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Structural Differences of Arterial Walls Which are Either Vulnerable or Resistant to Atherosclerosis

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Summary. **In order to clarify the mechanism of different vulnerability to atherogenesis between the apical and lateral wall at a branching, both regions of the inferior mesenteric artery in human autopsy cases were investigated electron microscopically.**

The lateral wall has been accepted as the most preferential site for the disease, in contrast with the apex which is considered the most resistant by many investigators. In newborns intimal thickness in the apex was greater than in the lateral wall due mainly to proliferation of SMC. After the 3rd decade, collagen fibers drastically increased in the whole apex and smooth muscle cells embedded between collagen fibers modulated their phenotype from synthetic to contractile. In the lateral intima smooth muscle cells still remained in the synthetic type. The synthetic smooth muscle cells have been considered to be capable of proliferation in the arterial wall.

The lateral intima was generally abundant in proteoglycans and lacked collagen (including subendothelial basement membranes) as well as elastic fibers, particularly in the upper part of the intima. This structural difference could bring favorable conditions for atherogenesis.

Results of *in vitro* studies revealed that collagen gel containing types I and III suppressed proliferation of SMC and changed their phenotype from synthetic to contractile. Therefore, laminar high shear stress would give resistance to the arterial wall in atherogenesis through phenotypic change of SMC.

Although rabbits showed different preferential regions for lipid deposition, it developed mostly in areas covered by oval round endothelial cells which might be exposed to relatively low mean shear stress. These shapes of endothelial cells were seen already in intact rabbits. Therefore, hemodynamic forces may play a very important role in determining the vulnerability of arterial walls to atherogenesis.

INTRODUCTION

The non-uniform development of atherosclerotic lesions has been recognized for many years.[1-4] It suggests that blood constituents are not the sole pathognomonics, but hemodynamic forces are major contributing factors in initiating and developing the disease. There are many papers reporting topographical localization of atherosclerotic lesions in large and medium-sized arteries which are observed at preferential sites, such as the arterial branches and curvatures.

The careful topographical investigation of early human atherosclerosis revealed that lesions developed in the outer lateral wall of bifurcations, the inlet of branches and the inner distal wall of curvatures of arteries.[1,5,6] It is believed that these regions are exposed to either low but turbulent shear stress, or separation and stagnation of the blood flow.

On the other hand, experimental animals such as rabbits, swines and dogs have shown topographical localization of atherosclerotic lesions different from that of human beings. For example, rabbits in acute dietary-induced hyperlipidemia have preferentially lipid deposition on flow dividers at branching sites which are expected to be laminar and high shear stress regions.[2,7] There is an apparent difference in geographical localization of the disease between human beings and rabbits. The reason for this difference is unknown. However, we can say that preferential sites for the disease may have the structure and function of the arterial wall vulnerable or sensitive to causative agents of the disease, while the unfavorable sites must have structures resistant or

insensitive to the agents.

To clarify these structural and functional differences of the arterial wall and to solve mechanisms of either resistance or vulnerability of the arterial wall against atherosclerosis is very important in understanding the actual processes of atherogenesis. In addition, knowledge obtained from these studies will be helpful in finding preventive methods for the disease.

During the development of atherosclerosis, particularly in the early phase, proliferation of smooth muscle cells in the intima can be observed before connective tissues increase predominantly. Although intimal smooth muscle cells (intimal SMC) have a high potential for proliferation because of autocrine or paracrine stimulation by growth factors produced by themselves,[8-10] replication of the cells will cease at some point; endless proliferation has never been observed in the vessel wall. One should consider that SMC proliferation must be regulated by some means. What is capable of suppressing the high proliferative activity of intimal SMC? There have been some reports related to this mechanism, in which confluent and quiescent endothelial cells,[11] heparin,[12,13] TGF-β,[14] and collagen have been pointed out as suppressive substances.

In this paper we will discuss two topics: first, the structural differences of arterial walls which are resistant or vulnerable to atherosclerosis in human as well as rabbit arteries, and secondly, the effects of collagen to suppress DNA synthesis of intimal smooth muscle cells *in vitro*.

MATERIALS AND METHODS

1. Studies on structural differences between apical and outer lateral intimas of bifurcations of the human aorta

Human aortae were obtained from 23 autopsy cases whose deaths were caused by various diseases and whose ages ranged from newborns to 51 years (Table 1). Both the flow divider (apex) and the outer lateral wall of the inferior mesenteric artery (IMA) branching from the abdominal aorta were investigated electron microscopically on longitudinal surfaces cut along the axis through the center of the vessel lumen (Fig. 1).

The IMA bifurcations were perfusion-fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 100 mmHg within 2 hours after death. Small tissue blocks from both the apex and outer lateral wall of the IMA were postfixed in 1% OsO_4 for 2 hrs and

Table 1. Number of cases for the electron microscopic study on IMA bifurcation

Decades	No. of cases
1	12
2	1
3	4
4	2
5	2
6	2
Total	23

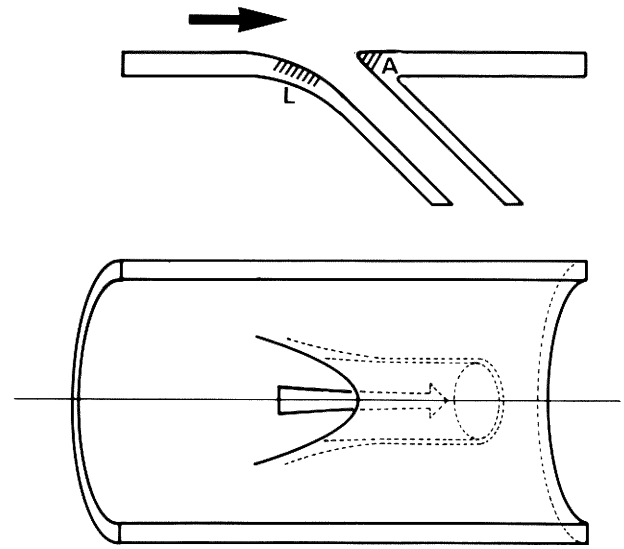

Fig. 1. Investigated areas at the bifurcation of the inferior mesenteric artery of the human abdominal aorta. Arrows: direction of the blood flow.

embedded in Epon 812. Ultrathin sections were stained with uranium-lead stain. Some sections were stained additionally with tannic acid, periodic acid methenamine silver (PAM) or ruthenium red stain.

2. Studies on effects of collagen on SMC morphology and proliferation

Atherosclerotic intimal SMC were investigated *in vitro* in order to clarify the effects of increased collagen fibers in the apical intima of the human artery of young individuals to determine whether they could suppress the proliferation of SMC and modulate their phenotype.

Atherosclerotic intimal SMC for the *in vitro* study were obtained from plaques of the thoracic aortae in male rabbits (New Zealand White strain) weighing approximately 2 kg which received daily 100 g of an

atherogenic diet (1 g of cholesterol, 5 g of lard and 100 g of the stock diet) for 4 months. The serum cholesterol level of the rabbits placed on the atherogenic diet rose to approximately 1.2 g/dl around the end of the first month, and it gradually increased to approximately 2.0 g/dl in 4 months. Normal medial smooth muscle cells (medial SMC) were prepared from explants of the tunica media of intact animals.

After removing both endothelia and adventitia, two kinds of explants were prepared from the normal thoracic aortas and the intimal plaques, respectively.

Preparation of medial explants has been described elsewhere.[15] Intimal explants were obtained from atherosclerotic plaques, which were stripped from the media at the internal elastic lamina in Hanks' balanced solution with forceps under a dissecting microscope. All tissues, both medial and intimal were cut into approximately 1 mm^2 pieces to be used as explants. Explants were placed separately in 750 ml plastic tissue culture flasks and grown in Dulbeccos' modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS). They were cultivated in a humid atmosphere at 37 ℃ in a 5% carbon dioxide - 95% room air mixture until large colonies of cells developed around the explants. During the primary culture for 3 to 4 weeks lipid-laden macrophages died and disappeared from the cultures. Cells were subcultured on 10 cm-dishes with the same medium in order to obtain enough cells for the experiment. Non-specific esterase staining was used to confirm the absence of macrophages in the subcultures.

DNA synthesis: To investigate the effects of collagen gel on DNA synthesis of SMC, normal medial and atherosclerotic intimal SMC in the third passage were plated at a concentration of $4.5-5\times10^4$ cells per well on type 1 collagen gel (Collaborative Research Inc. Bedford. Mass., CR.: Nitta Gelatin Co. Ltd., Tokyo, NG) containing 1.0 mg/ml of protein, in Falcon's 24-well dishes and incubated in 1 ml of DME with 10% FBS for a week. Control cells from two sources were seeded directly on plastic dishes. After 0.5 μCi/ml of tritiated thymidine was applied for 24 hours, on the 1st, 3rd, 5th, and 7th days the cells were harvested to count the number of cells and to measure their radioactivity. The purchased collagen solutions were applied to SDS gel electrophoresis (interrupted gel electrophoresis) to analyze what types of collagen they contained.

cAMP: The cells were plated either on collagen gel or on plastic dishes (35 mm in diameter) at a density of 2×10^4 cells per dish and cultured in the aforementioned manner. After the cultures were moved onto ice, they were fixed with 10% trichloroacetic acid, then harvested by a cell scraper for competitive radioassay of cAMP with a kit (Yamasa Shoyu Co., Choshi, Japan).

Fine structures: On the 2nd and 10th days of the experiment, the cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for one hour and post-fixed by 1% osmium tetroxide in the same buffer for an additional hour. The cells were then processed and embedded in Epon 812, sectioned vertically and observed under an electron microscope (H-600. Hitachi Ltd. Tokyo). The areas of both bundles of cytoskeletal filaments and whole cytoplasms were measured by the image analyzer to calculate the volume percentage of cytoskeletons in the cytoplasm. Widths of the filaments were measured along perpendicular lines drawn at two points of each cell, namely at one fourth and one half the cell length, which had been printed on photographic papers at a magnification of $\times$50,000.

3. Studies on morphological and functional differences between endothelial cells on the apex and shoulder of a flow divider at the bifurcation of the rabbit aorta

Male rabbits weighing approximately 2.0 kg which had been placed on either a normal stock diet or the atherogenic diet for up to 3 weeks were sacrificed to obtain specimens for scanning (SEM) and transmission electron microscopy (TEM) at the branching site of the brachiocephalic trunk from the aortic arch.

1) Preparatory procedures for SEM specimens: Rabbits were injected intravenously with 1 ml of a saline supplemented with 500 units of heparin per kg of body weight prior to sacrifice to avoid clotting of the blood on the endothelial surface of the arteries. Perfusion-fixation with 1.8% and then 2.5% glutaraldehyde in 0.18 M phosphate buffer (pH 7.4) was performed after rinsing thoroughly the lumen with the same buffer supplemented with heparin. Small specimens from the above-mentioned branchings were processed by the ordinary methods and observed under SEM (JSM T-220, JEOL, Tokyo).

The cell shape index, which was given by A/B (A: width, B: length), was measured by an image analyzer (IBAS 2000, Zeiss) to estimate the grade of roundness of endothelial cells.

2) Permeability studies of endothelial cells: Horseradish peroxidase (type II. Sigma, HRP), ferritin (type I, from horse spleen, Sigma) and autologous immunoglobulin against HRP were used as tracers to investigate permeability of endothelial cells. Horse-

radish peroxidase (250 mg/kg) was injected intravenously 4 or 15 min prior to sacrifice. Ferritin was injected intravenously 30 min before sacrifice. Autologous immunoglobulin against HRP was produced by immunization with HRP before the experiments. Permeability of apoprotein E was studied with an immunocolloidal gold method.[16]

RESULTS

1. Studies on morphological changes of the intima at IMA bifurcation of the human abdominal aorta

Autopsy cases of newborns had thicker intimas of the apex at the bifurcations consisting of 3-4 layers of SMC, as compared with the outer walls which had none or only 1 layer of SMC. In cases younger than one year, particularly newborns younger than one month old, the apical intima (A) was thicker than the outer lateral intima (L) of the IMA. Consequently, the intimal thickness ratio of apical to outer lateral walls (A/L) was greater than 1, and in the most extraordi-

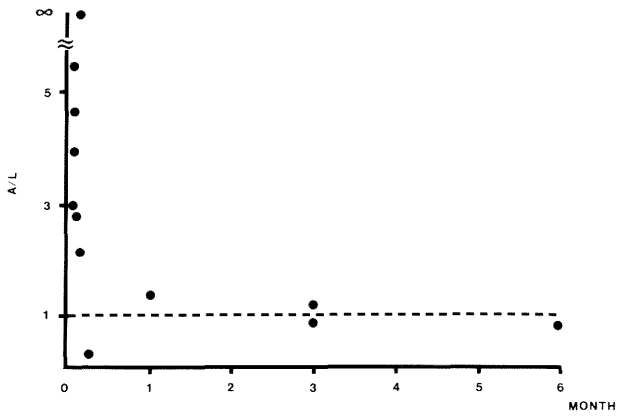

Fig. 2. Ratio of apical to outer lateral intimal thickness in cases younger than 6 months of age.

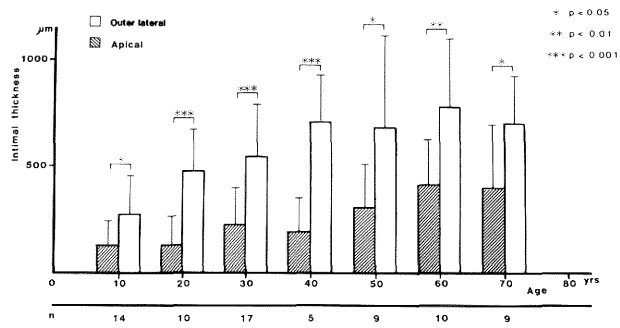

Fig. 3. Intimal thickness of bifurcation of the interior mesenteric artery.

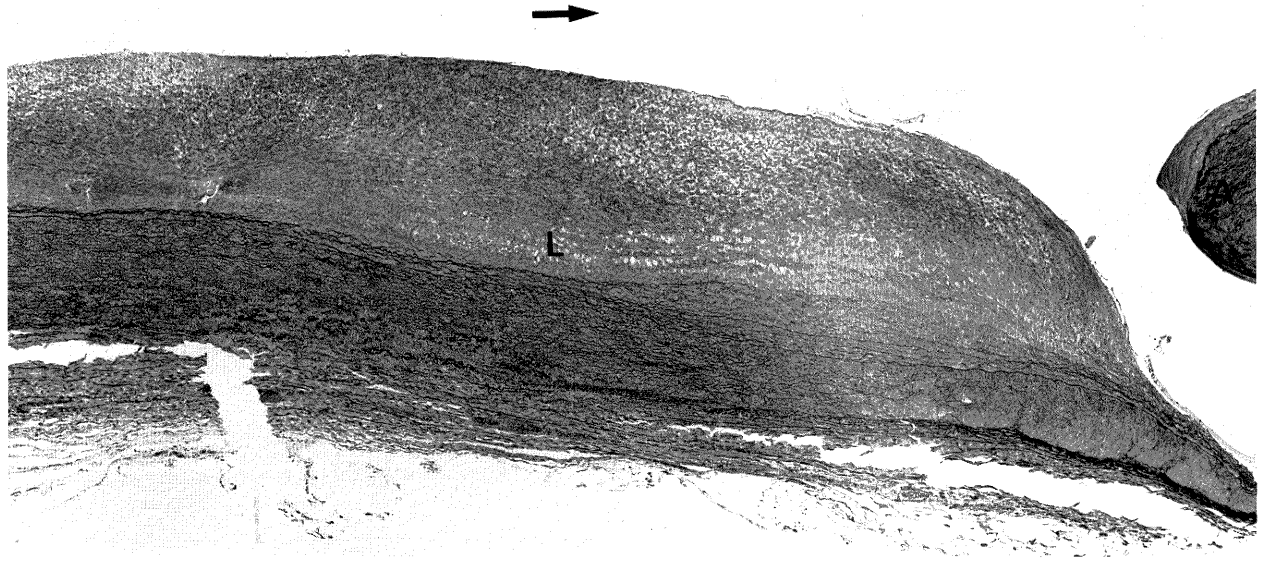

Fig. 4. An atheromatous plaque develops in the outer lateral wall of IMA branching. A 36-year-old female died of tuberculosis. Masson's trichrome stain.

Legends A: apex, L: outer lateral wall, EC: endothelial cells, IEL: internal elastic lamina, MC: monocyte/macrophage, SMC: smooth muscle cell.

nary case, infinity (Fig. 3). In that particular case, the apical intima was 15 μm thick while the outer lateral wall intima had no thickness, which means the endothelial cells lay directly on the internal elastic lamina. SMC in either the apical or lateral outer intimas of newborns had a moderate amount of rough surfaced endoplasmic reticuli occupying a large portion of cytoplasm. Therefore, such SMC were regarded as a synthetic type of SMC. Endothelial cells had no discernible changes. After one month, thickness in the outer lateral wall became greater than that in the apex of branchings, consequently lowering the A/L ratio (Fig. 2). In all ages after the second decade the

Table 2. Frequency of endothelial cells possessed microfilaments in 18 cases under 30 years old

	No. of cells with MF / No. of cells investigated	
Apex	39/131	29.8%
Outer lateral wall	4/133	3.0%

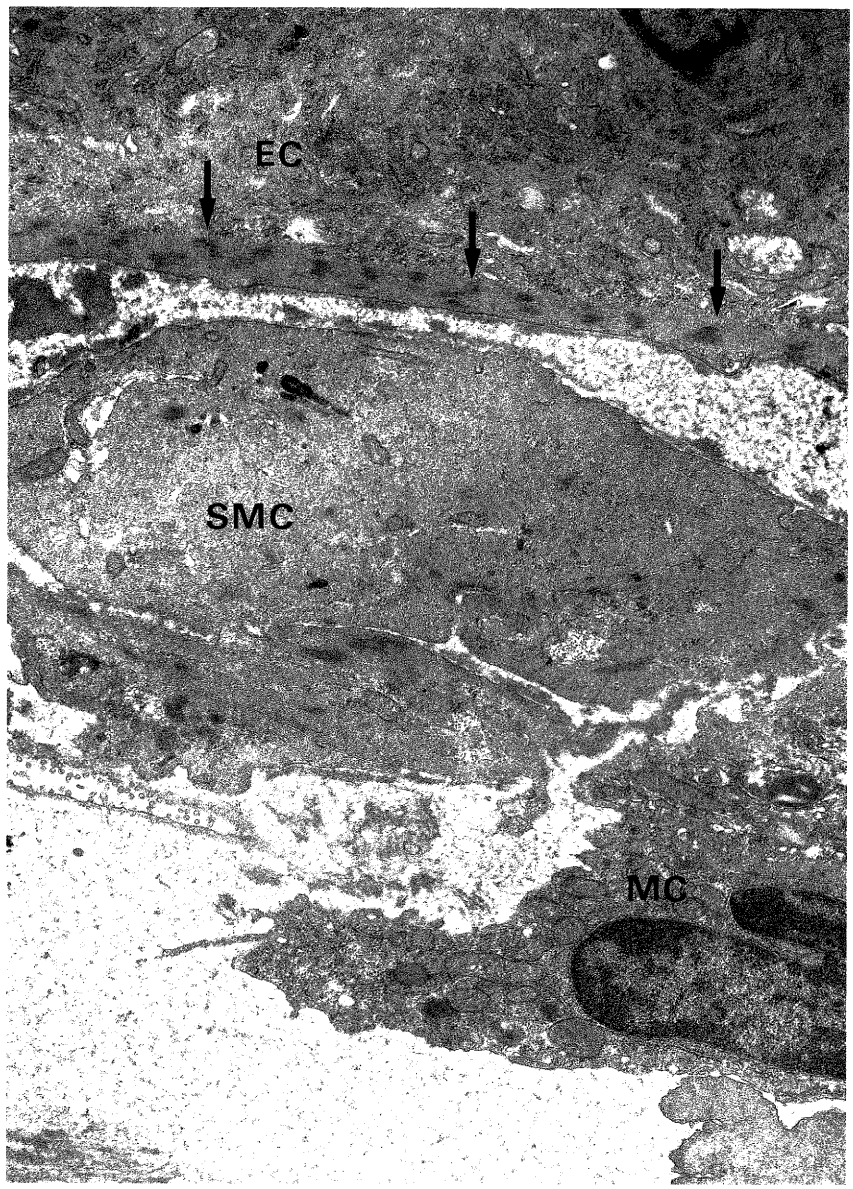

Fig. 5. Microfilament bundles run in the apical endothelial cell (arrows). There is a monocyte in the intima where flocculent materials accumulate. 14-year-old female. Uran-lead stain. ×12,000

mean maximal intimal thickness of the outer wall surpassed that of the apex with a statistically significant difference (Figs. 3, 4), although both intimas thickened more gradually as age increased.

In cases over 3 months old, stress fibers were observed more frequently in the endothelial cells of the apical intima than in those of the outer lateral walls (Fig. 5, Table 2).

Cases around 12 months old had basement membranes beneath endothelial cells forming a network structure, membranes which were more developed in the apex than in the outer lateral walls. SMC in both intimas still had characteristic figures of the synthetic phenotype and cytoplasmic vacuoles either empty or containing translucent homogeneous materials, supposedly lipids., which were in almost the same frequency in the SMC of both apical and lateral intimas. Collagen and elastic fibers were dispersed between SMC and ran almost parallel to the endothelial lining contrary to the lateral intima showing an arbitrary arrangement (Fig. 6). Also, we noticed relatively large amounts of collagen and elastic fibers in the apical intima.

A case of a 7-year-old had subendothelial edema accompanied by monocytes or lymphocytes in both apical and lateral intimas. There were no marked differences in the figures of the intimal SMC between the two regions. However, there was a tendency of fibrous structures being more developed and arrayed parallel to the endothelial lining in the apical intima, contrasting with the lateral intimas in which collagen and elastic fibers were shorter and fewer and irregularly arranged.

Fatty streaks were observed in the outer lateral wall of the bifurcation of an 8-year-old girl. Many lipid-laden macrophages accumulated in the intima. When examined under electron microscopy some elongated intimal cells were indistinguishable between monocyte—derived macrophages or SMC. There were some elongated cells surrounded by basement membranes and possessing dense ovoid areas along microfilament bundles in ectoplasms

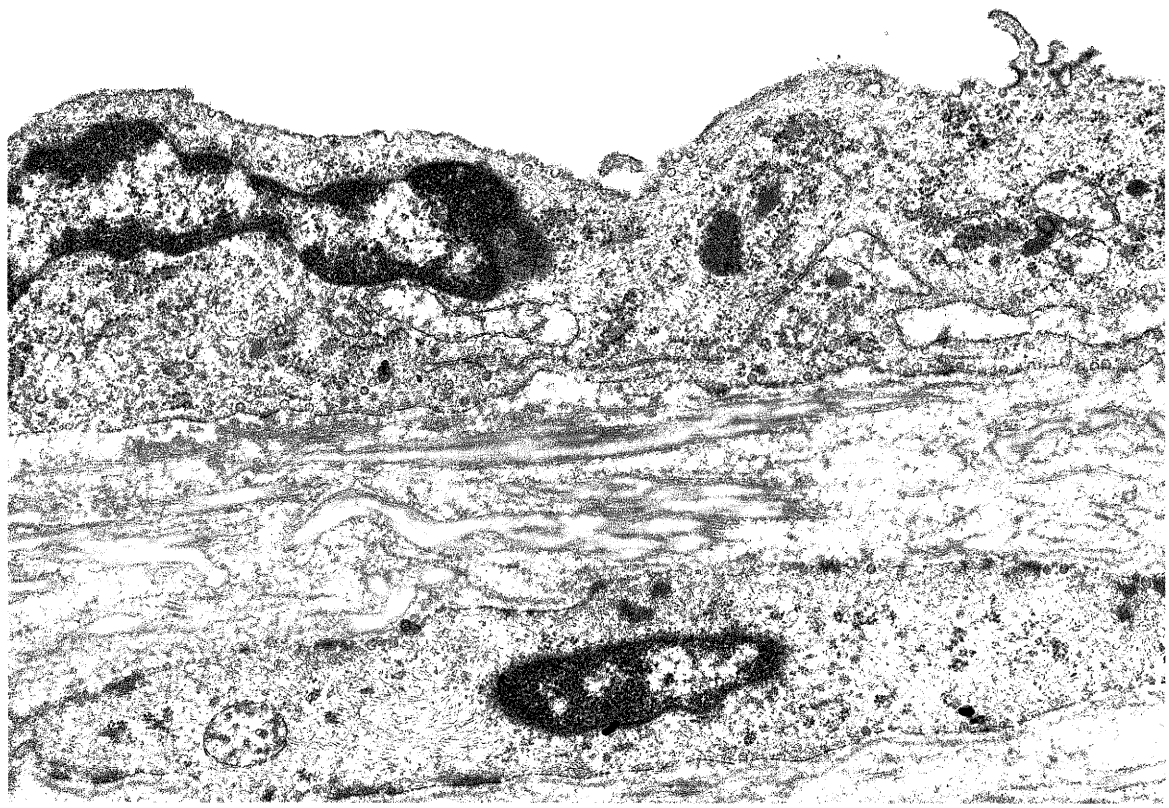

Fig. 6. A. Electron micrographs (6A, B) were obtained from IMA branching of a case of a 3-month-old boy. Apical intima shows that the subendothelial basement membrane develops well and elastic and collagen fibers run almost parallel to the endothelium. Uran-lead stain. ×20,000

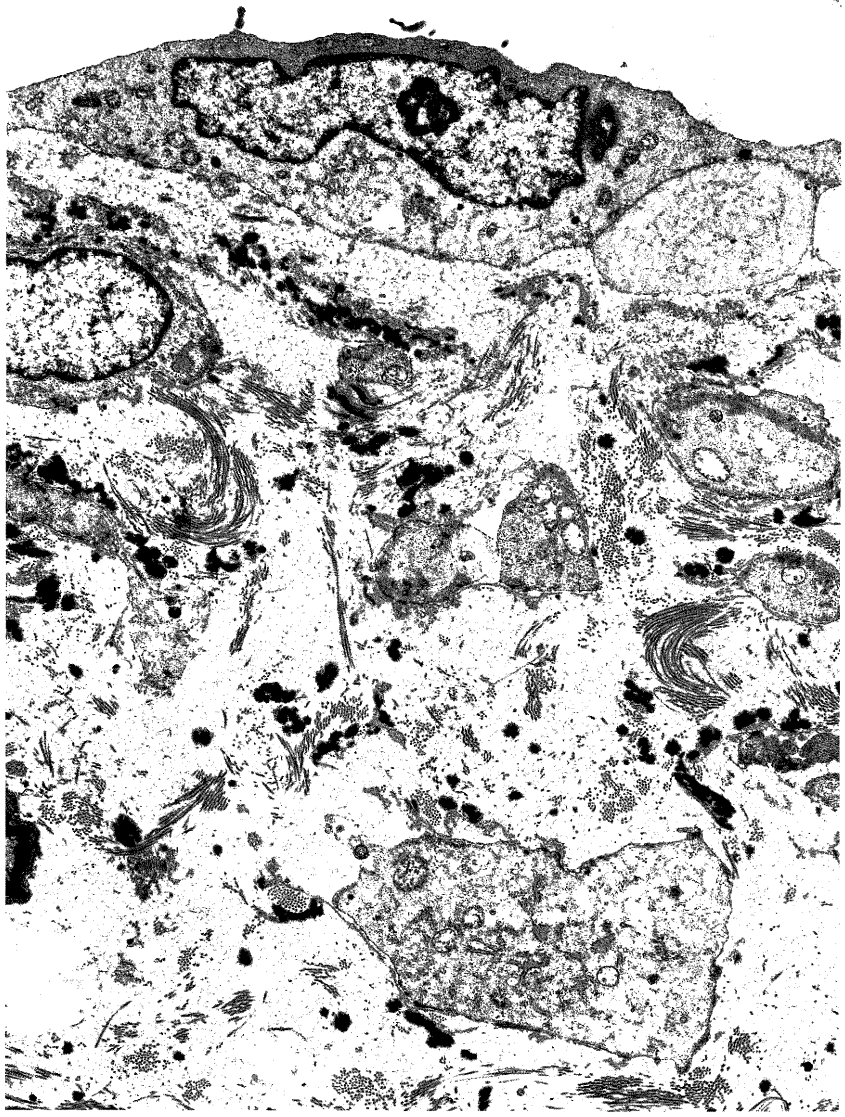

Fig. 6. B. Lateral intima has small fragments of elastic and collagen fibers running in arbitrary directions. Tannic acid-uran-lead stain. ×5,400

among lipid-laden macrophages. In the apical intima of the same case, the SMC scattered fibrous matrix containing lipid droplets in their cytoplasms. Endothelial cells covering the apex also showed apparent bundles of stress fibers in the cytoplasms with well-developed subendothelial basement membranes.

In those cases aged in the second decade, predominant findings in the apical intima were the occurrence of synthetic SMC accompanied by collagen and elastic fibers and an increase of basement membranes and basement membrane-like substances beneath the endothelium. Macrophage invasion beneath the endothelial cells was not infrequent in the outer lateral wall.

In the third and fourth decades, the apical intima, which was covered with flat endothelial cells, had a remarkable increase of collagen fibers giving a dense appearance. Intimal SMC embedded among collagen fibers were elongated and showed contractile phenotype (Fig. 7), being rich in microfilaments and poor in synthetic organelles in their cytoplasms, and they were surrounded by distinct thick basement membranes. Contrary to the apical intimas, the intimas of the outer lateral walls had a loose appear-

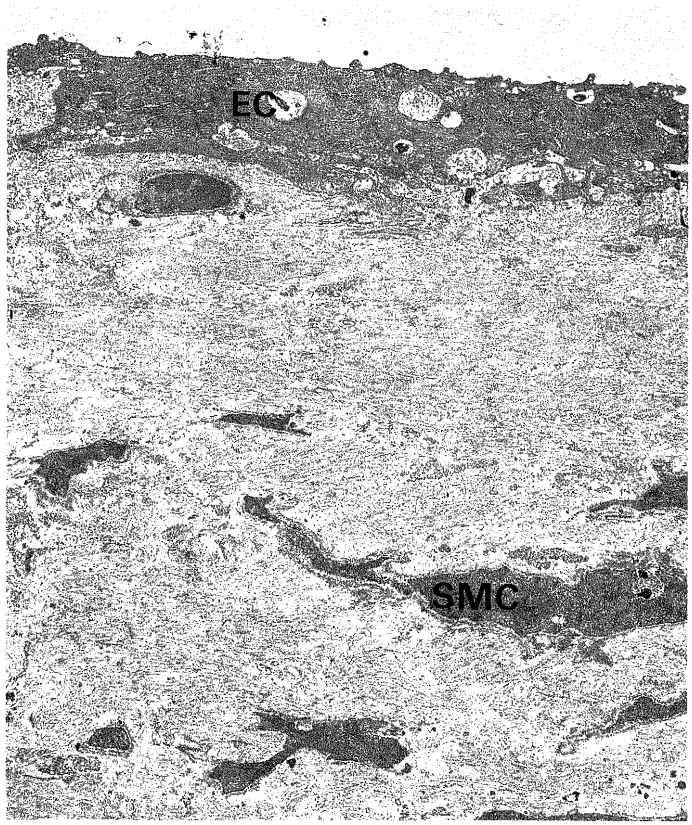

Fig. 7. Apical intima has contractile SMCs which are surrounded by collagenous fibers. A 25-year-old female. Uran-lead stain. ×4,500

ance due to the accumulation of either a matrix abundant in proteoglycans or flocculent materials and sparse collagen and elastic fibers. Intimal SMC of the lateral wall had wide cytoplasms filled with rough-surfaced endoplasmic reticuli (RER), so they were regarded as synthetic phenotype (Fig. 8). Basement membranes which surrounded SMC incompletely were fragmented and scattered in the intercellular matrix. Endothelial cells covering the mucinous intima of the outer lateral walls sometimes had many mitochondria and RER and appeared to bind unsteadily to the underlying tissues, as if floating on the mucinous intimas. Subendothelial basement membranes were thin and fragmented. In the subendothelial regions, erythrocytes and/or lymphocytes were frequently found.

After the fourth decade, the outer wall intima exhibited frequent denudation of endothelial cells, leading to platelet adhesion and enhancement of the insudation to accumulate blood constituents in the inner layer of the intima. There was infiltration of lymphocytes and macrophages, as well as deposition of fibrin threads. Subsequently, cellular and fibrous components in the inner layer of the intima appeared to have been dispersed by the accumulation of edema fluid. Lipid droplets appeared in synthetic SMC in the intima to form foam cells (Fig. 9). In the depth of intimas, there was the elastic muscular layer consisting of SMC and collagen and elastic fibers scattered between SMC. Usually the elastic muscular layer was not affected by intimal edema.

The distinct differences of intimal structures between the two regions in cases over the 5th decade were as follows; 1) the apical intima showed thicker basement membranes in the subendothelium, 2) stronger fibrous appearance in the intima consisting of strong bundles of thicker fibers and a lower population of synthetic SMC. In contrast to the apical intimas, lateral intimas had a loose appearance, due to the accumulation of proteoglycans, which was proven with ruthenium red staining or edema fluid, and a high population of synthetic SMC in which lipid droplets were stored. Lipid-laden macrophages were observed very often.

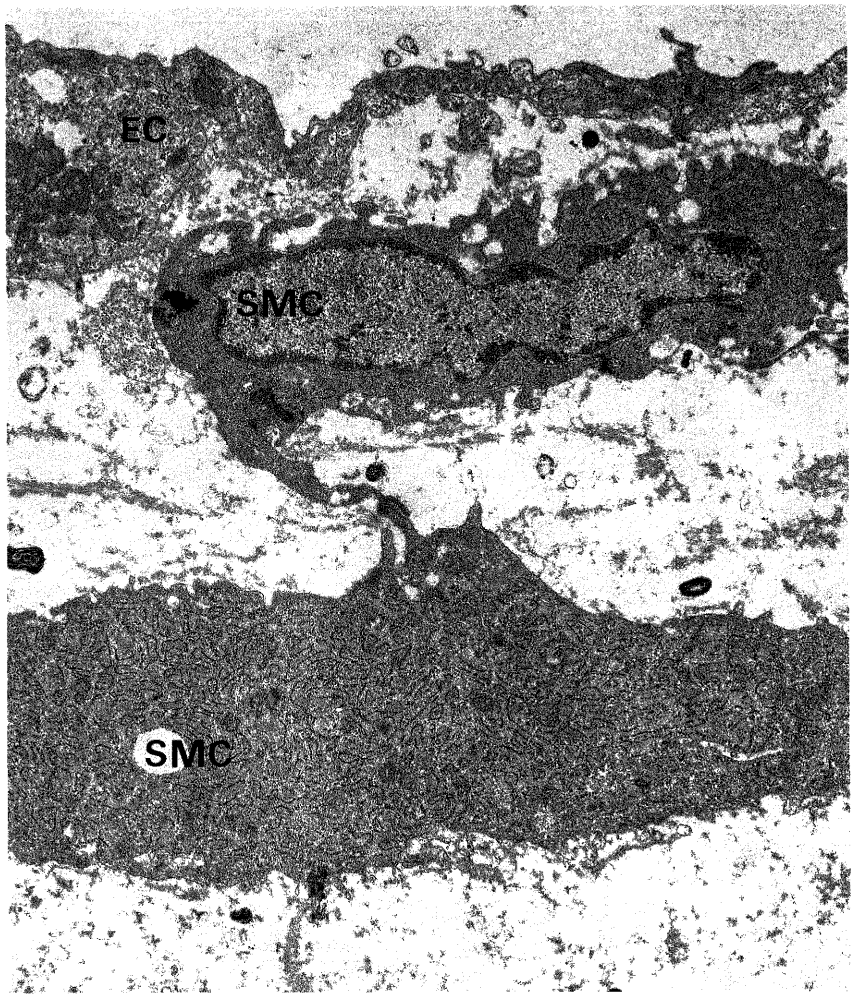

Fig. 8. Synthetic SMCs are observed in the lateral intima which is rich in proteoglycans. The same case to Fig. 7. Uran-lead stain. ×10,000

2. Effects of collagen gel on SMC function and morphology

Electrophoresis on collagen type I purchased from either CR or NG revealed that CR type I collagen contained approximately 90% of type I and 10% of type III, while NG type I collagen consisted of pure type I (Fig. 10).

When both intimal and medial SMC were plated on the CR collagen gel, their growth was suppressed as compared with SMC cultivated on plastic dishes (Fig. 11). The number of intimal SMC on plastic dishes on the 7th day of experiment reached $45.8 \pm 2.8 \times 10^4$/dish but that on the collagen gel gained only half of the number ($27.4 \pm 1.4 \times 10^4$ cell/dish), yielding significant differences in cell number between the two groups after the third day. The growth rate of medial SMC on collagen gel was suppressed more effectively than that of intimal SMC (Table 3). Thymidine uptake into either type of cell was also decreased when cultivated on the gel.

Intracellular cAMP levels of both intimal and medial SMC that had been cultivated on plastic dishes increased after the third day. Intimal SMC, showing higher proliferative activity, produced less cAMP than medial SMC. On the fifth and seventh experimental days, there were apparent differences between production of cAMP by both intimal and medial SMC. When both cells were inoculated on the CR collagen, cAMP levels increased more rapidly than they did on plastic dishes (Table 4). In contrast to the CR collagen, the NG collagen did not show any

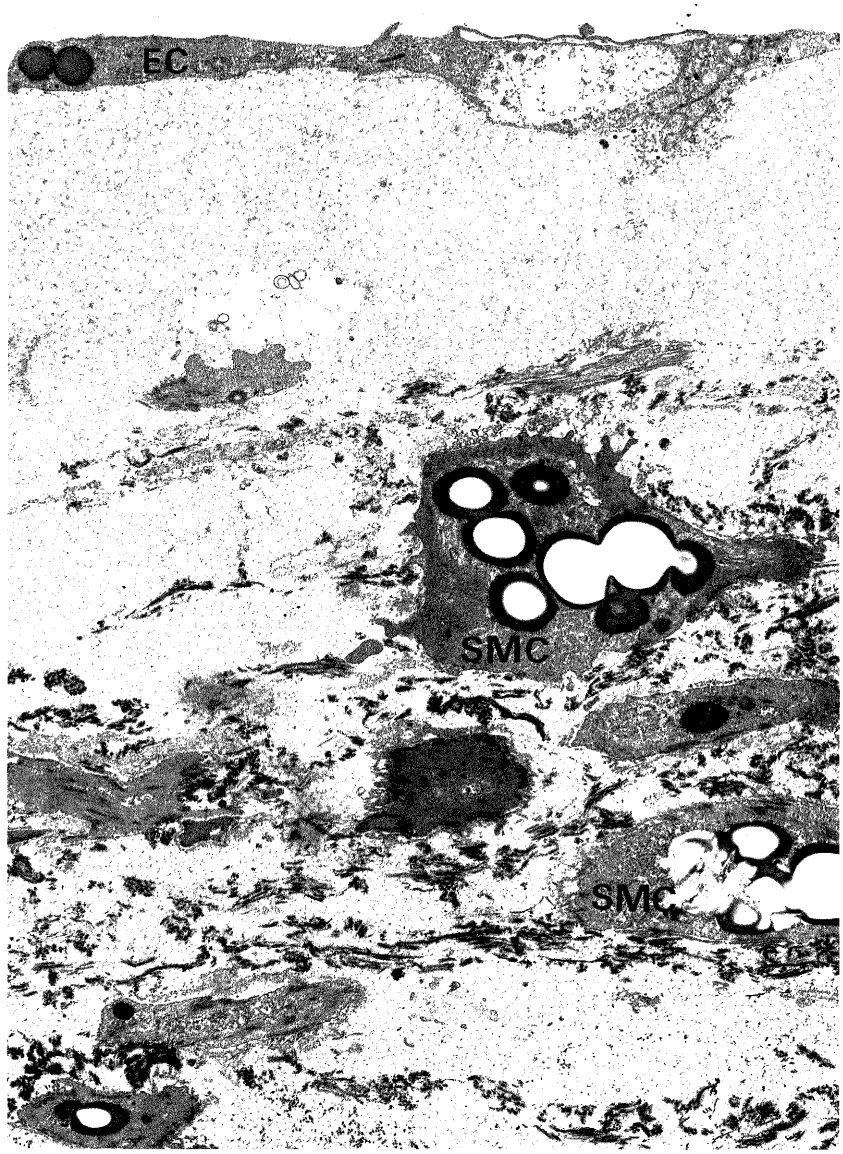

Fig. 9. A lateral intima has synthetic SMCs in which lipid droplets accumulate. Note that the subendothelial basement membrane poorly develops and fragments of fibrous components are dispersed in edematous intima. A 51-year-old man. Tunnic acid-uran-lead stain. ×6,000

effect on either cell number or cAMP content in SMC.

Under phase-contrast microscopy, on plastic dishes, intimal SMC with a monolayer structure, like fibroblasts, began to show a hill-and-valley growth pattern like typical SMC on the CR gel, but not on the NG gel. Electron microscopy disclosed that areas covered with cytoskeletal filaments increased in the cells grown on the CR gel after the 2nd day, but not in the cells grown on the NG gel (Table 5). Cultivation on the CR gel greatly increased thin filaments (75%). Microfilaments among cytoskeletal filaments in the cells cultivated on the CR gel increased more predominantly than intermediate filaments. The NG gel appeared beneficial to the growth of SMC rather than suppressive or non-effective on the cell growth. And it did not increase the cAMP level in the cells. The concentration of cAMP correlated versely with growth rates in the cell number per day.

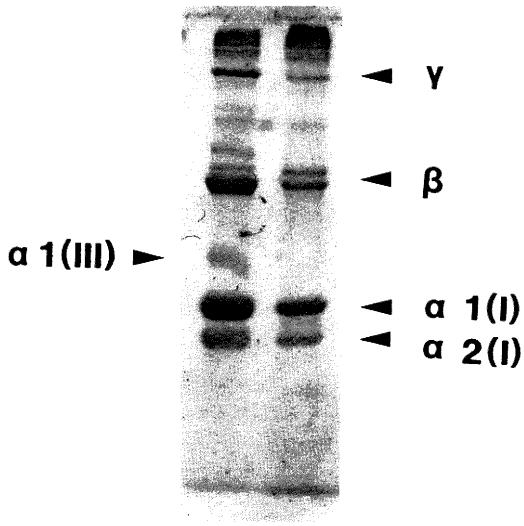

Fig. 10. Electrophoretic patterns of CR and NG collagens.

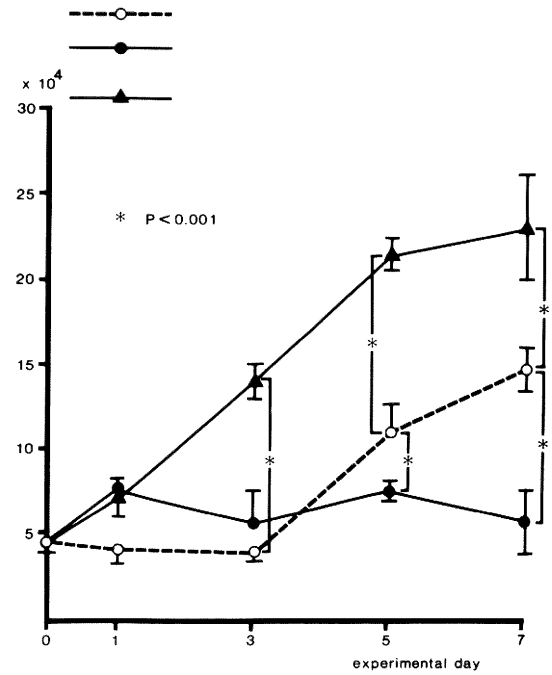

Fig. 11. Growth curves of atherosclerotic intimal SMC cultivated on either collagen gel or plastic dishes.

Table 3. Effects of CR collagen gel on cell growth of arterial SMC

	Atherosclerotic intimal SMC		Intact medial SMC	
Day	on gel	on plastic	on gel	on plastic
1	10.3±1.2	8.9±0.9	0.87± 0.1	11.5±1.3
3	20.9±1.1	27.2±1.2	2.72± 0.1	12.9±1.4
5	25.5±1.6	43.3±1.9	3.39± 0.2	16.2±1.3
7	27.4±1.4	45.8±2.8	2.96±0.08	15.8±2.1

Table 4. Effects of CR collagen gel on cAMP contents in arterial SMC

	Atherosclerotic intimal SMC		Intact medial SMC	
Day	on gel	on plastic	on gel	on plastic
1	3.23±0.31	3.15±0.23	5.35±0.44	3.10±0.42*
3	6.35±0.34	3.35±0.60*	8.20±0.80	3.45±0.43*
5	10.50±1.26	5.38±0.22*	10.45±0.91	8.05±1.29
7	11.60±0.33	8.38±1.16*	10.75±1.28	10.40±1.27

*$p<0.001$ SMC on gel vs on plastic

Table 5. Volume percentage of microfilament bundles in atherosclerotic intimal SMC cultured on CR collagen gel

Day	3	10
Cultures on plastic dish	8.0±1.1 n=18	13.2±3.3 n=10
collagen gel	14.2±1.2 n=24	24.3±4.0 n=15

3. Studies on morphological and functional differences between endothelial cells on the apex and shoulder of a flow divider at the bifurcation of the rabbit aorta

Rabbits placed on atherogenic diets for 2 weeks showed a special geographic pattern of lipid deposition around bifurcations of the aorta. Our investigation was focused on the bifurcation of the brachiocephalic trunk from the aorta.

Lipid deposits were crescent-shaped along the full arc of the flow divider and spared their leading edge, especially around the center of the arc. Such a pattern of lipid deposition was generally encountered in any bifurcation of the aortae. Crescent-shaped lipid deposits had various widths along the flow direction in individual rabbits, averaging a width of 1.1 mm at the center of the arc (Fig. 12). The area spared from lipid deposition on the flow divider of this bifurcation generally covered 160 to 250 μm from the apex downward in the 2-week-cholesterol-feeding cases.

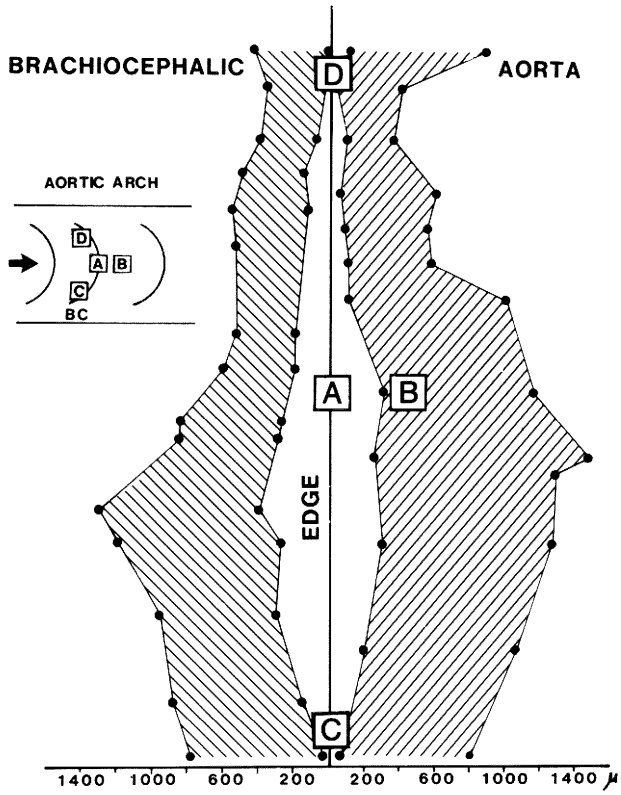

Fig. 12. Hatched areas have deposition of apoprotein E in the flow divider of the brachiocephalic trunk. Histlogical serial sections were stained immunohistochemically with an antibody to Apo E and the areas stained were measured. Apo B also deposits in similar crescent form.

Under a scanning electron microscope the form of endothelial cells on the flow dividers varied depending upon the distance from the edge. Namely, just the arc of the leading edge of the divider was covered by round endothelial cells. The neighboring region 110 to 240 μm downward, was covered by elongated cells and the next area was covered by oval cells (Fig. 13. Table 6). Lipid deposition occurred consistently in the area of oval endothelial cells arranged in a cobblestone-like fashion. Intact rabbits which did not receive the cholesterol diet showed similar forms of endothelial cells on flow dividers. The area preferred by lipid deposition was termed as a shoulder of the flow divider. The cell shape index seemed to depend on geographical localization of the cells, but independent of diet.

Investigation with a transmission electron microscope on intact rabbits revealed that spindle endothelial cells most frequently showed stress fibers in their cytoplasms (Table 7), less vacuoles, and thick subendothelial basement membranes (Fig. 14). On the contrary, oval endothelial cells covering regions which were expected to be involved in lipid deposition after switching to the atherogenic diet had swollen with many vacuoles with synthetic organelles and mitochondria, and less stress fibers (Fig. 14).

When intact animals were injected intravenously with horseradish peroxidase 4 min prior to sacrifice, large numbers of pinocytotic vesicles and intracellular junctions of endothelial cells were found (Fig. 15). Subendothelial deposition of HRP was distinctly larger. There was still a difference of the number of

Table 6. Cell shape index of endothelial cells on the flow divider of the brachiocephalic trunk

	Normal stock diet	Atherogenic diet	
		2w	4w
Apical round cell	0.38±0.11 n=30	0.46±0.16 n=61	0.52±0.20 n=15
Subapex elongated cell	0.18±0.11 n=61	0.09±0.03 n=60	0.13±0.06 n=60
Shoulder oval cell	0.35±0.23 n=60	0.34±0.12 n=60	0.22±0.07 n=63

Table 7. Volume percent of microfilament bundles in endothelial cells on the flow divider of the brachiocephalic trunk

Apex	Shoulder
9.09±6.05% n=19	2.17±2.95% n=18

$p<0.001$

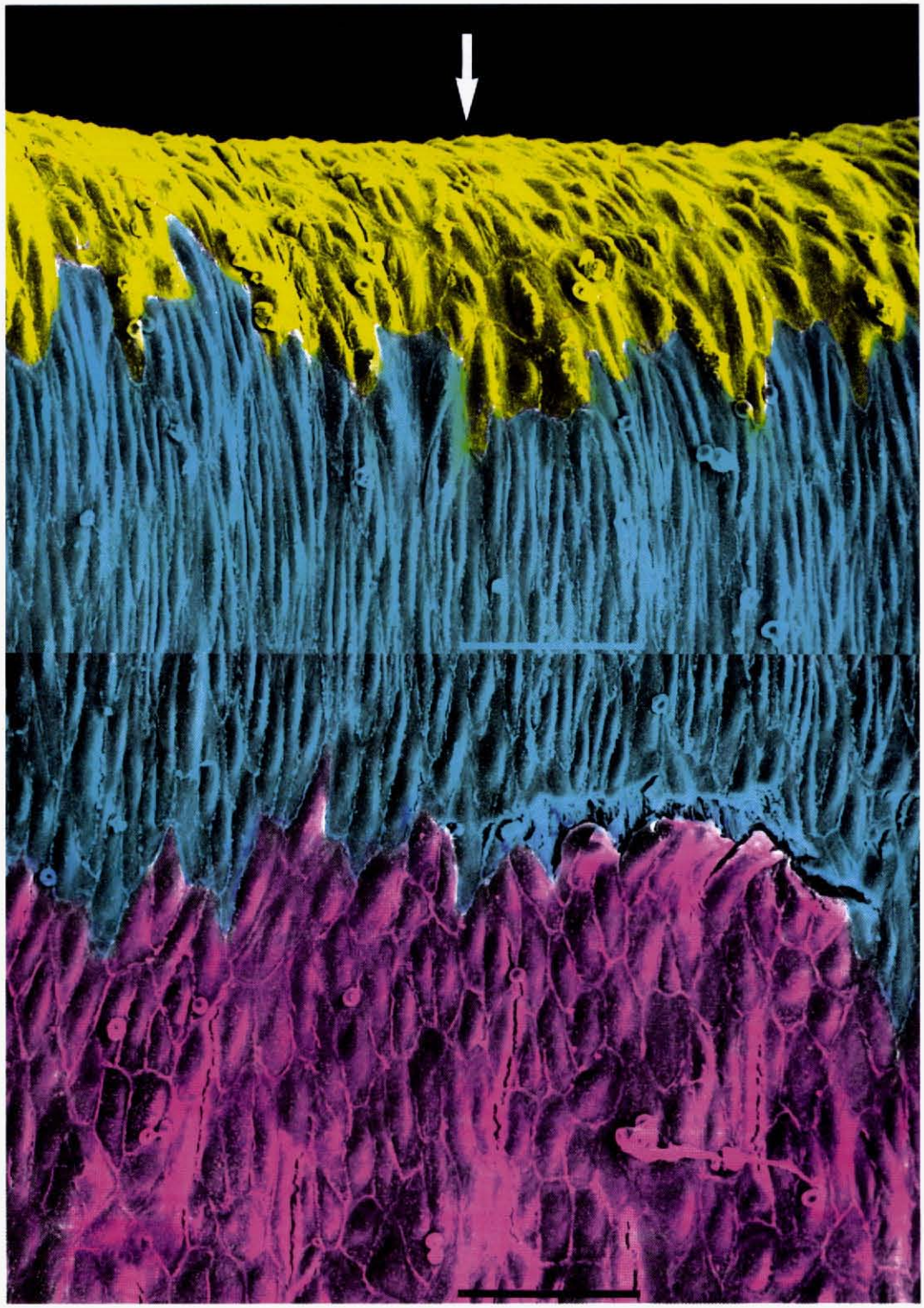

Fig. 13. Scanning electron micrograph shows different shapes of endothelial cells covering the flow divider on the brachiocephalic trunk. Apex has round cells (top), subapex elongated cells (middle) and shoulder oval cells (bottom). Lipid deposition of cholesterol-fed rabbits occurred consistently in the area having oval endothelial cells. Bar: 50 μ. Arrow: the flow direction.

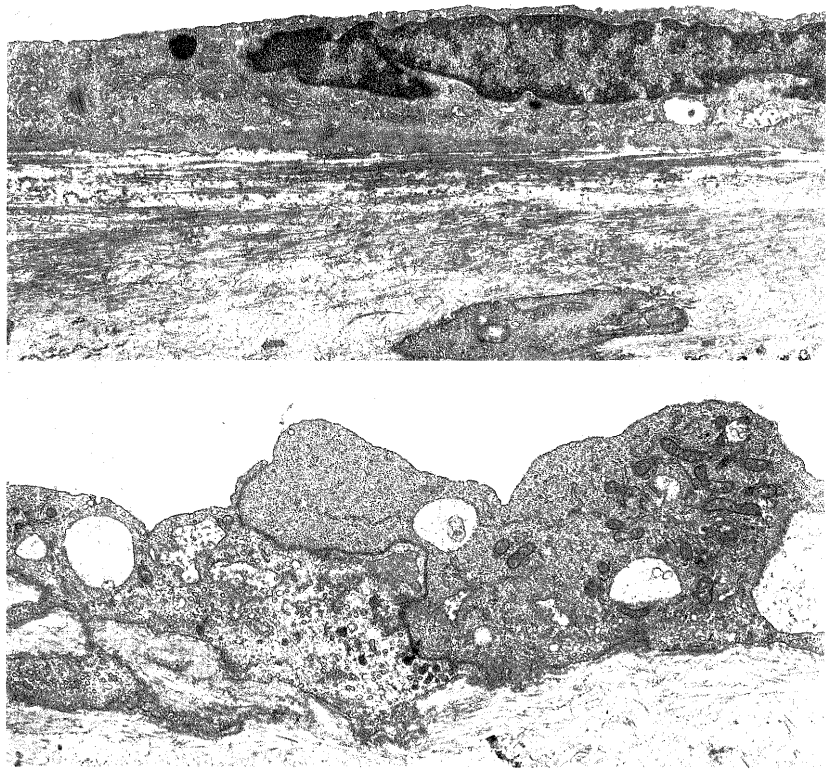

Fig. 14. Electron micrographs show elongated (upper) and oval cells (bottom) obtained from the subapex and shoulder of the flow divider at the brachiocephalic trunk in an intact rabbit. Uran-lead stain. ×8,000. Note that an elongated cell has microfilament bundles in the cytoplasm and thick basement membrane beneath the endothelial cell. Oval cels have vacuoles and mitochondria observed in thickened cytoplasms. Uran-lead stain. ×8,000

the vesicles and junctions between the endothelial cells of two areas after 15 min, although the numbers and staining intensity increased in both areas.

After switching the rabbits from the stock diet to the atherogenic diet, the number of vesicles and intercellular junctions containing HRP increased in both areas. Subendothelial connective tissues were stained more strongly, but endothelial cells at the shoulder were still a dominant area for the permeation of HRP rather than elongated cells at the flow divider.

When ferritin was injected intravenously 30 min before sacrifice it was found that oval endothelial cells on the shoulder had more vesicles containing ferritin in normal and cholesterol-fed rabbits than the endothelial cells covering the apical portion of the flow divider. When rabbits which had been immunized with HRP were placed on an atherogenic diet for 2 weeks immunoglobulins were detected more frequently in endothelial cells of the region with lipid deposition than the apex of the flow divider. Intact animals did not show any antibody endocytosis in either area.

DISCUSSION

Areas exposed to low and turbulent shear stress produced by local flow patterns have been adopted as preferential sites for human atherosclerosis.[2–4,6,7] On the contrary, however, high and unilateral laminar shear stress regions have been recognized as resistant areas for the disease in human beings.

Rabbits had an absolutely different geographical pattern of lipid deposition in the aorta than humans.[5,17] The lipid deposition in rabbits occurred in the flow divider of bifurcations which, in the human artery, was usually spared from atherosclerotic lesions. The human artery showed atherosclerotic lesions in the outer lateral wall of bifurcations in

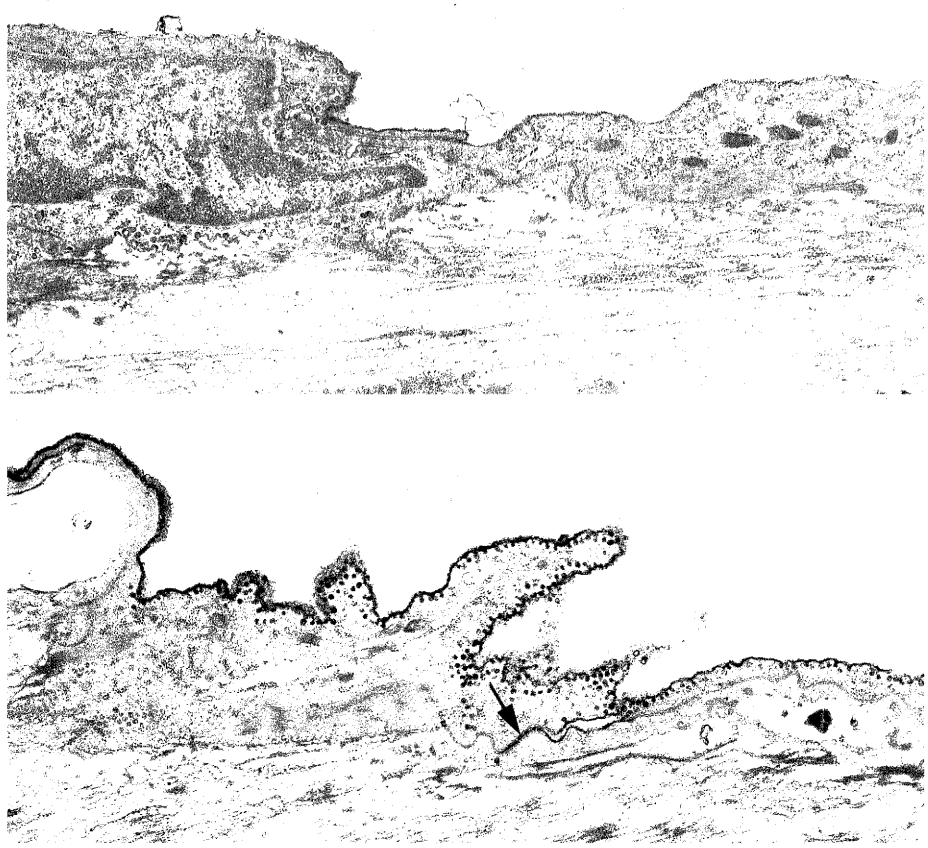

Fig. 15. An upper micrograph shows that elongated endothelial cells have less vesicles containing HRP, while oval cells have many vesicles with HRP (bottom). An intercellular junction is stained distinctly with HRP (arrow). A rabbit fed with the atherogenic diet for 2 weeks was injected with HRP 4 min prior to sacrifice. ×12,000

high probability but no rabbit showed lipid deposition. There has been no satisfactory explanation for this species difference of the localization of atherosclerotic lesions.

First, we will discuss the preferential sites for human atherosclerosis.

We selected the inferior mesenteric artery (IMA) for our investigation for three reasons: 1) The order of magnitude of Reynold's number at the branching site would be estimated at around a few hundred. This is because of A) the order of magnitude of the peak Reynold's number in the upper part of the abdominal aorta is at around 1,000,[4] and B) the blood volume at IMA ostium might be smaller than that of the upper abdominal aorta in which there are several major branches receiving considerable amounts of the blood from the upper abdominal aorta. Because of this low Reynold's number, the blood flow around this branching will be laminar unless there is marked irregularity of the luminar surface of the wall. 2) The IMA ostium locates in the area which has an enough length of the aortic segment without major branches. The ostium is usually downstream from the ostia of the renal arteries by two or three aortic diameters without any major branches between them. This length of segment can allow the blood flow to become consistent at the branch of IMA. 3) The inlet of the IMA branch has one of the highest probabilities of sudanophilia.[6]

It was confirmed in this study that intimal thickness at bifurcations was thicker in the lateral wall than in the apical wall in humans over 10 years old. Newborns, on the other hand showed a thicker intima in the apical wall rather than in the lateral wall. These findings were similar to the results obtained by Friedman.[1] He stated that the early stages of thickening were dominated by smooth muscle accumulation and with time increasing amounts of matrix were produced, particularly where the shear stress was low. The dependence of the intimal thickening

rate on mural shear was complex and changed with times. In particular, the intimal thickness at sites exposed to high unidirectional shear increased quickly to a modest value, growing slowly thereafter, while the thickness at sites exposed to low and more oscillatory shear increased more slowly but in time reached higher value.[1] The early stages of thickening is dominated by SMC, that is, proliferation of intimal SMC induced by vascular injury due to high shear stress. Intimal edema, which is caused by increased endothelial permeability, may be evidence of the vascular injury. Four hundred dyn/mm^2 is a critical magnitude of shear stress to lead to endothelial damage.[5] Even if the magnitude is less than critical it can stimulate endothelial cells to increase their permeability.

Injured or regenerating endothelial cells, after any kind of vascular injury promote not only an increase of endothelial permeability, but also a release of growth factors to stimulate SMC proliferation.

Long-standing mild unidirectional shear stress may produce adaptational structures, bundles of cytoskeleton and thick basement membranes in and beneath the endothelial cells. In experimental animals the elongated endothelial cells, which are expected to be exposed to unidirectional high shear stress, showed higher volume of cytoskeletal structures and less pinocytotic vesicles. It remains unclear whether an increase of cytoskeleton can affect endothelial permeability.

Smooth muscle cells in the intima of newborns showed synthetic phenotype. Arterial SMC have two phenotypes, contractile and synthetic, in both *in vivo* and *in vitro* conditions.[17,18] The different phenotypes of SMC show much different characteristics in not only morphological points but also functional behaviors. The cytoplasm of the contractile cell contains numerous myofilament bundles. Synthetic organelles, such as rough surfaced endoplasmic reticulum, Golgi complex, and free ribosomes are few in number and located in the perinuclear region. Their function is primarily contraction. On the other hand, the synthetic cells contain few filament bundles but large amounts of rough endoplasmic reticulum, Golgi complex, and free ribosomes.[19] Synthetic SMC are regarded as an essential form for cell division. Therefore, if suppressive regulation is not applied on the apical intima, atherosclerosis will develop equally in the apex as well as in the lateral wall or faster in the apex than in the lateral wall.

We focused our attention on the regulatory mechanisms for the proliferation of SMC which must exist in the apical intima of human bifurcations. After the 3rd decade, collagen fibers accumulated predominantly in the apical intima. There was no evident result whether wall shear stress could affect collagen synthesis of intimal smooth muscle cells. It is conceivable that endothelial cells can transmit some stimuli to smooth muscle cells to increase collagen synthesis, because there are gap junctions between endothelial cells and intimal smooth muscle cells which soluble mediators can pass through uni- or bi-directionally to modulate cell functions.[20] There are some regulatory systems reported by some researchers[18] to control SMC proliferation in the vessel wall, such as integral and quiescent endothelial cells, heparin and heparan sulfate.

To clarify the effect of collagen fibers on the SMC proliferation and morphology, we studied cultivated SMC on collagen gel. Our results showed that collagen gel was one potential substance to modulate SMC morphology from the synthetic type to contractile type and to suppress DNA synthesis. After cultivation on CR collagen gel for 2 days, synthetic SMC, obtained from atherosclerotic intima increased their cytoskeleton to change their phenotype to the contractile form and cAMP also increased.

The suppressive mechanism of collagen gel against SMC proliferation is still unknown. From these results it is assumed that type III collagen may be a contributing factor to suppressive effects on SMC proliferation. NG collagen gel, consisting of pure type I collagen, showed favorable effects on the proliferation to increase, but CR collagen containing 90% of type I and 10% of type III, decreased cell proliferation. This study needs further investigation.

Lateral wall intima had thinner and discontinuous basement membrane beneath the endothelium. Endothelial cells covering lateral intima were equipped with fewer stress fibers. It may be assumed that the endothelium on the lateral wall is likely to peel off because the anchoring structure of the endothelium to the subendothelium appeared looser. Heparan sulfate, which is one of the beneficial substances in modulating phenotypes of smooth muscle cells from synthetic to contractile, was found in basement membranes.[21]

Synthetic SMC existed in the lateral wall intima, where proteoglycans were abundant. Ruthenium red stained granules were present in an increased concentration in the inner layer of the lateral intima and a decreased concentration in the fibrous apex. It must be noted that some proteoglycans, especially dermatan sulfate proteoglycans, which is dominant in the intima as well as chondroitin sulfate, form insoluble precipitates with LDL[22] in the presence of cal-

cium irons. This chemical reaction is considered to be responsible for the extracellular deposition of lipid.

Lipid droplets appeared in synthetic SMC, but not in contractile SMC. Enhanced degradation of β-VLDL and VLDL and decreased specific activity of acid cholesteryl esterase as well as degradation of LDL of synthetic SMC were obtained in the contractile phenotype.[19] As a result of disordered lipid metabolism in synthetic SMC, lipid droplet presumably appeared in the synthetic cells.

Although it is clear that proteoglycans accumulate within lateral intima of human bifurcations, reasons for this accumulation are not clear. Some change of environment in the arterial wall induced by the flow conditions is assuredly responsible for the accumulation of glycoproteins. But many factors can be nominated as additional candidates to modulate glycoprotein metabolism during development of atherosclerosis. Cell culture studies have revealed that proteoglycan synthesis is increased when arterial smooth muscle cells are stimulated to proliferate and endothelial cells are stimulated to migrate.[22] Other possible influences of proteoglycan synthesis by arterial cells include cyclic stretching,[23] hypoxia,[24] prostaglandins,[25] interleukins,[26] lipids, lipoproteins,[27] and cell age.[28]

In contrast to human cases sudanophilic lesions in atherosclerotic rabbits occurred most commonly in the flow divider which should be an area of increased exposure to high shear stress. The intensity of shear stress on the endothelial surface tends to vary from point to point depending on the local flow patterns. The shear stress at a flow divider *in vivo* which has been assumed to be 0 at the very narrow apex of the flow divider, the stress becomes greatest a short distance from the apex and rapidly decreases downstream from this point on both sides of the junction.[5] Intimal lipid deposition occurs in a crescent form at the flow divider in early stages of atherosclerotic rabbits. The leading edge of the flow divider is spared from lipid deposition. This area covers approximately 160-250 μm from the apex of the flow divider downward in cases on the 2 week-cholesterol feeding. From the combination of macroscopic findings and distributions of magnitude of shear stress, we can easily make an erroneous conclusion that lipid deposition in dietary induced-atherosclerotic rabbits occurred in the area involved with the greatest high shear stress. The critical value of shear stresses necessary to damage the endothelial surface is about 400 dyn/cm.[2,5] Shear stresses on the endothelial surface of relatively uniform aortic segments under basal anesthetic conditions in dogs reached 100 dyn/cm^2. It is conceivable that the peak shear stress in branching regions could approach the critical value. Therefore, some believe theoretically that high shear stress may be triggering endothelial damage to induce lipid deposition in hyperlipidemic rabbits. Scanning electron microscopic investigations of cell shape revealed that lipid deposition occurred in the area covered by oval endothelial cells, not round or elongated cells. *In vitro* studies on the endothelial cells have shown that the cells which exposed to higher stress became longer and arrange themselves parallel to the flow. And also cytoskeleton in the cells became developed and aligned parallel to the cell's long axis.[29] Results of *in vitro* studies could be applied to the explanation on *in vivo* phenomena; lipid deposition in rabbits should occur in the areas exposed to relatively low shear stress, not to the highest shear stress.

When horseradish peroxidase (HRP) was intravenously administered to rabbits, HRP leaked through oval endothelial cells at the flow divider, both in the space of cell junctions and in their pinocytotic vesicles. Ferritin was also endocytosed more in the oval cells than in the other elongated endothelial cells. Endogenous immunoglobulin was not endocytosed into endothelial cells in intact rabbits, but in the hyperlipidemic state, those leaky endothelial cells can permeate immunoglobulin and apoprotein E through pinocytotic vesicles, not through endothelial junctions.

Spindle endothelial cells exposed to high shear stress developed stress fibers in them. Activated endothelial cells may be primary events in an early process of atherogenesis.

The oval endothelial cells at the flow dividers had more vacuoles, rough surfaced endoplasmic reticuli, and mitochondria than the elongated cells. Endothelial cells which showed similar fine cell structures have been regarded as activated endothelial cells. Morphology and functional characteristics of activated endothelial cells have been studied *in vitro,* particularly in conditions stimulated with interleukin 1.[30] The activated endothelial cells secrete growth factors which might stimulate smooth muscle cells and enhance their permeability. Intact rabbits have "activated endothelial cells" in the limited areas located approximately 300 to 1300 μm from the leading edge of the flow divider. Therefore, activation of endothelial cells might be induced by normal blood flow, but activation by flow has been reported only by turbulent flow.[31]

In the initial stage of atherogenesis, endothelial injury has been stressed. Under electron microscopy,

it is difficult to observe apparent injuries such as denudation of endothelial cells or opening intercellular junctions. Endothelial activation might be the initial stage of development of atherosclerosis, but little and turbulent shear are much more effective in stimulating the replicability of endothelial cells without rearrangement of cell alignment. What is contributing to activate endothelial functions? Further studies are required.

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