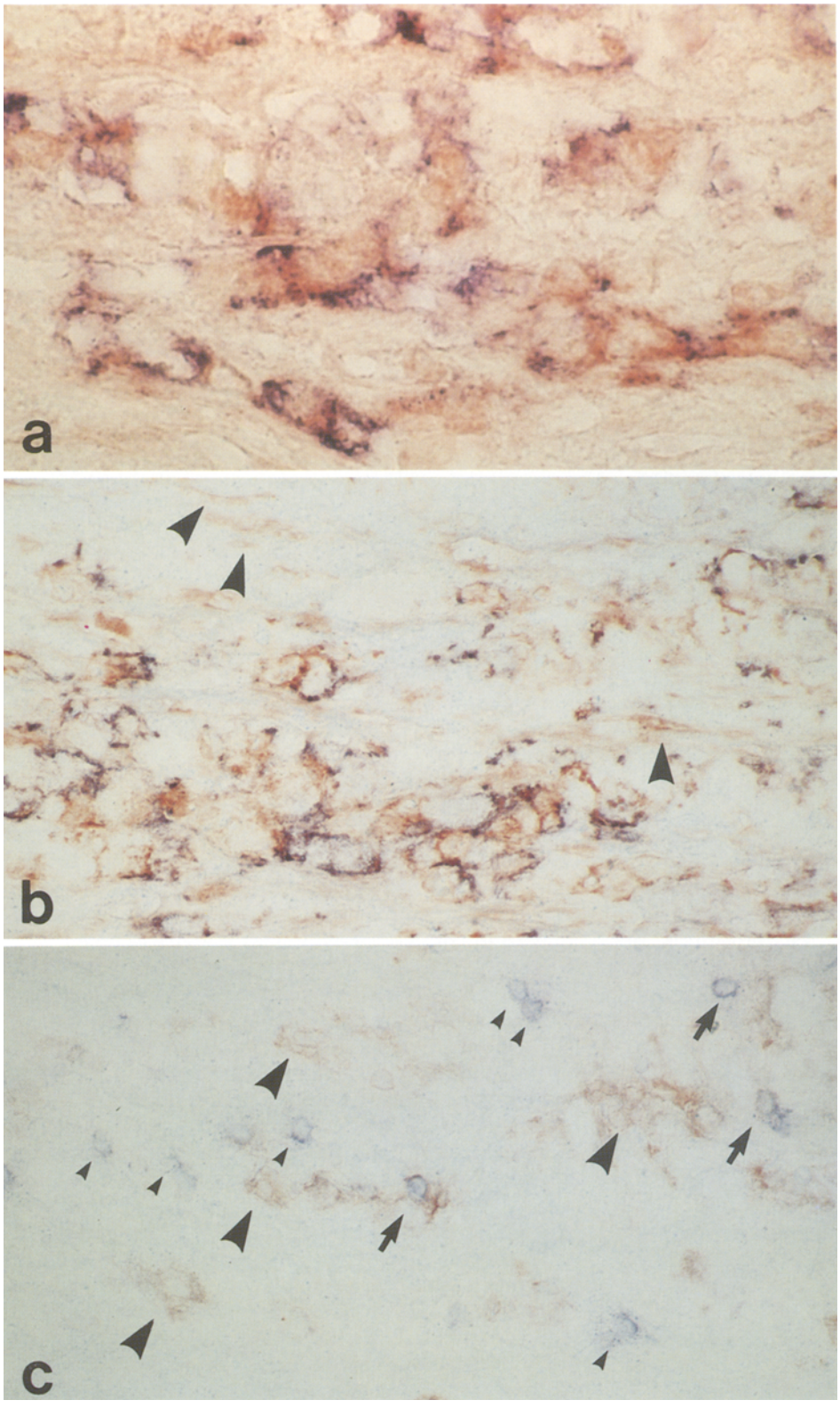

Fig. 5a–c. Double immunostaining of fatty streak lesions. **a** Foam cells show the combined staining with IL-1 (*red* with peroxidase method) and OKM1 (*blue* with alkaline phosphatase method). Double-stained cells (*purple*) represent IL-1 producing monocytes/macrophages. **b** Purple-stained cells (double expression of IL-6 and OKM1) are macrophage foam cells. Single-stained red cells (IL-6) most likely are of smooth muscle cells from their morphological features (*arrowheads*). **c** The combined staining with IL-2R (*red*) and Leu 4 (*blue*). IL-2R expressing T lymphocytes (double-stained cells, *arrows*) lie embedded within the foam cells. Single-stained blue cells are IL-2R negative T lymphocytes (*small arrowheads*). Single-stained red cells are IL-2R positive macrophages (*large arrowheads*). Original magnification ×100

either anti-monocyte/macrophage or anti-lymphocyte antibodies were regarded as being smooth muscle cells. In the same way, the majority of IL-6 (Fig. 5b) and TNF positive cells were shown to be macrophages. Smooth muscle cells also comprised part of them, whereas IL-2R bearing cells were composed of a mixed population of T lymphocytes and macrophages (Fig. 5c).

Atherosclerotic plaques were taken from 38 patients ranging in age from 54 to 83 years. The lesion could be divided into cap, shoulder and necrotic core. In 10

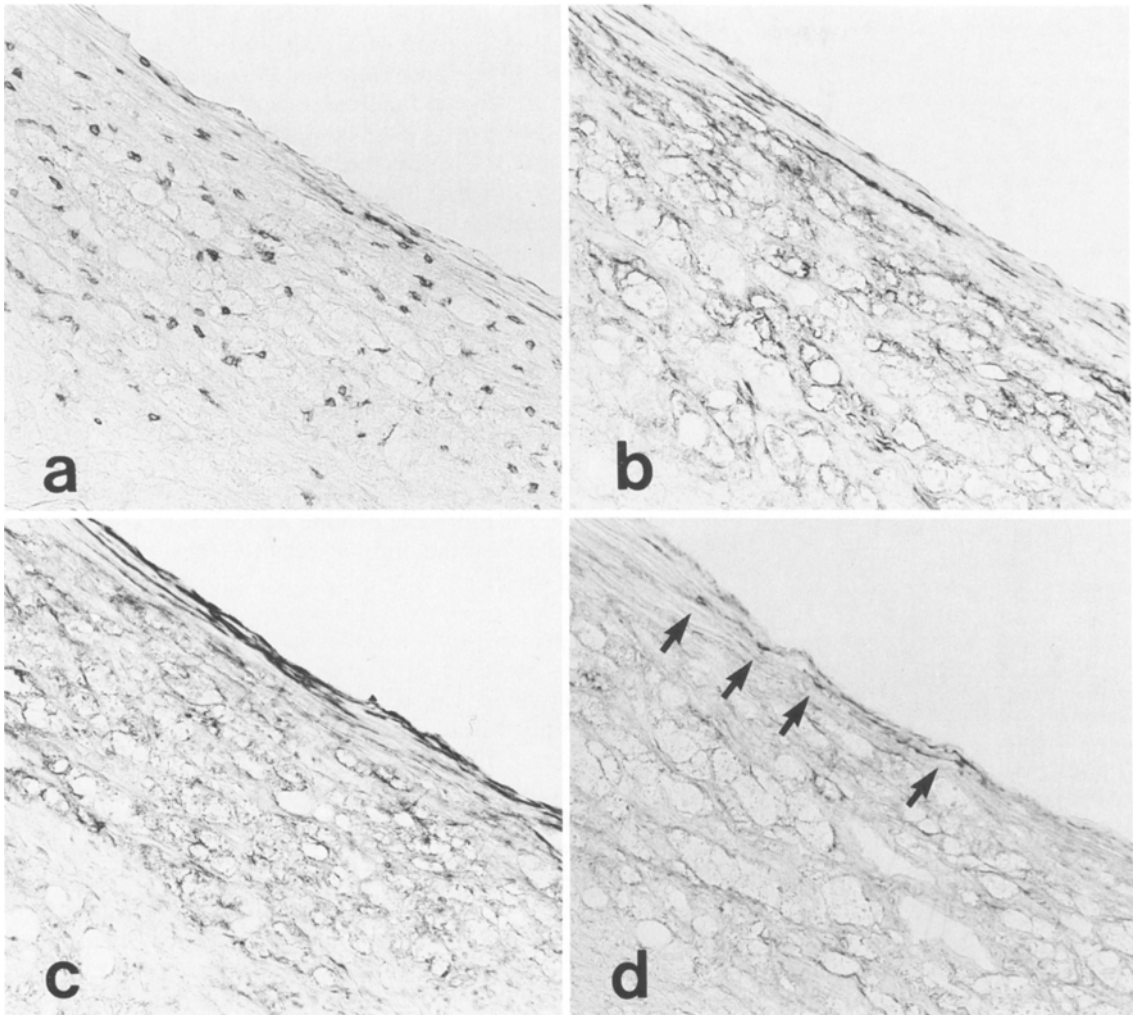

Fig. 6a–d. Atheromatous shoulder region obtained from a 62-year-old male. The necrotic core was not included in the figure at the lower left side. **a** Aggregates of macrophage foam cells are dotted with Leu 4 positive T lymphocytes. **b, c** Spindle-shaped mononuclear cells in the upper portion show cytoplasmic positive reaction against anti-IL-1 (**b**) and IL-6 (**c**) antibodies. Foam cells in the deeper portion reveal pericellular or membranous positive reaction against these antibodies. **d** IL-2R positive cells (*arrows*) are few in number regardless of cell-populated region. Original magnification ×80

cases, the plaque displayed a highly cellular shoulder region where constituting cells were arranged immunohistologically in a similar pattern as that observed in fatty streaks (Fig. 6a). Cells expressing cytokines IL-1, IL-6 and TNF were often identified in such a cellular area (Fig. 6b, c). IL-2R positive cells were also present, but their number was invariably small (Fig. 6d). The numbers of macrophages and T lymphocytes in the cap varied from case to case. In general, the cap from the old subjects was fibrous and cellular elements were scanty, whereas atheromatous plaque from the young subjects was often covered by a cellular cap containing considerable numbers of macrophages and T lymphocytes. These cellular caps variably contained IL-1, IL-6 and TNF positive cells (data not shown). The number of IL-2R positive cells were generally small in cap regions.

Figure 7 shows the distribution of IL-1, IL-6, TNF and IL-2R positive cells in the three different types of aortic lesions. The results concerning IL-2, LT and IFN-γ are not included, since cells expressing these cytokines were not evident in all sections studied. IL-1 and TNF were detected in all three lesions. Their percentage distribution showed considerable individual variations. On the whole, however, the numbers of these cytokine positive cells were lower in atheromatous plaques (mean; IL-1 51.3%, TNF 43.4%) than those in grossly normal areas (mean; IL-1 65.9%, TNF 58.8%) and in fatty streaks (mean; IL-1 60.7%, TNF 58.8%). IL-6 and IL-2R expression showed interesting features with disease progression. The relative number of IL-2R positive cells was much greater in grossly normal areas (mean; 53.3%) than that in both fatty streaks (mean; 15.6%) and atheromatous plaques (mean; 6.9%). In atheromatous plaques, the shoulder region contained a small but a distinct number of IL-2R positive cells. In contrast, IL-6 expressing cells were not detected in any of the grossly normal areas examined, whereas fatty streaks and atheromatous plaques contained variable numbers of IL-6 positive cells (mean 55.3% and 44.7%, respectively).

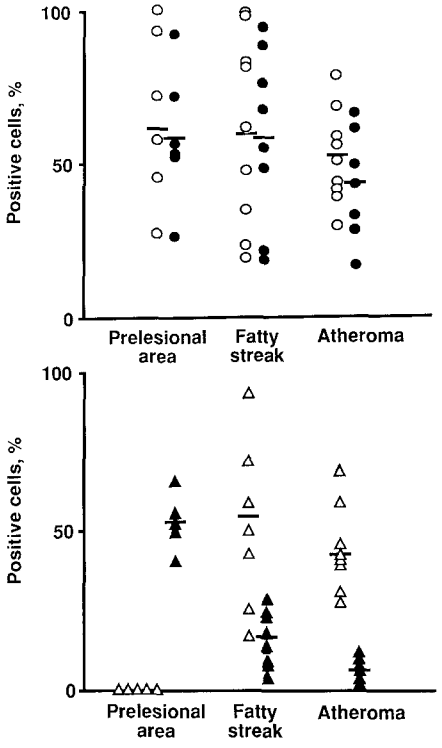

Fig. 7. Distribution of IL-1, IL-6, TNF and IL-2R expressing cells in three stages of human atherosclerosis: grossly normal aorta (prelesional area), fatty streak, and atheromatous shoulder region (atheroma). The upper panel shows the distribution of IL-1 (*open circles*) and TNF (*filled circles*) positive cells. The lower panel shows IL-6 (*open triangles*) and IL-2R (*filled triangles*) positive cells. Horizontal lines indicate the mean values

Discussion

Recent investigations have clarified the specific cellular constituents in human vascular lesions with modern methods including immunohistological techniques. Our findings presented here demonstrated that T lymphocytes and macrophages not only coexist in intimate relation, but also function in a similar fashion as evidenced by cytokine production. Especially important is that those observations were made in morphologically distinct types of atherosclerotic lesions. There is general acceptance that fatty streak is the first pathologically defined lesion of atherogenesis (Stary 1987; Ross 1992). To the best of our knowledge, however, the question of the presence or frequency of "prelesional" change in normal-looking intima, and its relation to ubiquitous fatty streaks had either not been asked or had not been pursued. One of the most intriguing findings of this study was the demonstration of cell-rich areas in normal-looking intima of children. It seems noteworthy that such prelesional areas consisted of rows of macrophages and T lymphocytes accumulated in the intima near the ostia of intercostal arteries and in the posterior wall of the thoracic aorta, which are well-known to be the lesion-prone areas of fatty streaks and atherosclerosis (Schwartz and Mitchell 1962; Strong 1992). Immunohistochemical studies disclosed that these intimal cells expressed IL-1, TNF and IL-2R. Since these antigens were induced by activation of T cells and macrophages (Davies 1986; Libby and Hansson 1991), the findings suggested that T cell-macrophage interactions occurred even in the grossly normal intima of young subjects. Further studies by electron microscopy supported this notion by showing subendothelial migration and intimate co-localization of macrophages and T lymphocytes. Intracellular lipid uptake was not remarkable so that macrophages did not yet take on the appearance of foam cells. We consider that the findings obtained from such human prelesional areas were practically identical to those of the early events observed in experimental atherosclerosis (for review, see Watanabe et al. 1989). In spite of such similarities in morphology and location, however, at present we have no direct evidence that such lesions develop fatty streaks in the same location. What is more important, we feel, is to study prelesional areas of the intima because they may well be showing the earliest, or incipient, changes of atherosclerosis.

The present study revealed in all phases of atherosclerosis the presence of T cells and macrophages which, when activated, can secrete potent biological mediators that modify vascular and immune cell function (Munro and Cotran 1988; Davies 1986; Libby and Hansson 1991). In human atherosclerotic lesions, TNF was found in the cytoplasm of macrophages and smooth muscle cells (Barath et al. 1990), and also as granular deposits in the connective tissue matrix (Rus et al. 1991). Moyer et al. (1991) studied iliac lesions of *Cynomolgus* monkeys and reported that the predominant cells expressing IL-1 mRNA were foam cells in the intima. Apart from the findings of grossly normal intima, our observations on the distribution of IL-1 and TNF positive cells are compatible with these reports. Ikeda et al. (1992) recently demonstrated IL-6 gene transcription in fibrous plaques but not in normal intima of WHHL rabbits. In their report, however, it was unclear which cell types produced IL-6 in the plaque. In the present study, the double immunostaining method revealed that both macrophages and smooth muscle cells were major cells expressing IL-6.

The roles played by cytokines in the arterial wall and their relationship to atherogenic events remain to be defined. Synthesis and biological function of cytokines are most likely different at the initiation of atherogenesis, in fatty streaks and established plaques. From our findings in grossly normal intima, it seems probable that, similar to cellular events widely observed in experimental animals, the adherence of T lymphocytes and macrophages to endothelium and the substantial migration of these leukocytes into the intima occur in an early stage of atherogenesis in humans. As evidenced by the expression of IL-2R and HLA-DR, those intimal T lymphocytes and macrophages were allegedly in an activated state. IL-1 and TNF produced by activated macrophages would induce a variety of responses, resulting in expression of adhesion surface proteins on leukocytes and endothelium, metabolic changes, and secretion of platelet-derived growth factor by smooth muscle cells (Le and

Vilcek 1987; Pober 1988; Raines et al. 1989; Libby and Hansson 1991). Lack of IL-6 expression in the cells infiltrated in grossly normal intima may suggest that an increase of smooth muscle cells is not a potent phenomenon in these nascent lesions, since this cytokine is assumed to be one of the principal growth-regulatory molecules responsible for migration and proliferation of smooth muscle cells (Loppnow and Libby 1990; Ikeda et al. 1992). In contrast, fatty streaks and plaque lesions that contained significant numbers of smooth muscle cells invariably revealed expression of IL-6 by macrophage foam cells and smooth muscle cells.

There were individual variations in cytokine expression in fatty streaks and atheromatous plaques. Particularly in the latter cases, IL-1, IL-6, TNF and IL-2R positive cells appeared in different numbers in different regions of a given lesion. It is perplexing to explain precisely what these variations imply. However, as mentioned above, since these cytokines are produced by immunocompetent cells in an active state (Davies 1986; Hansson and Jonasson 1989b; Libby and Hansson 1991), the variations of cytokine expression may to some extent reflect those of the lesion activity. In this respect, the increase of the fibrous matrix found in lesion areas may contribute to the lesion activity potentially affecting the migration and the subsequent interaction of T lymphocytes and macrophages. Further, the penetration of injurious agents or antigens would be retarded by the thickened intima. This notion is partly supported by the finding that IL-2R positive cells were invariably small in number and localized only in the subendothelial space of fibre-rich areas.

In summary, the present study suggested that cytokine-mediated cellular interaction plays a pivotal role in the grossly normal prelesional intima, fatty streaks, and shoulder regions of the atheromas, where T lymphocytes and macrophages are located in close proximity. In addition, our electron microscopic observations demonstrated the cellular interactions via the cognate cell-to-cell contact, as shown by the direct apposition of lymphocytes to macrophages. It is conceivable that a specific in situ cell-mediated hypersensitivity would particularly take place in the areas where atherosclerotic lesions are growing or being active. Further studies are needed on the pathogenetic potential of immunological factors in atherosclerosis.

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