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Endothelial cell heterogeneity in experimentally-induced rabbit atherosclerosis. Demonstration of multinucleated giant endothelial cells by scanning electron microscopy and cell culture

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Abstract We investigated the aortic endothelial cells of cholesterol-fed rabbits, using scanning electron microscopy and a cell culture technique. Rabbits were given a 1% cholesterol diet intermittently for up to 40 weeks. In these animals, the area of endothelial cells was increased and the cells showed polymorphism in relation to the progression of atherosclerosis. In animals fed the cholesterol diet for 12, 28 and 40 weeks, the average area of the endothelial cells was 436±15, 762±153, and 836±165 μm^2, respectively. In the cholesterol-fed 40-week group, in particular, giant endothelial cells, measuring more than 1200 μm^2, accounted for 14% of the population. In animals fed a standard diet there was no significant difference in endothelial cell morphology between control 0-week and control 40-week groups; in both, the luminal surface of the thoracic aorta formed a homogeneous sheet covered by small rhomboidal endothelial cells, the area of most being less than 400 μm^2. Primary cultured endothelial cells harvested from those control groups were mononuclear typical small cells with a centrally located nucleus; the proportion of binucleated cells was less than 2% and no multinucleated giant cells with three or more nuclei were detected. Endothelial cells from the cholesterol-fed groups, however, contained larger numbers of binucleated cells, with the number increasing in proportion to the duration of cholesterol feeding. The major distinguishing feature of the endothelial cells in the cholesterol-fed groups was the presence of multinucleated giant cells with three or more nuclei; these accounted for 2.3% and 3.3% of the total cell population in the cholesterol-fed 28- and 40-week groups, respectively. No bromodeoxyuridine uptake was found in the nuclei of the cultured multinucleated giant cells. Heterogeneity of endothelial cells, with the concomitant appearance of multinucleated giant cells, emerges with the progression of diet-induced atherosclerosis. The morphological alterations of endothelial cells observed in the present study intimately reflect changes in their function associated with the progression of atherosclerotic lesions.

Key words Atherosclerosis · Hypercholesterolemia
Cell culture · Scanning electron microscopy
Endothelial cell heterogeneity

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Introduction

Endothelial cells have a number of functions which contribute to the maintenance of homeostasis in the blood vessels [4, 14]. Endothelial dysfunction or injury may be a key event in the initiation and progression of vascular diseases, including atherosclerosis [14, 19, 23]. Numerous studies have investigated alterations in endothelial morphology and function relevant to the formation of atherosclerotic lesions. From the 1940s to the early 1960s, using the Häutchen technique, a number of researchers [5, 7, 16, 25] examined endothelial arrangement in human vessels and demonstrated the heterogeneity of endothelial cells and the emergence of multinucleated giant cells in certain conditions. However, this relationship was not addressed further until Repin et al. [1, 22] and we [29] reaffirmed the relationship between the number of giant cells and the severity of atherosclerotic lesions. The relevance of these findings to atherogenesis remains unclear, however, since studies have been limited to analysis in human autopsies. We have therefore turned to an experimental model to investigate the temporal relationship between alterations in endothelial morphology and the development of atherosclerotic lesions.

Few experimental studies [8, 24, 36] have addressed this issue. Here, using scanning electron microscopy (SEM) and cell culture methods, we examined qualitative and quantitative patterns in aortic endothelial cells of the cholesterol-fed rabbit model. We have demonstrated that heterogeneity of endothelial cells, with the concur-

rent appearance of multinucleated giant cells, occurred with the progression of experimental atherosclerosis.

Materials and methods

Twenty-nine male albino rabbits weighing 2–2.5 kg (10-weeks-old) were used. To produce various stages of experimental atherosclerotic lesions under long term hyper-cholesterolaemia, 17 animals were given standard laboratory chow (Clea Japan, Tokyo) coated with cholesterol (Wako Pure Chemical Industries, Osaka), following the method reported by Constantinides et al. [3] with some modification. The animals were fed the cholesterol diet (1.0 g cholesterol/day) for 12 weeks and were then given the standard diet and the cholesterol diet alternately every 8 weeks. The animals were sacrificed at 12, 28, and 40 weeks. There were two control groups: the control 40-week group, consisting of six animals given the standard diet for 40 weeks; and the control 0-week group which consisted of six age-matched animals at the beginning of the experiment. Plasma cholesterol levels in all animals were determined with a Hitachi 7250 automatic analyser (Hitachi, Tokyo).

For SEM heparin (1000 units/kg) was administered intravenously before the animals were anaesthetized (Nembutal; Abbott Laboratories, North Chicago, IU.), following which they were canulated into the left ventricle. Blood was washed out of the aorta with an infusion of phosphate-buffered saline (PBS) and 5% glucose solution. Silver nitrate solution (0.15% in 4.8% glucose solution) was then infused for 1 min and excess silver nitrate was washed off with 5% glucose solution. Perfusion fixation was then carried out with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4). For all steps, the perfusion was adjusted to a physiological level, 120 mmHg. After perfusion fixation was completed, the aorta was excised and adventitial adipose tissue was removed. The aorta was then further fixed with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4), for 12 h. Specimens of the thoracic aorta were cut longitudinally, dehydrated in graded ethanol, and freeze-dried with T-butyl alcohol, after which they were coated with gold palladium in a GEKO IB-3 ion coater (Eiko Engineering, Ibaraki, Japan) and observed in an S-700 SEM (Hitachi). All specimens were obtained from the same upper portion of the descending thoracic aorta.

The area of each endothelial cell was measured in SEMs at ×500–800 magnification; 300 cells on the surface of the dorsal side of each descending thoracic aorta, excluding the orifices of the intercostal arteries and the surrounding regions, were examined, using soft disk Cosmozone 1S analysis system (Nikon, Tokyo).

For transmission EM (TEM) the thoracic aorta was cut into small pieces after being washed with PBS, following which it was fixed in cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Epon 812 (Taab Laboratories Equipment, Aldermaston, UK). Ultrathin sections, double-stained with uranyl acetate and lead citrate, were then examined in a JEM-100CX TEM (JEOL, Tokyo).

Aortic endothelial cells were isolated and cultured by a previously described method [29]. Briefly, the descending portion of the thoracic aorta was excised and the adventitial adipose tissue was removed, after which the aorta was gently washed with PBS from one end. The piece of the aorta with both ends open was incubated in Dispase solution (1000 units/ml; Sanko Pharmaceuticals, Tokyo) for 30 min at 37° C. After being cut open longitudinally, the aortic lumen was gently washed with PBS, using a syringe with a 23-gauge needle. The harvested cells were then centrifuged at 180 g for 10 min. Sedimented cells were resuspended in the culture medium composed of Eagle's minimal essential medium (Nissui Pharmaceuticals, Tokyo) supplemented with L-glutamine (0.294 g/L; GIBCO, Grand Island, N.Y.), 15% fetal bovine serum (Microbiological Associates, Logan, Utah), 30 μg/ml of endothelial-cell growth supplement (Collaborative Research, Lexington, Ky.), 100 units/ml of heparin, and 50 μg/ml of Kanamycin. The isolated endothelial cells were seeded in 35 mm tissue culture dishes, or on coverslips that were coated with 10 $\mu g/mm^2$ fibronectin and incubated in a humid 5% carbon dioxide/95% air atmosphere at 37° C.

After isolation of the endothelial cells was completed, oil red O staining was performed on the aorta, for the gross evaluation of atherosclerotic lesions. The aorta was fixed with 10% formalin, stained with oil red O, and thoroughly rinsed with 70% ethanol.

In preliminary studies, endothelial cells harvested from the rabbit thoracic aorta were exhibited no staining for anti-human von Willebrand factor antibody (Dakopatts, Glostrup, Denmark). Therefore, we used acetylated low density lipoprotein labelled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine-perchlorate (Dil-ac-LDL; Biomedical Technologies, Stoughton, Mass.) as a marker for the identification of endothelial cells [32]. Endothelial cells were cultured on a coverslip for the first 3 days of primary culture. On day 4 of incubation, the old medium was replaced by labelling medium consisting of 1 ml culture medium, including 50 μl of Dil-ac-LDL solution. After incubation for 4 h, the endothelial cells were rinsed in PBS and fixed with 4% formalin, followed by counterstaining of nuclei with bisbenzimide solution (Hoechst dye 33258; Polyscience, Warrington, Pa.). The cells were observed under a fluorescence microscope. In each sample, the ratio of concomitant multinucleated endothelial cells was determined by counting 300 Dil-ac-LDL-positive cells.

For the detection of contaminating smooth muscle cells and macrophages, cells cultured on coverslips were examined with a murine anti-smooth muscle actin monoclonal antibody (anti-SMA ab; MA-933, Enzo, New York, N.Y.) and a murine anti-macrophage monoclonal antibody [34]. The cells were fixed with 4% formalin in PBS and stained with anti-SMA ab and a Histofine SAB-PO kit (avidin-biotin-complex kit; Nichirei, Tokyo). For staining with anti-macrophage antibody, the indirect immunoperoxidase method with peroxidase-conjugated anti-mouse IgM goat IgM (N. L. Cappel Laboratories, West Chester, Pa.) was used.

Primary cultured endothelial cells on a coverslip were examined to determine 5-bromo-2′-deoxyuridine (BrdU; cell proliferation kit, Amersham International, Amersham, UK) uptake [10]. The medium was replaced by labelling medium (1:1000 dilution) on day 4 of incubation, and the cells were incubated for 5 h, following which they were washed in PBS, fixed with acid-ethanol, and then incubated with anti-BrdU antibody for 1 h. They were then exposed to peroxidase-conjugated anti-mouse IgG, followed by the development of peroxidase activity with diaminobenzidine.

For in vivo uptake of BrdU by endothelial cells, two rabbits fed the cholesterol diet for 40 weeks were injected intravenously with BrdU (50 mg/kg body weight) at 3 and 24 h before sacrifice. Aortic endothelial cells were isolated and cultured by the method described above. The cells were incubated for 3 days and stained with the above immunocytochemical method.

The results of observations were expressed as mean±standard deviation. Statistical significance was determined by Student's *t*-test. A P value of <0.05 was considered as significant.

Results

Plasma cholesterol levels

During feeding of the cholesterol diet, plasma cholesterol levels were elevated and, after 12 weeks, the mean serum cholesterol level exceeded 2000 mg/dl. The levels then remained at 1000–1800 mg/dl until the animals were sacrificed. Control animals showed no difference in serum cholesterol levels during the experiment (Fig. 1).

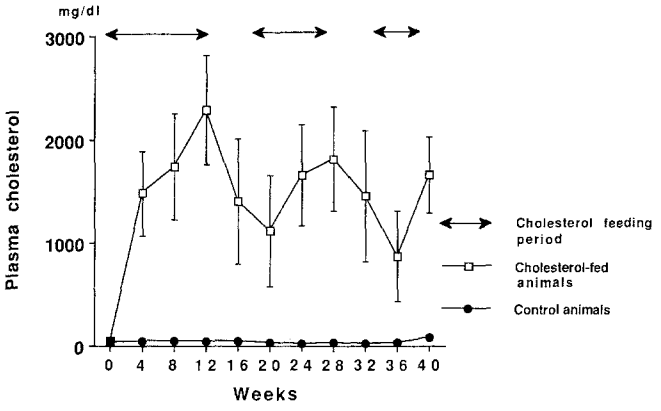


Fig. 1 Plasma cholesterol levels

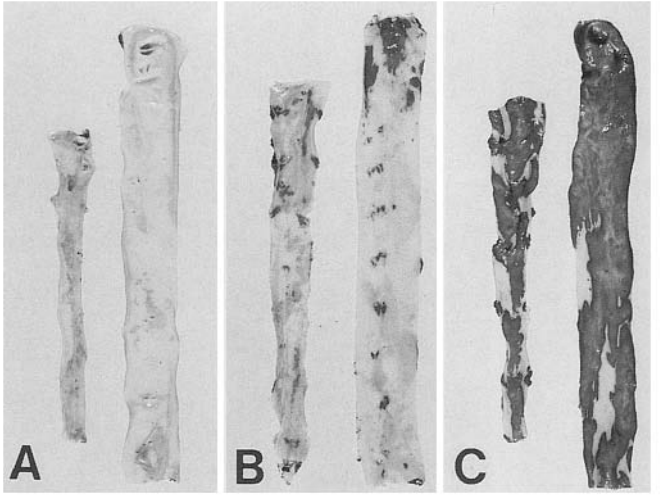


Fig. 2A–C Gross finding of aorta stained with oil red O. **A** Control 40-week rabbit. The luminal surface of the aorta is smooth and oil red O-positive foci are absent. **B** Cholesterol-fed 12-week rabbit. Small atherosclerosis foci are observed in the aortic arch and branching portions. **C** cholesterol-fed 40-week rabbit. The luminal surface of the aorta is diffusely and deeply stained with oil red O, corresponding to severe atherosclerotic changes. (×0.9)

Gross observation of aorta

The inner surface of the aorta of the control 0- and 40-week animals was smooth and flat, and showed no oil red O staining. In the cholesterol-fed 12-week group, small discrete atherosclerotic foci were observed in the aortic arch and branching portions. In the cholesterol-fed 40-week group, the luminal surface of the aorta was diffusely elevated and deeply stained with oil red O, corresponding with severe atherosclerotic changes (Fig. 2). Microscopically, these advanced lesions consisted of marked intimal thickening due to accumulation of numerous foamy macrophages and proliferation of smooth muscle cells. In places, extracellular deposition of lipid materials and calcium was present.

Scanning electron microscope observations

The appearance of the endothelial surface of aortas of the control 0- and 40-week groups on SEM was essentially the same. Rhomboidal endothelial cells appeared as a confluent sheet outlined by silver salt deposition at the intercellular junctions; the cells were arranged with their long axes oriented in the direction of blood flow (Fig. 3A, B). The mean area of the endothelial cells was 374±12 in the control 0-week group and 348±29 μm^2 in the control 40-week groups ($P>0.05$). In cholesterol-fed 12-, 28- and 40-week groups, the mean area of the endothelial cells was 436±15, 762±153, and 836±165 μm^2, respectively (Fig. 3C, D). All these values were significantly different from those of the control 0- and 40-week groups. In the cholesterol-fed 28- and 40-week groups considerable morphological changes were noted in the endothelial cells overlying the elevated lesions of the aorta. The cells were often chaotically oriented and their shapes were altered; polygonal, triangular, square and star-shaped cells were frequently observed. Some of the cells were extremely large, measuring more than 1200 μm^2 (Fig. 3D). In contrast, endothelial cells overlying the flat areas of the aorta were generally small in size and arranged in a fusiform manner along the axis of flow; the features were similar to those of control groups.

Figure 4 shows random distribution of endothelial cells by area in the thoracic aorta in all groups. In both control 0- and 40-week groups, more than 70% of the endothelial cells were small (cell area less than 400 μm^2). There was no difference in the distribution of endothelial cells by area between these control groups. In the cholesterol-fed groups, the distribution was shifted towards populations of cells with a larger area. In the cholesterol-fed 40-week group, in particular, small cells accounted for only 3% of the population, while medium-sized cells (area 400–800 μm^2) accounted for 49%, large cells (area 800–1200 μm^2) for 34%, and giant cells (area over 1200 μm^2) for 14% of the population.

Morphology of primary cultured endothelial cells

In all animals studied, more than 95% of primary cultured cells took up fine granules of Dil-ac-LDL. Cultured endothelial cells can be classified into two distinct types in terms of morphology: a small type and a larger type. The small type is round or polygonal in shape and has a centrally located single nucleus; we found cells of this type to be arranged in a pavement pattern. Their diameters were fairly constant, ranging from 30 to 60 μm. Endothelial cells harvested from the control 0- and 40-week groups appeared as monolayers of typical small cells (Fig. 5A). Endothelial cells of the larger type were more heterogeneous in size and shape. They were 80–180 μm in diameter, and often contained more than

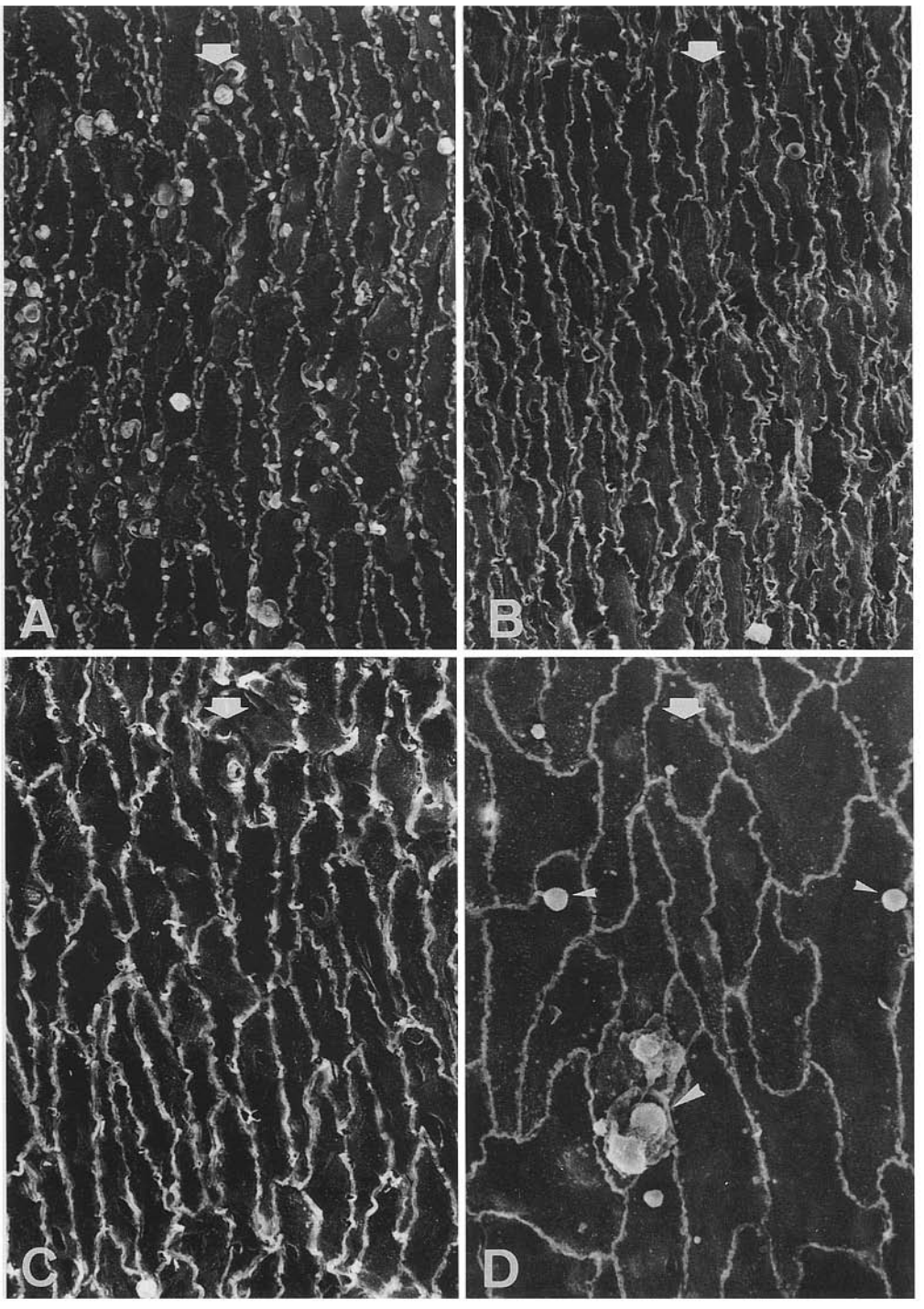


Fig. 3A–D Scanning electron microscopy of endothelial cells in the thoracic aorta. Inner surface of thoracic aortas from control 0-week (**A**) and 40-week (**B**) rabbits is covered by a homogeneous sheet of fusiform cells. In a cholesterol-fed 12-week animal (**C**), endothelial cells with preserved fusiform shape are larger than the cells of control animals. Endothelial cells in a cholesterol-fed 40-week rabbit (**D**) vary in shape and are extremely large. Note attachment of leukocytes to the surface (*small arrowheads*) and focal exposure of subendothelial foam cells (*large arrowhead*). *Arrows* show the blood flow direction. All photographs were taken at the same magnification (×570)

two nuclei, up to seven being seen. Giant endothelial cells with three or more nuclei bear striking similarities to the variant endothelial cells previously demonstrated in human aortic endothelial cell populations [29]. Endothelial cells from the cholesterol-fed groups were characterized by a mixture of typical small cells and larger cells, including the multinucleated giant endothelial cells (Fig. 5B). The percentage of the larger cells decreased as time passed since only small cells proliferated.

Distribution of multinucleated endothelial cells in primary culture

The ratio of multinucleated endothelial cells that appeared concomitantly with increased heterogeneity was determined by counting the number of Dil-ac-LDL-positive multinucleated cells per 300 Dil-ac-LDL-positive cells under an immunofluorescent microscope (Fig. 6). The primary cultured endothelial cells of control 0- and 40-week groups consisted almost exclusively of mono-

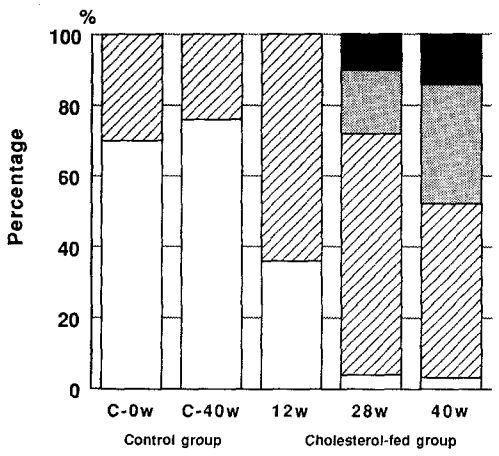


Fig. 4 Distribution of aortic endothelial cells by area. Values represent the mean of 300 cells from three animals. □ cell area less than 400 μm²; ▨ area 400–800 μm²; ▩ area 800–1200 μm²; ■ area over 1200 μm²)

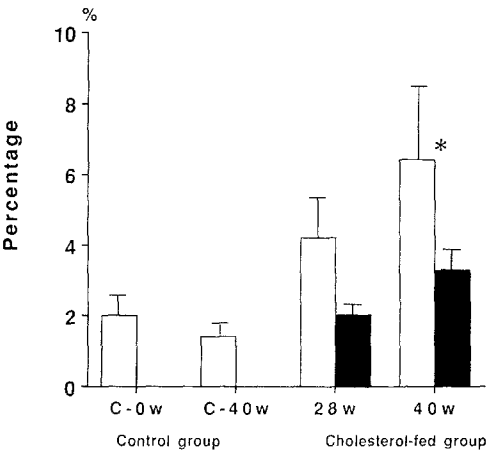


Fig. 6 Distribution of binucleated (□) and multinucleated endothelial cells with three or more nuclei (■) in primary culture. Values represent the mean±standard deviation of 300 cells from three animals. * The percentage of binucleated cells is significantly different from those in control 0-week and 40-week groups ($P<0.05$, Student's t-test)

nuclear cells; the population of binucleated cells was less than 2% and no multinucleated giant endothelial cells with three or more nuclei were detected. However, the endothelial cells of the cholesterol-fed groups contained larger numbers of binucleated cells, with the number increasing in proportion to the duration of cholesterol feeding; the population of binucleated cells was 4.2% and 6.5% in cholesterol-fed 28- and 40-week groups, respectively. The major distinguishing feature of the endothelial cultures from the cholesterol-fed groups was the presence of multinucleated giant endothelial cells with three or more nuclei; these cells accounted for 2.3% and 3.3% of the total cell population in cholesterol-fed 28- and 40-week groups, respectively.

Characterization of multinucleated giant endothelial cells

The presence of multinucleated giant cells was the most striking feature of the present study. Characteristically, they emerged in the cultures of aortas from cholesterol-fed animals, and could be found in cultures immediately after seeding and at all stages of cell growth and monolayer formation. Such cells took up fine granules of Dil-

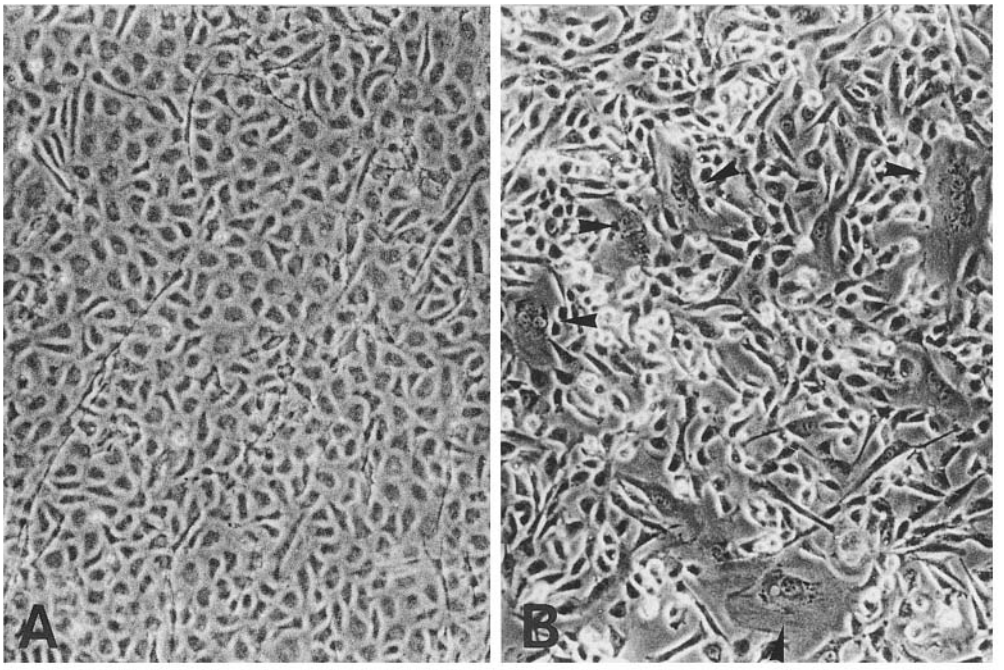


Fig. 5A, B Light microscopy of primary cultured endothelial cells, **A** Endothelial cells from a control 0-week group rabbit are uniform in size and polygonal in shape. **B** In a cholesterol-fed 40-week rabbit, multinucleated giant endothelial cells (*arrowheads*) are common. (×120)

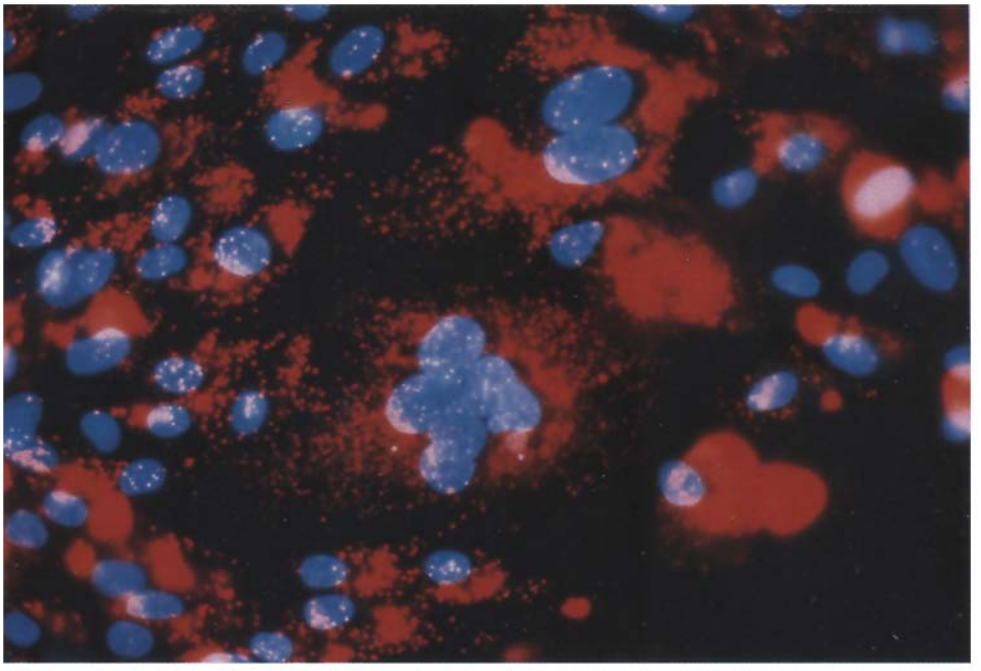
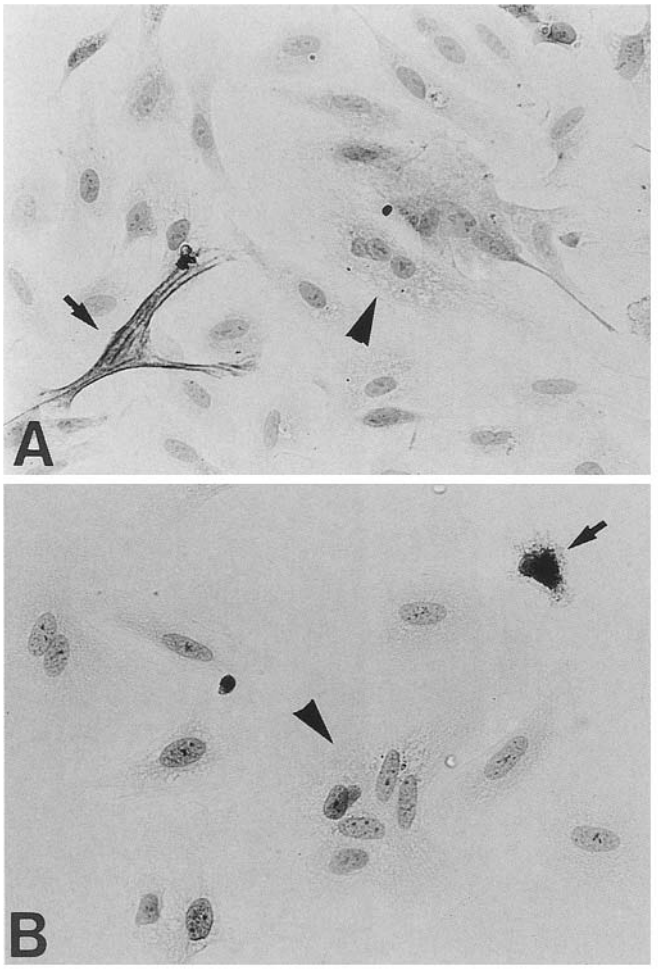


Fig. 7 Multinucleated endothelial cells taking up fine granules of Dil-ac-LDL in the same way as mononuclear endothelial cells. The centrally-located cell has six nuclei, clearly counterstained with bisbenzimide. (×380)



ac-LDL in the same manner as typical mononuclear endothelial cells (Fig. 7). They were invariably negative for staining with both anti-smooth muscle actin antibody and anti-macrophage antibody (Fig. 8).

Proliferation potency of endothelial cells in vitro and in vivo

In primary culture with labelling medium, many mononuclear cells showed incorporation of BrdU. However, none of multinucleated cells was labelled (Fig. 9). Further, we examined the endothelial cells from rabbits administered BrdU before sacrifice. A small number of mononuclear cells demonstrated in vivo uptake of BrdU, while harvested multinucleated cells invariably showed negative staining (Fig. 10).

Transmission electron microscopy

In control groups, the luminal surface of the thoracic aorta was lined by flattened mononuclear endothelial cells on TEM. Weibel-Palade bodies were not seen in the cy-

Fig. 8A, B Immunohistochemical staining with anti-smooth muscle actin antibody (**A**) and anti-macrophage antibody (**B**). **A** Centrally-located giant cell with three nuclei (*arrowhead*) is negative for anti-smooth muscle actin antibody. *Arrow* shows positively-stained muscle cell. **B** Multinucleated giant cell (*arrowhead*) shows negative reaction to anti-macrophage antibody, while a macrophage (*arrow*) shows positive staining. **A**: ×420, **B**: ×520

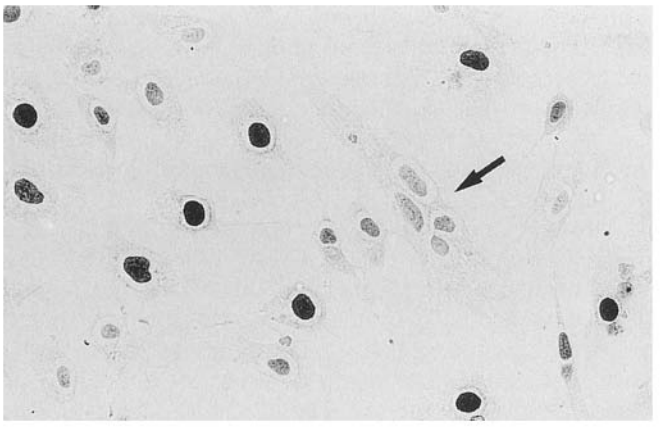


Fig. 9 Bromodeoxyuridine (BrdU) labelling in primary culture demonstrates positive staining of many mononuclear endothelial cells. Note a giant cell with four nuclei (*arrow*) showing no uptake of BrdU. (×340)

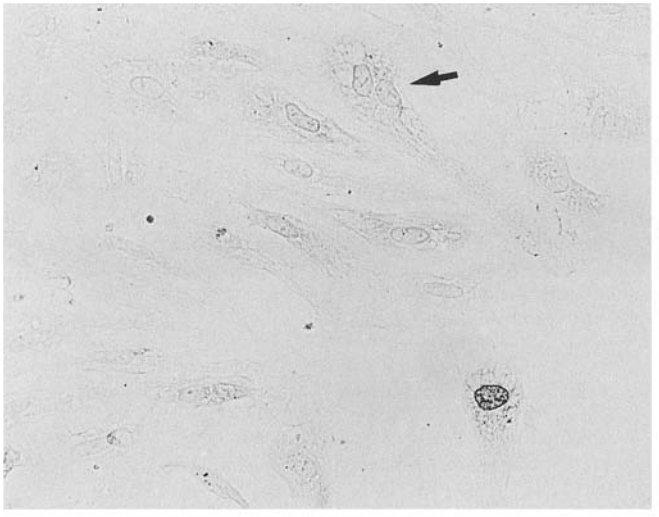


Fig. 10 In vivo uptake of BrdU demonstrates one mononuclear endothelial cell with positive labelling. Nuclei of a binucleated endothelial cell included (*arrow*) show no positive staining. (×410)

toplasm. Observation of the aortas of cholesterol-fed 28- and 40-week group animals revealed apparently multinucleated endothelial cells on advanced atherosclerotic lesions (data not shown). Apart from the nuclear configuration, however, their ultrastructural features were not different from those of the mononuclear endothelial cells. On the flat areas of the same animals, multinucleated endothelial cells were not detected.

Discussion

Aging is a major factor in the pathogenesis of atherosclerosis [2]. In our previous study of human aortas [29], large and multinucleated giant cells were seen not only in relation to the severity of atherosclerosis but also in relation to aging. Atherosclerosis in humans, however, results from long-term exposure to various atherogenic factors [19] and it is difficult to examine the role of aging alone in relationship to endothelial cell morphology. To estimate the effect of aging as a contributing factor we prepared a second control group that was given a standard diet for 40 weeks (control 40-week group) to serve as a comparison with animals sacrificed at the beginning of the experiment (control 0-week group). As shown in the Results the endothelial cells in both control groups had essentially the same features; enlargement with the concomitant emergence of multinucleation was not seen in the aged control animals. The morphological alterations of endothelial cells observed in the present study were not simply due to the biological aging of the experimental animals.

The most important finding in this study was that of abnormalities in endothelial cell morphology, with the variations in cell size and arrangement occurring in accordance with the progression of atherosclerosis. Further, there was a close relationship between the number of multinucleated giant cells in culture and the severity of the atherosclerotic lesions. These multinucleated giant cells were found in isolated suspensions and at all stages of monolayer formation. They quickly took up Dil-ac-LDL, and were not stained by monoclonal antibodies that identified macrophages and smooth muscle cells. Moreover, the in vivo presence of these cells was demonstrated by both TEM and SEM.

We found, along with progression of atherosclerotic lesions, progressive enlargement of mononuclear endothelial cells with concurrent appearance of multinucleated giant endothelial cells. Enlargement of endothelial cells overlying atherosclerotic lesions has been reported by Taylor et al. [28] where endothelial cells were seen to be attenuated and spread over bulging foam cell lesions without increase in cell number. The presence of multinucleated giant endothelial cells was not shown in these studies and the mechanism by which they are formed remains unclear. In earlier studies using the Häutchen technique, McGovern [16] and Poole et al. [20] demonstrated that the presence of giant endothelial cells was often associated with sites of cellular damage; they considered the emergence of these cells to be evidence of endothelial cell regeneration following vascular injury. Recently, a number of investigators [11, 33] have found that increased endothelial cell replication was closely related to intimal lesions in experimentally induced atherosclerosis. It is conceivable that the capacity of the endothelium to regenerate might be limited, since cells proliferating in the lesion might eventually become senescent. The repair of defects would then proceed at the expense of cell spreading or fusion [22]. In our previous study [28], we demonstrated that tritiated thymidine was not incorporated into the nuclei of multinucleated variant endothelial cells cultured from human aorta. In primary culture from cholesterol-fed rabbits, none of the multinucleated endothelial cells were labelled, although mononuclear small

cells proliferated and showed incorporation of BrdU. Further, we failed to demonstrate in vivo uptake of BrdU by multinucleated endothelial cells harvested from cholesterol-fed 40-week rabbits. These findings support the conclusion that the multinucleated endothelial cells have no replication ability in vivo or in vitro. It is likely that the cells were formed by fusion of adjoining cells rather than by intracytoplasmic nuclear multiplication without cell division. Such accelerated aging of endothelial cells may lead to loss of integrity of the endothelial lining of the arterial wall and may contribute to progression of atherosclerosis.

In the cholesterol-fed 40-week group, the surface of the aorta was widely covered with atherosclerotic lesions where the large cells measuring over 800 μm^2 accounted for 48% of the population. In contrast, the percentage of binucleated and multinucleated cells in vitro was less than 15% in the same group. We consider that this difference was, at least in part, caused by loss of proliferative capability in the multinucleated endothelial cells. Mononuclear endothelial cells could take up BrdU in their nuclei and showed exponential increase in primary culture, while the multinucleated cells failed to display proliferative capability. The percentage of multinucleated cells, therefore, would increase progressively during primary cultivation.

The endothelial monolayer contributes to the maintenance of homeostasis in vessel walls and lumens via the formation of an efficient barrier to plasma constituents or leukocytes, the prevention of platelet activation and blood coagulation, and the production of biologically active substances [4, 14]. Many investigators have studied functional alterations of endothelial cells as a predisposing factor in the development of atherosclerosis. Endothelial cells determine the nature of the lipoproteins and other plasma constituents that reach the subendothelial space [4]. Kurozumi [15] showed that rabbit endothelial cells overlying fatty lesions showed increased permeability to ferritin and horseradish peroxidase. One of the earliest cellular events in hypercholesterolaemia is the adhesion of monocytes to the arterial endothelial surface and their subsequent migration into the subendothelial space [13, 34, 35]. As the lesion advances, accumulated intimal macrophages engulf lipids by their scavenging function, and are converted to foam cells [27, 35]. The expression of leukocyte adhesion molecules has been demonstrated in both human and experimental atherosclerosis [6, 21] and Molenaar et al. [17] have demonstrated that the adherence of monocytes to endothelial cells is significantly increased by in vitro aging of the endothelial cells. Prostacyclin, which is the most potent inhibitor of platelet aggregation (thrombus formation) [18], is produced by vascular tissue, primarily by the endothelium. A number of studies [26, 31] have shown that atherosclerotic vascular tissue and sera from elderly subjects generate less prostacyclin than is found in the samples of younger subjects and a recent study [30] demonstrated an age-related decline of prostacyclin synthesis in human aortic endothelial cells. An enhanced capacity to release plasminogen activators has also been observed in serially-propagated endothelial cells from human aorta, in parallel to the emergence of enlarged endothelial cells [12]. It seems likely that the functional alterations that occur in endothelial cells with aging play an important role in the development and progression of atherosclerosis and thrombosis.

Most previous studies of endothelial cells have been performed with cells obtained from non-atherosclerotic vessels, such as the human umbilical vein and animal blood vessels. Our present study, however, demonstrated a different picture, which should not go unheeded: in vivo endothelial cells undergo a variety of alterations as atherosclerosis progresses. These changes differ greatly from those in non-atherosclerotic vessels. It is inferred that endothelial cells overlying the lesion area are damaged by various risk factors, the most notable of which is hyperlipidaemia [14, 19, 23, 27]. This damage manifests itself as endothelial dysfunction, since there is a close correlation between endothelial structure and function [9]. We consider that the morphological alterations of endothelial cells observed reflect changes in function associated with the progression of atherosclerosis.

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