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Absence of atherosclerosis evolution in the coronary arterial segment covered by myocardial tissue in cholesterol-fed rabbits

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Abstract The evolution of atherosclerotic lesions is suppressed in the intima of the human coronary artery, beneath myocardial bridges. To elucidate the mechanism of the protective effect, we investigated morphological changes using the rabbit coronary artery as a model. Rabbits fed a 1%-cholesterol diet were killed at intervals up to 20 weeks. Two short segments of the left coronary arteries running in the epicardial adipose tissue (EpiLAD) and subsequently running in the myocardium (MyoLAD) were compared morphologically. The intima of the EpiLAD had flat endothelial cells with a polygonal shape, and demonstrated raised atherosclerotic lesions with increase in serum cholesterol level. In contrast, the intima of the MyoLAD was free of atherosclerotic lesions throughout the study, and the endothelial cells were spindle-shaped and engorged. While ferritin particles reached only the surroundings of the internal elastic lamina in the MyoLAD, they permeated into the media of the EpiLAD. We suggest that myocardial bridges suppress coronary atherosclerosis by an alteration of endothelial permeability, which may be due to changes in haemodynamic force tending towards a higher shear stress. The data provide an insight into the relationship between haemodynamics and the development of coronary atherosclerosis.

Key words Myocardial covering · Rabbit coronary artery · Atherosclerosis · Vascular permeability · Shear stress

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Introduction

The epicardial human coronary arteries are occasionally covered by myocardial tissue for part of their course. This section is known as a myocardial bridge (MB) and is most frequently recognized in the left anterior descending coronary artery (LAD) [12, 18, 26, 31]. By coronary arteriography, MBs have been shown to produce transient luminal narrowing due to a milking effect or constriction during systole [1, 2, 30]. In addition, it has been described that the presence of MB is related to myocardial ischaemia [21, 30], myocardial infarction [9], ventricular fibrillation [11], sudden death [28] and A-V block [8].

Histopathological studies of human coronary arteries have disclosed that atherosclerosis evolution is suppressed in the intima beneath MBs [12, 18, 19, 26, 31]. Scanning electron microscopy in situations of altered haemodynamic stress in various animal experiments [15, 25, 27, 32, 36] have shown that endothelial cells of the intima stressed by low shear force are polygonal-shaped, and are spindle-shaped where there is a high shear force. Our previous study of human LADs suggested that alteration of haemodynamics, through the presence of MBs, may greatly influence the evolution of atherosclerosis within the LAD [18]. It is also suggested that the intima beneath the MB is stressed by high shear forces on the basis of comparison with the endothelial cell shape of the intima proximal or distal to the MB [20]; however, exploration of the mechanism has been overlooked except for a single experiment using canine heart [3].

Atherosclerotic lesions are much more common in the extramural than in the intramural coronary arteries of Watanabe hereditary hyperlipidaemic rabbits [34]. In contrast, in hypercholesterolaemic rabbits, the intramural coronary artery was found to be more stenotic with severe intimal lipidosis than was the extramural vessel [16]. In these experiments [16, 34], however, the intramural coronary segments included peripheral arterioles the diameter of which was less than 0.2 mm. The rabbit's LAD, after branching of the left circumflex coro-

nary artery, always runs in epicardial adipose tissue for a short distance and is then, without exception, directly covered by myocardial tissue. This anatomical similarity to man led us in the present study, to observe the two segments of intraepicardial (EpiLAD) and intramyocardial LAD (MyoLAD) from each individual rabbit heart. We compared these two segments of rabbit LAD by immunohistochemical and ultrastructural methods, and examined the effect of myocardial covering on the evolution of atherosclerosis through changes of lipid permeability caused by alterations in haemodynamic force.

Materials and methods

The experiment was carried out on 137 male Japanese White rabbits weighing between 2.2 and 2.5 kg which were maintained under specific pathogen free conditions. Of the 137 rabbits, 108 were fed with pelleted chow containing 1% cholesterol (Funabashi Farm Incorporated, Funabashi, Japan) as the experimental group (ChoR). The other 29 rabbits were fed with standard pelleted chow, as the control group (ConR).

The blood of all rabbits was taken for determination of serum total cholesterol (TC) level by an ear vein puncture once per week. ChoR and ConR rabbits were sacrificed at the intervals up to 20 weeks shown in Table 1 by an overdose of pentobarbital sodium (Dinabot Incorporated, Osaka City, Japan) via an ear vein. All rabbits used for transmission electron microscopic observations were injected intravenously with ferritin (50 mg/kg weight; Nutritional Biochemicals, Cleveland, Ohio, USA) 30 min before sacrifice as a tracer to examine the permeability of endothelial cells.

For light microscopic examination, 10% neutral buffered formalin was perfused into the left coronary artery at the pressure of 100 mmHg while the heart was still beating. After in situ fixation, the LAD was carefully removed with the surrounding myocardial tissue. The specimen of the LAD was cut perpendicularly to its flow axis, into the two segments, the EpiLAD and MyoLAD segments. Three millimetres of the EpiLAD was taken just before the myocardial covering, and 3 mm of the MyoLAD was taken just after the EpiLAD (Fig. 1a,b). Each specimen was embedded in paraffin. Thin sections obtained from each paraffin block were stained by haematoxylin and eosin and elastic van Gieson methods. In addition, they were stained immunohistochemically with antibodies against rabbit apolipoprotein B (ApoB, a gift from Dr. Nobuyoshi Hirose, Department of Geriatrics, School of Medicine, Keio University, Tokyo, Japan), alpha-smooth muscle actin (1A4, Dakopatts, Denmark) and proliferating cell nuclear antigen (PCNA, Dakopatts) by a labelled streptavidin biotin method (Dakopatts). In each ChoR rabbit, the intimal thickness was measured by light microscopy at the thickest portion of each EpiLAD and MyoLAD.

For transmission and scanning electron microscopy, the LAD was perfusion-fixed by 0.1 M cacodylate buffer and 2% glutaraldehyde in cacodylate buffer. The LAD was cut into the two segments of Epi- and MyoLAD, and each was postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer. For transmission electron microscopy, the fixed LAD was embedded in epoxy resin, and ultrathin sections were stained with 4% uranyl acetate-5% silicotungstic acid and lead citrate. These stained sections were observed with an electron microscope (JEM 1200 EX-II, JEOL, Tokyo, Japan), as were unstained specimens of the above epoxy resin blocks for endothelial permeability through detection of ferritin particles permeating into LAD walls. For scanning electron microscopy, the fixed LAD after dehydration was subjected to critical point drying and coated with evaporated gold-palladium. The

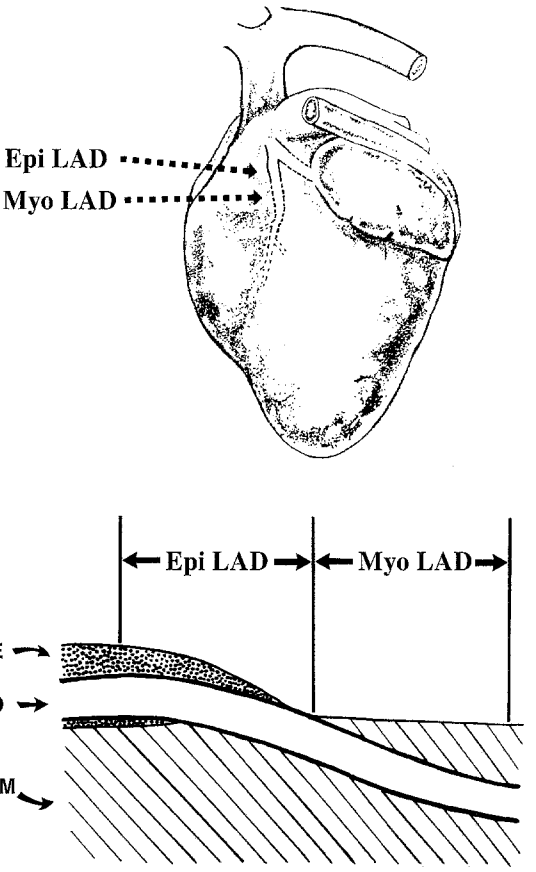


Fig. 1a, b Schematic representation of tissue sampling from rabbit heart. **a** EpiLAD is the intraepicardial segment of the left anterior descending coronary artery covered by adipose tissue. MyoLAD is the intramyocardial segment of the left anterior descending coronary artery covered by myocardium. **b** EpiLAD is 3 mm in length just before myocardial covering, and MyoLAD is the same length just after EpiLAD

Table 1 Number of rabbits sacrificed at given intervals for light microscopic examination (*LM*), transmission electron microscopic examination (*TEM*) and scanning electron microscopic examniation (*SEM*) in cholesterol-fed (*ChoR*) and control (*ConR*) groups

Weeks	1	2	3	4	8	10	12	14	16	20	Total
ChoR											
LM	5	5	5	5	5	4	4	4	4	4	45
TEM	4	4	4	4	4	4	4	4	3	3	38
SEM	3	3	3	3	3	2	2	2	2	2	25
ConR											
LM	5				5					2	12
TEM	4				4					2	10
SEM	3				3					1	7
Total	24	12	12	12	24	10	10	10	9	14	137

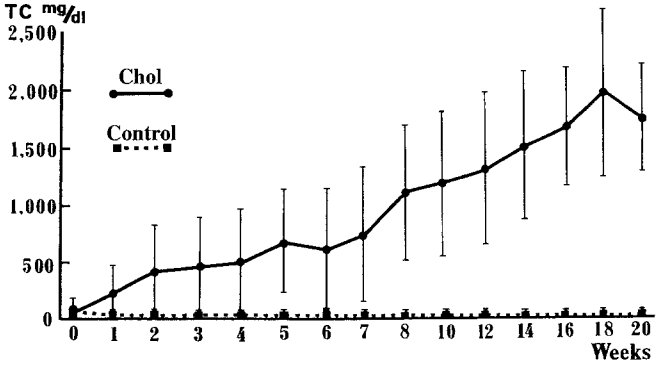


Fig. 2 The mean values of serum total cholesterol (*TC*) at each interval. The TC levels of cholesterol-fed rabbits (*Chol, ChoR*) are significantly elevated from week 1 ($P<0.01$). The TC levels of control rabbits (*Control*) remained at the basal level. Means ± SD shown

intimal surface of each LAD was observed with a scanning electron microscope (JSM5400, JEOL). The luminal diameter of the Epi- and MyoLAD segments was measured at the middle point of each by scanning electron microscopy, and the ratio of luminal diameter between the two segments of each individual (MyoLAD/EpiLAD) was calculated. Further, the medial thickness of the Epi- and MyoLAD segments was measured at the most thickened portion of each by scanning electron microscopy.

Results

The mean values of the TC of both the ConR and ChoR in each week are indicated in Fig. 2. The TC of the ConR remained steady at the basal level up to 20 weeks, while that of the ChoR was consistently elevated over the whole experimental period.

In control rabbits both the Epi- and MyoLADs remained normal throughout the study, and no lesions suggesting an atherosclerotic process were present. ApoB and PCNA stainings were negative in the whole arterial structure of both the Epi- and MyoLADs. The only positive reaction observed was for 1A4, which was in the cytoplasm of smooth muscle cells of the media exhibiting a normal appearance.

Histopathological examination of the EpiLAD in high cholesterol rabbits revealed a few foam cells aggregating in the intima at the 4th week after introduction of the cholesterol diet. The intimal cell population increased with elevation of the TC level, and intimal raised lesions gradually developed and extended up to 20 weeks (Fig. 3a). In contrast, despite the consistent increase in the TC level, the intima of the ChoR MyoLAD exhibited no atherosclerotic change whatsoever throughout the study (Fig. 3b).

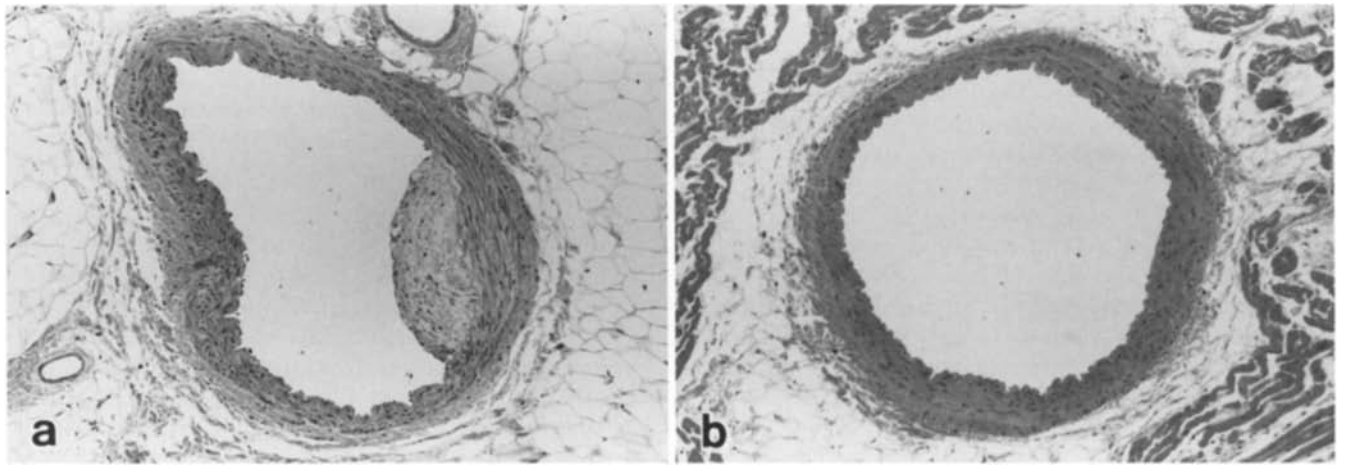
Immunohistochemically, ApoB was recognized in the cytoplasm of foam cells in the intimal raised lesions of the EpiLAD at the 4th week. From the 8th week after the introduction of a cholesterol diet, ApoB was also demonstrated in the stroma of the raised lesions, and in the surrounding internal elastic lamina below the raised lesions (Fig. 4a,b). No ApoB was demonstrated in the entire blood vessel wall of the MyoLAD in any rabbit in the ChoR group, up to 20 weeks (Fig. 4c).

PCNA-positive nuclei were often shown in the EpiLAD. They were seen in a few smooth muscle cells of the media even during the early stages where raised lesions had not yet been formed in the intima, and also in some foam cells in the intimal raised lesions of dvanced atherosclerosis. In contrast, in the MyoLAD, PCNA-positive nuclei were not found in any of the cellular components of the arterial wall structure.

In the EpiLAD, 1A4 was positive in the cytoplasm of several foam cells within the intimal raised lesions, from the 8th week of cholesterol feeding, as well as the normal medial smooth muscle cells (Fig. 4d). In the MyoLAD, 1A4 was positive only in the cytoplasm of normal smooth muscle cells in the media.

The mean values of intimal thickness of the ChoR during the experimental period are indicated in Fig. 5. The intima of the EpiLAD became significantly thick-

Fig. 3a, b Light micrographs of EpiLAD (**a**) and MyoLAD (**b**) in the same LAD of a 20-week ChoR (TC = 1,775 mg/dl). **a** An atheromatous plaque is observed in the intima of EpiLAD. Haematoxylin and eosin (H&E) ×25. **b** The intima of MyoLAD covered by myocardium is intact. H&E ×25



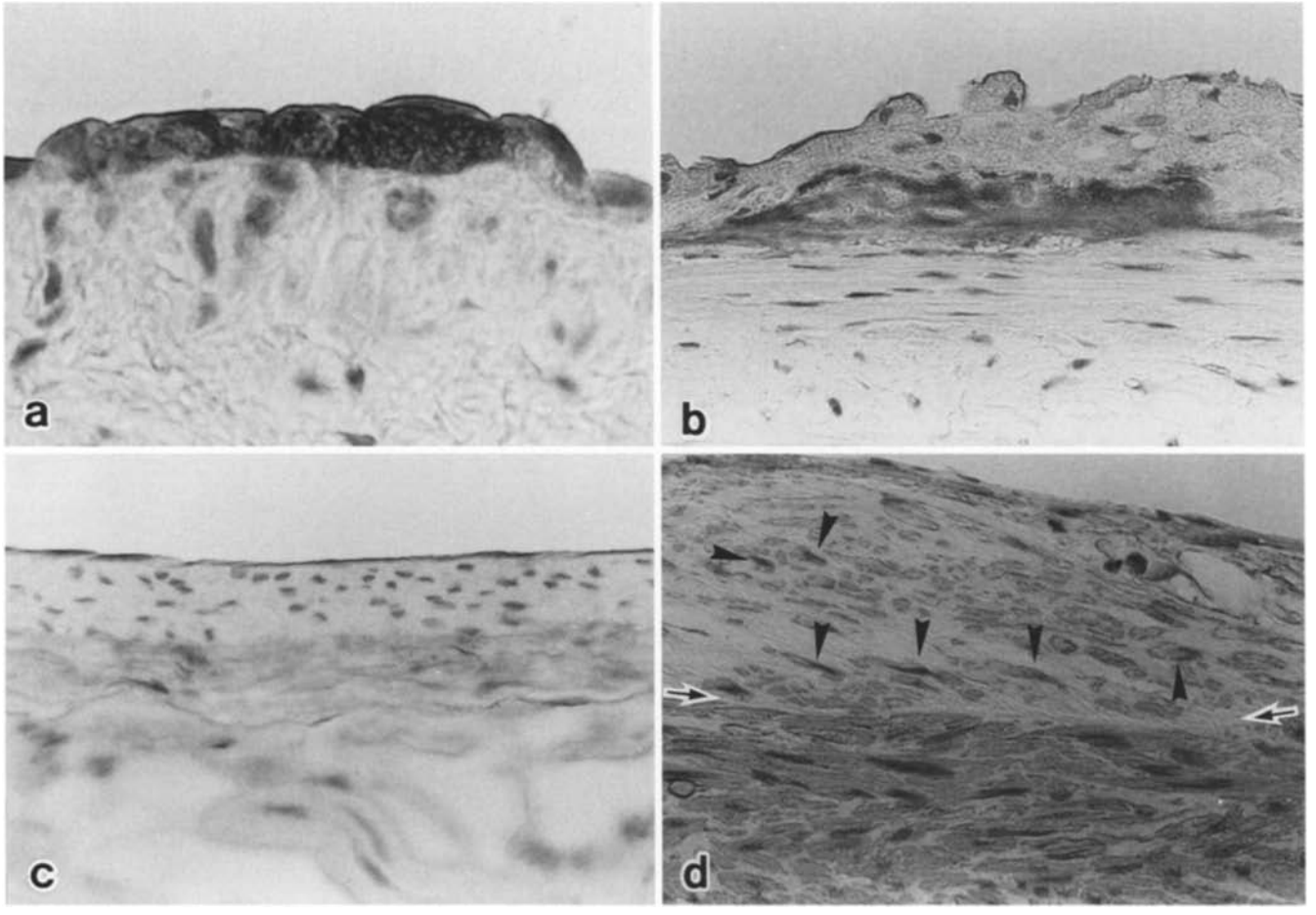


Fig. 4 **a** EpiLAD of a 12-week ChoR (TC = 1,075 mg/dl). Immunohistochemistry against ApoB shows positive staining in the intimal foam cells. ×300. **b** EpiLAD of a 12-week ChoR (TC = 1,095 mg/dl). ApoB is demonstrated in the surrounding internal elastic lamina below the raised lesion. ×250. **c** MyoLAD of the same ChoR as **Fig. 4a**. An atherosclerotic lesion is not observed, and ApoB is not detected in the arterial wall. ×200. **d** EpiLAD of a 10-week ChoR (TC = 2,250 mg/dl). Immunohistochemical staining for 1A4 is positive in cells (*arrow heads*) in the intimal raised lesion and normal media. *Arrows* show the internal elastic lamina. ×300

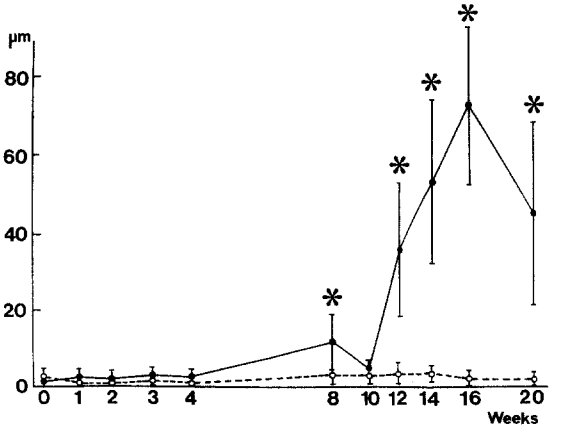


Fig. 5 The mean values of intimal thickness at each interval. In EpiLAD (*solid line*), the intima becomes thickened, while the intimal thickness of MyoLAD (*broken line*) shows no significant change. Asterisks in the table indicate the significance ($P<0.01$). Means ± SD shown

ened with the advance of the TC level. The intimal thickness of the MyoLAD showed no significant change.

There were no significant transmission electron microscopic changes in the EpiLAD or MyoLAD segments up to 20 weeks in ConR. Ferritin particles were recognized in the unstained ultrathin sections only in phagocytotic vesicles of endothelial cells and they were not seen in other sites.

In ChoR, however, a few foam cells appeared in the intima of the EpiLAD at the 4th week, and fusiform foam cells were also present at 8 weeks. After the 10th week, intimal raised lesions of the EpiLAD consisted almost entirely of fusiform foam cells which exhibited the phenotype of synthetic smooth muscle cells with abundant intracellular organelles [5]. Ferritin particles were scattered in the medial stroma among smooth muscle cells in the unstained ultrathin sections of the EpiLAD, even when the intima was free from any atherosclerotic change. They further permeated into the medial stroma of the EpiLAD in which intimal raised lesions had been formed, and were internalized in phagolysosomes of the smooth muscle cells (Fig. 6).

Conversely, in the MyoLAD of all the of the ChoR, no atherosclerotic change like that recognized in the Epi-

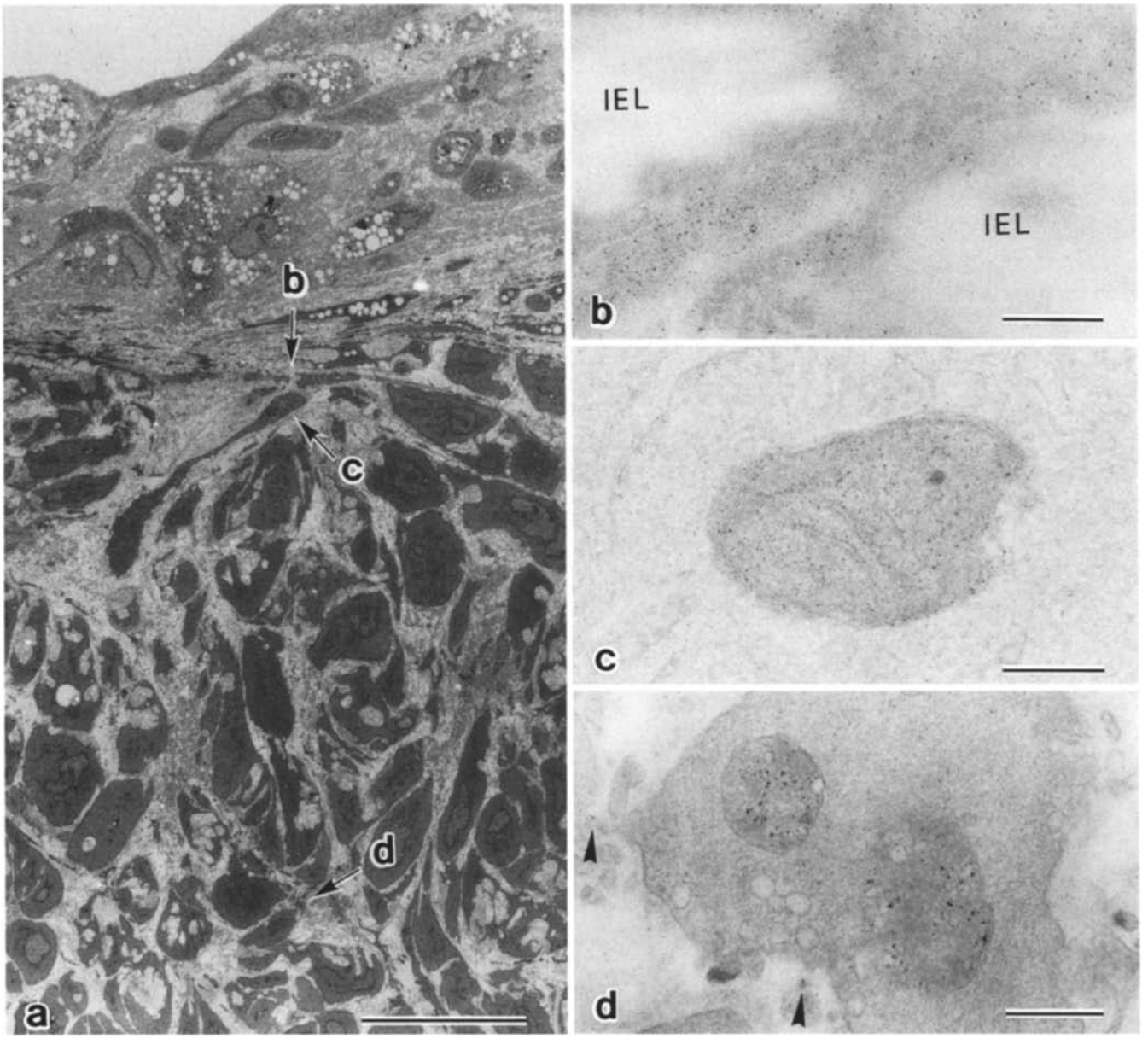


Fig. 6a–d TEMs of EpiLAD in a 12-week ChoR (TC = 594 mg/dl). **a** Numerous foam cells shown in the subendothelial space. *Arrow portions* of *b*, *c* and *d* are magnified in **b**, **c** and **d** of unstained sections, respectively. ×1,250. *Bar* = 20 μm. **b** Photograph of an unstained section of the *arrow portion-b* in **a**. There are many ferritin particles permeating a fenestrum. *IEL* internal elastic lamina. ×75,000. *Bar* = 200 nm. **c** Photograph of an unstained section of the *arrow portion-c* in **a**. Several ferritin particles are shown in a phagolysosome in a smooth muscle cell of the upper media. ×75,000. *Bar* = 200 nm. **d** Photograph of an unstained section of the *arrow portion-d* in **a**. Ferritin particles are internalized in phagolysosomes in a smooth muscle cell of the media and scattered in the medial stroma (*arrow heads*). ×75,000. *Bar* = 200 nm

LAD was found in intima of the MyoLAD throughout the whole experimental period. No phagocytotic vesicles were present in the endothelial cells.

Despite thorough observations of the unstained ultrathin sections, only a limited number of ferritin particles were demonstrated in the intima adjacent to the internal elastic lamina; however, they were never detected in the medial stroma or in the medial smooth muscle cells (Fig. 7).

On scanning electron microscopy of the ConR, endothelial cells were irregularly arranged at the luminal surface of the EpiLAD and their shape was polygonal and flat. In the MyoLAD, the endothelial cells were regularly arranged along the blood flow axis, and they were spindle-shaped and engorged for the whole intimal surface of the MyoLAD (Fig. 8).

In the EpiLAD of ChoR, 2 weeks after the introduction of cholesterol diet, a few monocytes adhered to the endothelial surface (Fig. 9a). The number of adherent monocytes increased with increase in the TC level. At the 20th week, numerous monocytes adhered to the endothelial surface, and the endothelial cells were focally denuded with monocytic attachment (Fig. 9b).

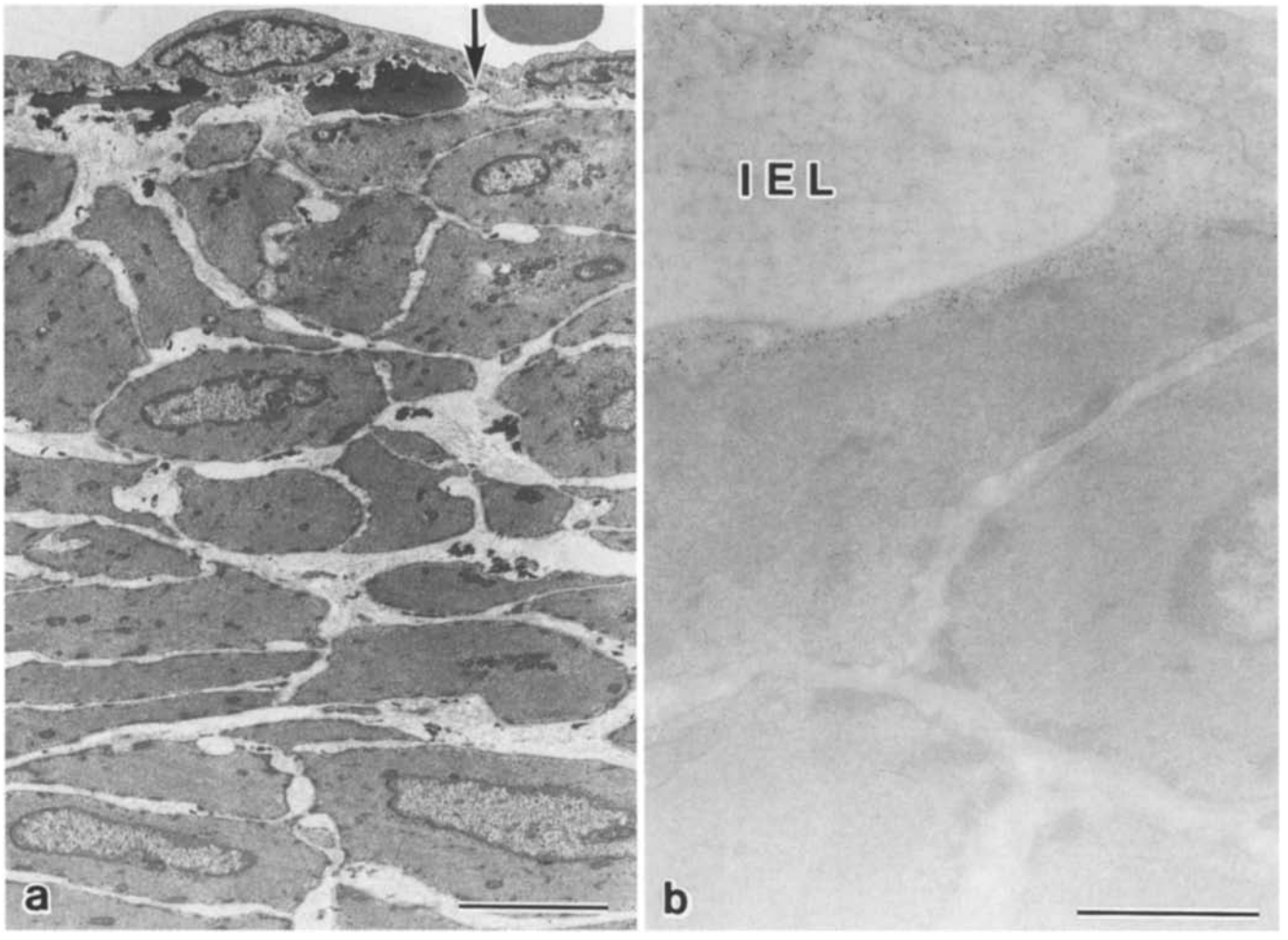


Fig. 7a, b TEMs of MyoLAD in the same ChoR as **Fig. 6** (TC = 594 mg/dl) **a** Normal structures of the intima and media of MyoLAD are demonstrated. *Arrow portion* is magnified in **b**. ×5,000. *Bar* = 4 μm. **b** Photograph of an unstained section of *arrow portion* indicated in **a**. Ferritin particles are limited around the IEL. Note the stroma of the media where there were no tracer particles. ×24,000. *Bar* = 1 μm

In the MyoLADs, despite the increased TC level at the 20th week, the endothelial cells remained normal, and monocytes were not recognized on the luminal surface (Fig. 9c,d).

The comparisons of luminal diameter and medial thickness between the two segments in all of the 32 rabbits observed by scanning electron microscopy are indicated in Table. 2. The luminal diameter ratio (Myo-/EpiLAD) showed a maximum of 1.000 and a minimum of 0.864. However, there was no statistically significant difference between the two segments. As to the medial thickness of LAD, the mean thickness of MyoLAD was not significantly different from that of the EpiLAD (Table. 2)

Discussion

Our previous studies demonstrated remarkable atherosclerotic suppression in a part of the human LAD which was covered by a MB [18, 20]. This suggests that the anatomical situation surrounding the coronary artery (myocardial covering) may influence atherosclerotic evolution. In the present study, the two short segments of the Epi- and MyoLAD in cholesterol-fed rabbits were examined anatomically and compared with respect to the evolution of atherosclerosis.

In the EpiLAD of the ChoR, raised atherosclerotic lesions appeared in the intima into which ApoB infiltrated; migrated smooth muscle cells and PCNA-positive foam cells were evident; intimal thickness was conspicuously increased with increase in the TC level. In contrast, despite the increased TC level, the intima of the MyoLAD was completely free from any atherosclerotic change, and lacked ApoB infiltration in the arterial wall throughout the study. Furthermore, the capacity for lipid traffic through the MyoLAD wall was considered to be almost the same as that of the EpiLAD, because the medial thickness of the MyoLAD was not significantly different from that of the EpiLAD. These results indicate suppression of atherosclerosis in the MyoLAD segment, which is covered by the surrounding myocardial tissue. In

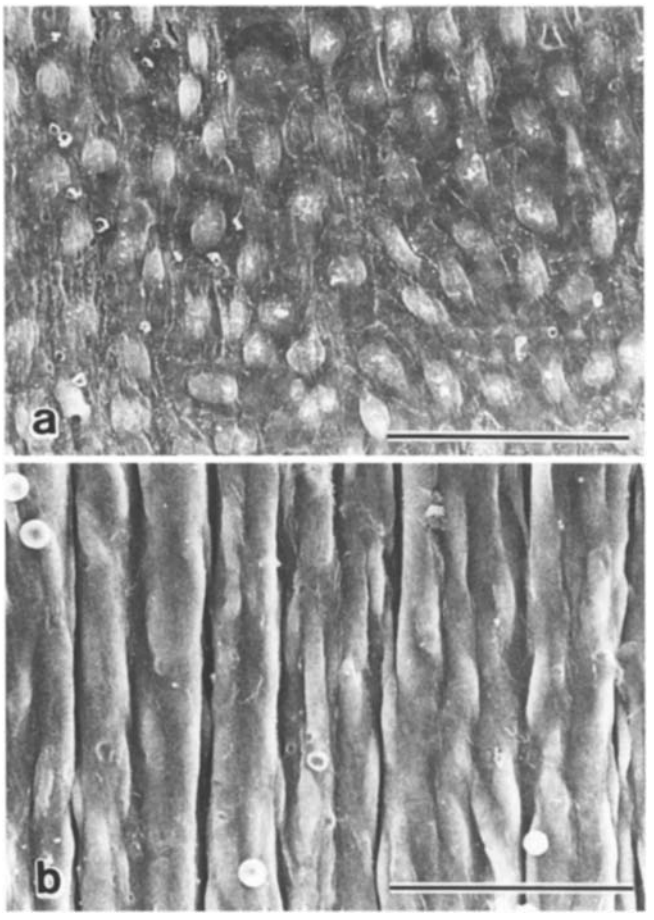


Fig. 8a, b SEMs of LAD of a ConR (TC = 32 mg/dl). ×640. *Bar* = 50 μm. **a** EpiLAD. The intima is covered by polygonal and flat endothelial cells. **b** MyoLAD. The endothelial cells in the same LAD as **a**, spindle-shaped and engorged

Watanabe hereditary hyperlipidaemic rabbits bred from Japanese White Rabbits, atherosclerotic lesions in the intramural coronary artery were less evident than those in the extramural coronary artery [34]. These phenomena in the MyoLAD correlate with suppression of atherosclerosis in the human LAD beneath a MB [18, 20]. In the MyoLAD segment of ChoR, it is suggested that, despite hypercholesterolaemia, there may be a mechanism suppressing permeability of ApoB which is a cholesterol-carrying protein of low density lipoprotein [14].

In the present study, the localized non-susceptibility was further evident by transmission electron microscopy. In both the Epi- and MyoLAD of the ConR permeation of ferritin molecules was confined to the endothelial cells and ferritin particles permeated into the media of the EpiLAD under the hyperlipidaemic condition. In contrast, in the MyoLAD, permeating ferritin particles were confined to the vicinity of the internal elastic lamina, even with hypercholesterolaemia. It is generally considered that endothelial permeability increases with the elevation of serum low density lipoprotein [33]. Ferritin permeability into the media of the present EpiLAD was accelerated with the elevation of the TC level, as revealed by examination of the results of ferritin permeability in ChoR compared with that in ConR. Ferritin particles are 9.4 nm in mean diameter [10], and ferritin molecules in circulating blood are usually transferred into the subendothelial space not by junctional transport, but exclusively by vesicular transport of endothelial cells [4, 17]. From this restricted pathway, it is suggested that endothelial permeability of the MyoLAD differs from that of the EpiLAD regardless of hypercholesterolaemia. It is thus considered that despite the finding that both short LAD segments had blood containing the same levels of serum lipids, the endothelial cell permeability in the MyoLAD is suppressed by another factor, such as the altered haemodynamic force produced by myocardial covering.

By scanning electron microscopy, the endothelial cell shape of the EpiLAD was found to be polygonal and flat, and spindle-shaped and engorged in the MyoLAD. These two morphological types of endothelial cell were first described in the rat coronary artery [35]. In in vivo experimental studies of the aortic flow divider [32], the anastomosed vein of arteriovenous fistulae [15] and aortic coarctation [25] in the rabbit, rat aortic stenosis [36] and stenosed dog aorta [27], the polygonal-shaped endothelial cells were found in areas of low shear stress, and the spindle-shaped endothelial cells were found in areas of high shear stress. Alterations of endothelial cell shape have been postulated to occur as a result of changes in haemodynamic forces on endothelium. For our present observations, the endothelial cells lining the surface of the EpiLAD are subject to haemodynamic forces producing lower shear stress than those in the MyoLAD. Low shear stress allows longer contact time between serum lipoproteins and the endothelial surface, and results in a facilitation of lipoprotein-uptake by endothelial cells [37]. In addition, it has been generally accepted that low shear stress may lead to the development of atherosclerosis by mass transfer of lipids into the subendothelial space [6]. The intima of the MyoLAD segment may be

Table 2 Luminal diameter and medial thickness (mean ±standard deviation, *n* number, *EpiLAD* epicardial left coronary artery, *MyoLAD* myocardial left coronary artery)

	EpiLAD(μm)	MyoLAD(μm)	Myo-/EpiLAD
Luminal diameter (*n*=32)	1,020 ± 180	960 ± 164	0.954 ± 0.038
Statistical significance[a]	(–)		
Medial thickness (*n*=32)	53.7 ± 9.1	52.3 ± 9.3	0.973 ± 0.024
Statistical significance[a]	(–)		

[a] The statistical significance was examined by Student's *t*-test

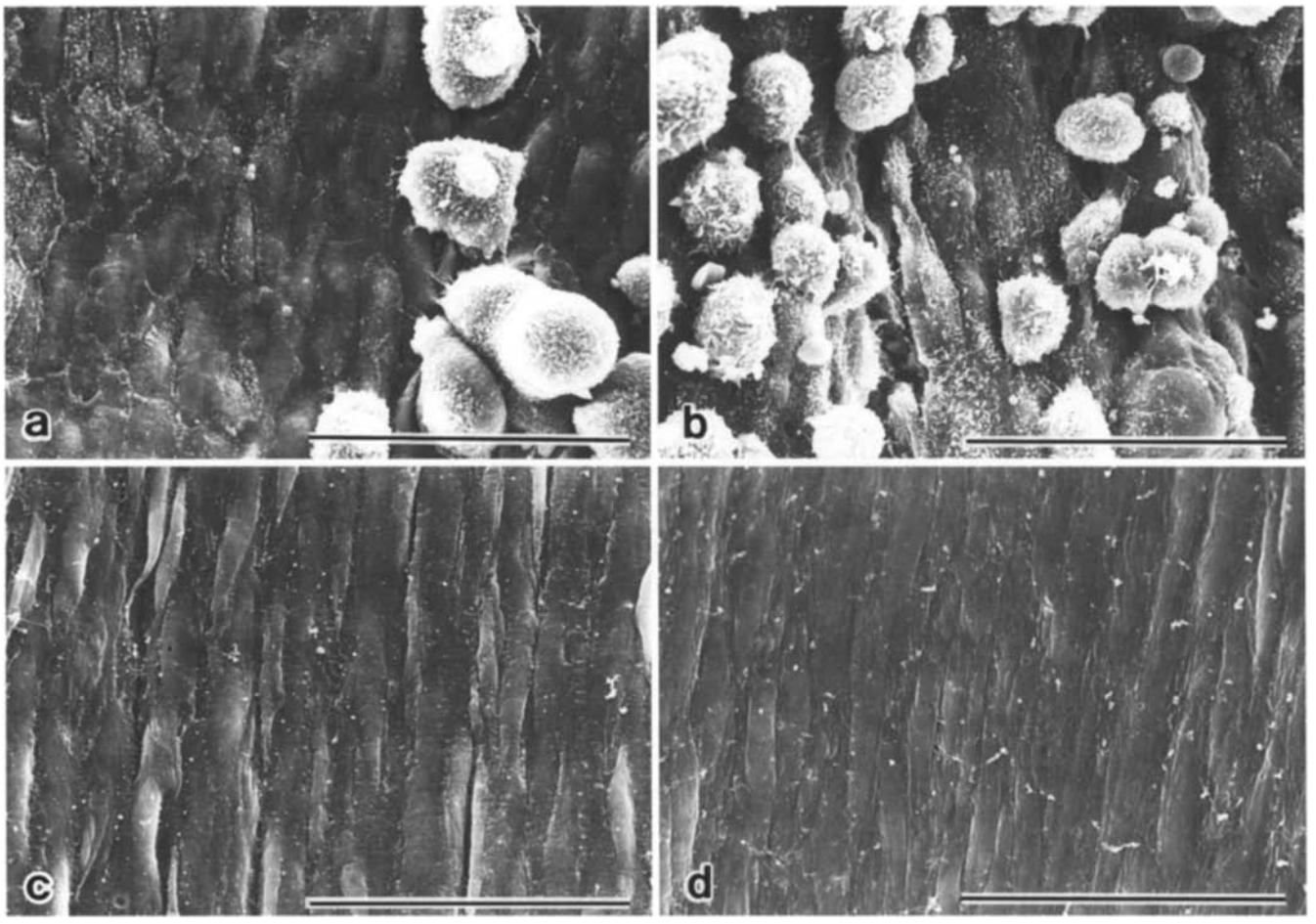


Fig. 9a–d SEMs of a ChoR. ×840. Bar = 50 μm. **a** The intima of an EpiLAD of a 2-week ChoR (TC = 704 mg/dl). Some monocytes adherent to the endothelial surface are observed. **b** The intima of an EpiLAD of a 20-week ChoR (TC = 1,800 mg/dl). Many monocytes are attached to the endothelial surface which is partially raised up due to the invasion of monocytes under the endothelial cells. **c** The intima of a MyoLAD of the same ChoR as **a**. The endothelial surface is intact. **d** The intima of a MyoLAD of the same ChoR as **b**. No evidence of monocyte attachment to the endothelial surface is shown in spite of hypercholesterolaemia

resistant to lipid infiltration, which is possibly based on locally high shear stress in the MyoLAD covered by myocardial tissue. The shear stress acting on endothelial cells is proportional to the blood flow velocity and blood viscosity, and is inversely proportional to the luminal diameter of the vessel [13, 22]. The viscosity of blood flowing in the MyoLAD is the same as that in the EpiLAD, because both the Epi- and MyoLADs are consecutive short segments of one LAD. In our study, the difference between the luminal diameters of the EpiLAD and MyoLAD segments is too little to change the overall gradient of shear stress pressing the endothelial surfaces of the two LAD segments significantly. Further, in an experimental study using canine coronary artery, incorporation of ^{35}S labelled sulphate into glycosaminoglycans synthesized by smooth muscle cells was increased in the intima proximal to myocardial covering, and it was suggested that myocardial covering could contribute to an alteration of blood flow in the EpiLAD [3]. It is thus considered that the apparent difference of intimal permeability between the Epi- and MyoLADs may be caused by blood flow alteration due to the difference in shear stress level of both segments, which is based on the presence of myocardial covering.

With regard to the localization of atherosclerotic lesions, haemodynamic factors have recently been considered to be a key factor, especially at arterial bifurcations [24, 29, 38]. Haemodynamic factors influence not only the cell shape but the physiological behaviour of vascular endothelial cells [7, 23, 25, 37]. The relationship between haemodynamic force and atherosclerosis was further evident in the present study. It is concluded that the myocardial covering of the rabbit LAD as well as the MB surrounding the human LAD present a useful lead to aid in the understanding of the relationship between haemodynamics and coronary atherosclerosis development. Coronary arterial lesions can be easily produced without any artificial surgical operation and the course of the rabbit LAD which we investigated is almost straight, in comparison with the branching or curved area of the artery usually used in the experimental studies. Finally, the myocardial covering can be easily defined as an objective anatomical landmark.

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