American Journal of Pathology, Vol. 135, No. 6, December 1989

Atherosclerosis- and Age-Related Multinucleated Variant Endothelial Cells in Primary Culture from Human Aorta

Osamu Tokunaga, Jianglin Fan, and Teruo Watanabe

From the Department of Pathology, Saga Medical School, Nabeshima-Machi Saga, Japan

Endothelial cells were cultured from human aortas and inferior venae cavae of autopsied subjects ranging in age from infancy to 85 years. Endothelial cells in 32 of more than 100 attempted cultures were pure enough for evaluation. Emerged endothelial cells in primary culture were classified into two types: typical endothelium and variant endothelium. Typical endothelial cells were small, round to polygonal shaped, and were arranged uniformly. Their diameter ranged from 50 to 70 μm. Variant endothelial cells were larger, ranging from 100 to 200 μm in diameter, and giant endothelial cells measuring more than 250 μm in diameter were scattered among them. Variant endothelial cells were usually multinucleated and possessed endothelium-specific markers of vWF and Weibel-Palade bodies. No incorporation of [^{3}H]thymidine was found in the nuclei of cultured variant endothelial cells. Although most cultured endothelial cells were of the typical type, variant endothelial cells were interspersed throughout the culture. The ratio of variant endothelial cells to typical cells correlated well with the severity of atherosclerosis, but less so with aging. The number of variant endothelial cells in cultures from inferior venae cavae was slight and constant throughout all age groups. The presence of multinucleated endothelial cells in in vivo ***aortas was confirmed by both scanning and transmission electron microscopy. They sometimes existed in colonies in the aortas from elderly subjects with intimal-thickened or advanced atherosclerotic lesions. These results indicate that variant endothelial cells were present*** in vivo ***and their ratio in primary culture reflected the*** in vivo ***population. It is likely that these cells were formed by adhesion of adjacent typical endothelial cells and that this process was affected more by atherosclerosis than by aging. Although it is not clear if the multinucleated variant cells were formed before the formation of atherosclerotic plaque or after the plaque formation, they will contribute to further development of atherosclerotic lesions, which in turn cause malfunction of the cell membrane. We suggest that there is a cyclic effect of these processes for multiplication of the variant endothelial cells and advancement of atherosclerotic lesions. (Am J Pathol 1989, 135:967–976)***

The recent rediscovery of heterogeneity in human aortic endothelium[1,2] suggests an alternative relationship between the role of endothelial cells and the development of vascular disease, especially atherosclerosis. Vascular endothelial cells have long been thought to be polygonal and to comprise a homogeneous population forming an *en face* pavement arrangement. Although giant endothelial cells were found in a Häutchen preparation in the 1940s[3] and 1950s,[4,5] this topic was not reviewed until Repin et al[6] reaffirmed a significant relationship between endothelial heterogeneity and the presence of atherosclerotic lesions. Recent biologic advances, especially in cell-culture techniques,[7,8] have elucidated a new approach to simultaneous comparative study of cell functions and morphologic changes. We previously reported that human aortic endothelium consisted of a heterogeneous population *in vitro* and reacted differently from venous endothelium when cultured at various ambient pressures.[9] Since then more data have been obtained from human aortic endothelial cells cultured at ages ranging from infancy to 85 years.

We report here on the morphologic heterogeneity of human aortic endothelial cells, their nature, comparison with venous endothelium, and the relationship between endothelial heterogeneity and atherosclerosis.

Supported in part by a Grant-in-Aid for Special Project Research: "Metabolic Researches of Blood Vessels" from the Ministry of Education, Science and Culture, Japan (61132004, 62122004).

Accepted for publication July 21, 1989.

Address reprint requests to Osamu Tokunaga, M.D., Ph.D., Department of Pathology, Saga Medical School, Nabeshima-Machi Saga 840-01, Japan.

Materials and Methods

Endothelial Cell Culture from Human Thoracic Aorta and Inferior Vena Cava

Endothelial cells were harvested from human thoracic aorta within two hours after death at pathologic autopsy using a slightly modified version of our previously reported method.[2] The aorta was removed and the adventitia was peeled off inside from the media, after which the aorta was gently washed with cold phosphate-buffered saline (PBS, pH 7.4) from one end. A piece of aorta with both ends open was incubated in Dispase solution (1000 units/ml; Sanko Pharmaceutical Co., Tokyo, Japan) for 60 minutes at 37 C. The aorta lumen was gently washed with cold PBS using a syringe with a 23-gauge needle. Using this treatment the endothelial cells were harvested from the subendothelial bed, and harvested cells were centrifuged at 800 rpm for ten minutes. The sedimented cells were resuspended in autoclavable RPMI 1640 (Nissui Pharmaceutical Co., Tokyo) supplemented with L-glutamine (0.294 g/L; GIBCO, Grand Island, NY), 15% fetal bovine serum (FBS; Microbiological, Associates, Logan, UT), 15% Nu-serum (No. 50,000; Collaborative Research, Lexington, KY), 15 μg/ml endothelial-cell growth supplement[10] (ECGS; Collaborative Research, Lexington, KY), and 100 μg/ml of heparin.[11] An antibiotic cocktail supplement consisting of 15 μg/ml of gentamicin, 1.5 μg/ml of amphotericin B, 50 μg/ml of ampicillin, and 2 μg/ml of minomicin was added to the medium for primary culture. At the end of day 2 of culture, the old medium was removed by suction and the culture was replenished with fresh medium containing only gentamicin. When the cultured cells became confluent they were subcultured with 0.2% trypsin (GIBCO) and 0.02% EDTA treatment.

Endothelial-cell culture from the inferior vena cava was performed simultaneously in some cases, as in aorta, for comparison. In three cases, however, the cultured aortic endothelial cells were highly contaminated with nonendothelial cells, and therefore only venous endothelial cells were used for the study.

[^{3}H]Thymidine Uptake and Autoradiography

Endothelial cells were cultured on a cover glass for examination of [^{3}H]thymidine uptake in the primary culture. The old medium was replaced with fresh medium containing 1 μCi/ml [^{3}H]thymidine (New England Nuclear, Boston, MA) on day 3 of incubation, and the cells were incubated for 12 hours. When culture was stopped, the cells were washed with PBS and fixed with 10% formaldehyde. They were covered with Sakura autoradiographic emulsion and exposed for 3 weeks in a cold, dark room before development.

Immunofluorescence for vWF

Endothelial cells were cultured on a cover glass for the first three days of primary culture. The cells were then fixed with cold acetone (−20 C) for ten minutes and rinsed thoroughly with cold PBS. The cells were incubated with rabbit anti-von Willebrand factor (vWF) serum (Medical and Biological Laboratory, Nagoya, Japan) for one hour at room temperature and subsequently with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG serum (MBL). A control staining was made to react with PBS and nonspecific rabbit serum. The reacted cells were observed with an Olympus fluorescence microscope. Unlabeled antibody peroxidase-antiperoxidase (PAP) method[12] was used for detection of vWF in the cell pellets (Dakopatts, Santa Barbara, CA).

Atherosclerotic Index of the Aorta

The atherosclerotic index (AI) was calculated according to the method of Gore and Tajada,[13] based on the involved surface of the thoracic aorta from which endothelial cells had been harvested. Atherosclerotic lesions of the aorta were then stained with oil red 0 and thoroughly rinsed with 70% alcohol to illuminate them (to distinguish them from noninvolved areas). Each aorta was photographed before and after the oil red 0 staining. Scores for atherosclerotic lesions such as fatty streaks, fibrous plaques, and calcification were multiplied according to the standard formula, using indices of individual severity and involved areas.

Preparation for Scanning Electron Microscopy

Either the thoracic or the abdominal aorta adjoining the aorta used for endothelial-cell culture was used for observation by scanning electron microscopy. The aortic surface was gently washed with cold 5% sucrose solution, treated with 0.1% silver nitrate for seven minutes to outline the cell border, and rinsed with the sucrose solution. Sections measuring 5 × 5 mm were randomly cut, fixed in 3% glutaraldehyde for two hours, postfixed in 2% osmium tetroxide, and dehydrated in a graded series of alcohols. The preparations were brought to the critical drying point out of liquid CO_2 in an HCP-2 critical-point dryer (Hitachi, Tokyo) and coated with gold-palladium in a GIKO IB-5 ion coater (Eiko Engineering, Tokyo). They were examined in an S-700 scanning electron microscope (Hitachi).

Preparation for Transmission Electron Microscopy

For transmission electron microscopic observations, the washed aorta was cut into small pieces, fixed in cacodyl-

Table 1. *List of Cases and Relationship of AI to Variant Endothelial Cells*

Number	Age	Sex	Log (AI × 100)	AI	Variant end.
1	0.2	F	0	(0.01)*	±
2	14	M	0	(0.01)	±
3†	24	M	0.30	(0.02)	±
4†	27	F	0	(0.01)	±
5	30	F	0.60	(0.04)	±
6†	39	M	0.78	(0.06)	±
7	45	M	0	(0.01)	n.t.
8	48	M	2.05	(1.12)	++
9†	49	F	1.08	(0.12)	+
10	52	F	1.66	(0.46)	+
11	53	F	1.00	(0.10)	±
12	53	F	2.53	(3.46)	+++
13‡	56	F	—	—	—
14	57	F	1.88	(0.75)	++
15	57	F	0.70	(0.05)	+
16‡	59	M	—	—	—
17	60	F	1.11	(0.13)	±
18	60	M	2.40	(2.50)	(+++)
19‡	61	M	—	—	—
20	62	M	1.15	(0.14)	n.t.
21	62	M	2.57	(3.73)	++
22	63	M	1.18	(0.15)	+
23	63	M	3.10	(12.53)	+++
24	70	M	0.70	(0.05)	±
25	72	F	2.18	(1.50)	+++
26	72	F	1.53	(0.34)	n.t.
27	74	F	2.67	(4.68)	++
28	74	F	1.95	(0.89)	±
29	75	F	2.85	(7.02)	+++
30	77	M	3.14	(13.80)	n.t.
31	81	M	3.33	(21.17)	+++
32	83	M	2.73	(5.40)	+

* Parentheses show actual AI. Ratio of variant endothelial cells is expressed as follows: ±: 0% to 5%, +: 5% to 10%, ++: 10% to 15%, +++: 15% to 30%. Endothelial cells were cultured independently from both aorta and inferior vena cava in cases marked by + and from only inferior vena cava in cases marked by ‡. For simplification of statistical analysis and scattergrams, AI in case 1 and ratio of variant endothelial cells in case 18 are temporarily placed at 0.01 and +++, respectively.

ate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated in a graded series of alcohols, and embedded in Epon 812. Silver-gray ultrathin sections were cut on an ultramicrotome (Reichert, Vienna, Austria). The specimens were washed with distilled water and double stained with uranyl acetate and lead citrate. They were examined in a JEM-100 CX transmission electron microscope (JEOL, Tokyo) at 75 kV.

Statistical Analysis

The significance of differences between the number of cultured endothelial cells and the grade of AI and of aging was examined by Student's *t*-test in an STAX computer program applicable to the NEC PC-9801 series (Tokyo).

Results

Morphology of Cultured Human Aorta-Derived Endothelium

Human endothelial-cell culture was initially very difficult. However, with the addition of ECGS and heparin to the culture medium it has been achieved successfully. Among more than 100 cases attempted, only 29 cases, ranging from a 2-month-old infant to an 83-year-old, were used in this study, according to the purity of harvested endothelial cells (Table 1). In 25 of those 29 cases, vWF-positive endothelial cells accounted for more than 95% of the primary cultures. In the remaining four cases, the cultured endothelial cells were pure enough for evaluation by phase-contrast microscopy, but detailed morphological analysis was not performed. Other cases attempted proved inadequate, as vWF-positive endothelial cells accounted for a smaller percentage of the total, and therefore were not used in this study.

The cultured endothelial cells could be classified into two distinct types: the typical small type and the larger type, as described previously.[2] The typical type had round or polygon-shaped cytoplasm with a single nucleus, and cells of this type were arranged in a pavement pattern (Figure 1). This type was generally present in cultures of aortas from infants or children and in cultures of veins from a given specimen. Their diameters were fairly uniform, ranging from 50 to 70 μm. Endothelial cells of the larger type were more varied in size and shape. They were generally larger than the typical cells, measuring from 100

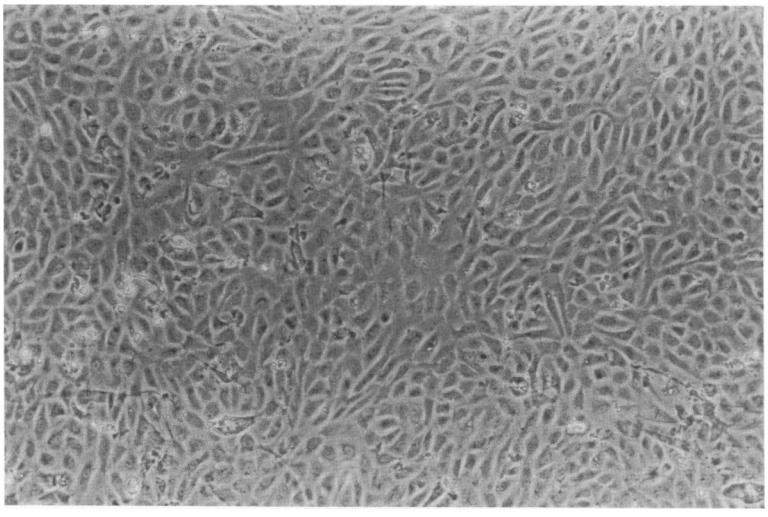

Figure 1. *Typical aortic endothelial cells at primary culture taken with an inverted phase-contrast microscope. They are small and uniform in size and round to polygonal in shape. A few nonendothelial spindle cells grow overlappingly (case 2, ×360).*

to 200 μm in diameter and had more than two nuclei. Giant endothelial cells larger than 250 μm in diameter were intermingled among the typical endothelial cells and were usually multinucleated in the primary cultures of even adolescent aortas (Figure 2). They sometimes emerged in colonies in cultures of aortas from elderly persons or of aortas with advanced atherosclerotic lesions, and occasionally had ten or more nuclei (Figure 3). In this study, vWF-positive multinucleated cells containing more than three nuclei were designated as variant endothelium. Binucleated cells were not considered to be variant cells because their possible formation as a result of dispase digestion during culture could not be ruled out. The ratio of the concomitant variant endothelial cells was determined by counting vWF-positive multinucleated cells per total vWF-positive cells under an immunofluorescent microscopy in three to five fields at 20 × 10 magnification and was expressed as follows: ±, 0% to 5%; +, 5% to 10%; ++, 10% to 15%; +++, 15% to 30%. Although the greater the severity of aortic atherosclerosis, the higher the number of emerged endothelial cells, there were no cases in which the ratio of variant endothelial cells exceeded 30%, except for a case of periarteritis nodosa (case 18). The ratio of this case was nearly 40%, but it was graded at +++ for convenience of statistical analysis.

Characterization of Variant Endothelium

Multinucleated cells with more than three nuclei were classified as variant endothelium, as mentioned above. More than ten nuclei were occasionally encountered, especially in cases with severe atherosclerosis or in older persons. The multinucleated variant endothelial cells were vWF positive by immunofluorescence (Figures 2 and 3) and contained Weibel-Palade bodies in the cytoplasm when observed by transmission electron microscopy. No uptake of [^{3}H]thymidine in any of these multinuclei was demonstrated by autoradiography (Figure 4). [^{3}H]Thymidine

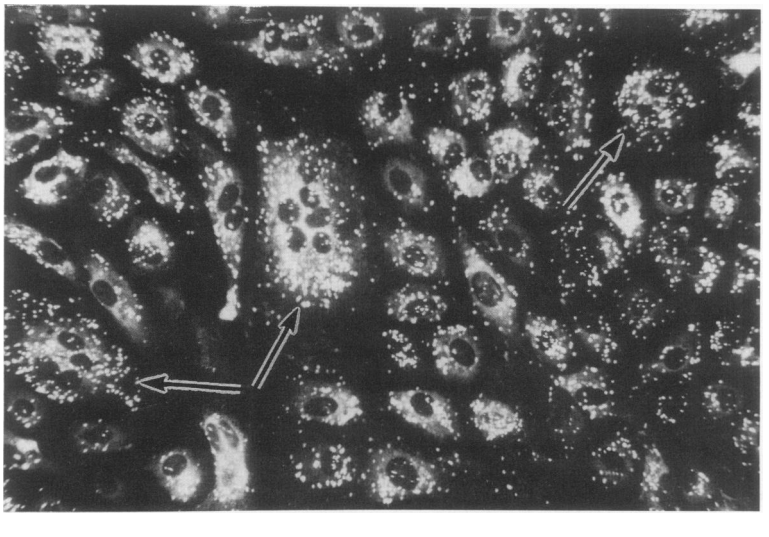

Figure 2. *A mixture of typical endothelial cells and variant endothelial cells. Multinucleated giant endothelial cells (arrow) are present among many mononuclear typical endothelial cells. More than 95% of the cells are vWF positive by indirect immunofluorescence (case 3, ×510).*

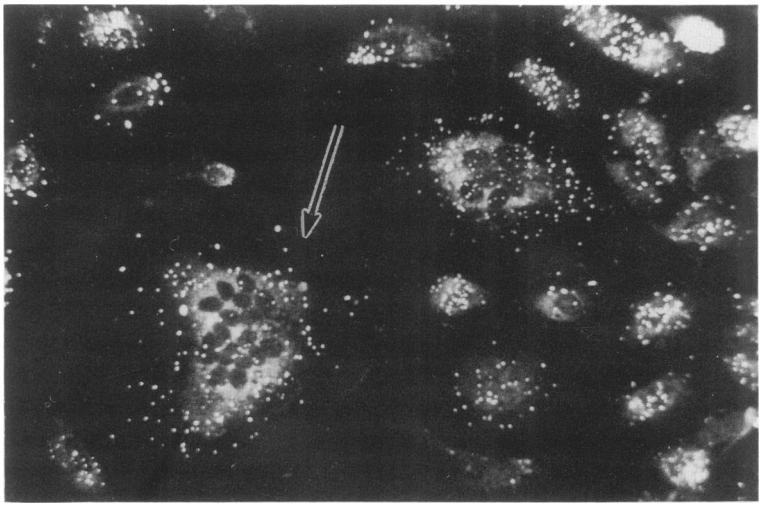

Figure 3. *Giant endothelial cells are vWF positive and one of them contains 19 nuclei (case 31, arrow. ×510).*

uptake was found only in the nuclei of mononuclear endothelial cells. The multinucleated cells survived for at least five passages, although the ratio decreased as the number of subcultures increased because only mononuclear cells could proliferate.

Demonstration of Presence of Variant Endothelial Cells in Preculture

Harvested endothelial cells from the aorta were centrifuged to form cell pellets, which were then examined for cell morphology and vWF expression. Precipitated cells were not affected by culture manipulation because they were not seeded yet. The constituent cells, which were from the aorta with severe atherosclerotic lesions, varied in size, were vWF positive by PAP method, and had two or more nuclei (Figure 5) or lobulated nucleus (possibly nuclei). The cell pellets from the aorta of children consisted of small and regular-sized endothelial cells that were well preserved after several passages. No large or multinucleated cells were seen.

Electron Microscopic Observation of Aorta and Vein

Endothelial cells in aortas from infants and children were small, round, and regular in size, measuring from 15 to 20 μm in diameter (Figure 6A) when observed by scanning electron microscopy. In the orifice of the vascular branch the cells tended to become spindle shaped. In young adults in their 20s, larger endothelial cells, surrounded by successively smaller endothelial cells, were occasionally seen. In adults, colonies of giant endothelial cells, displaying an abrupt transition from the surrounding cells, were

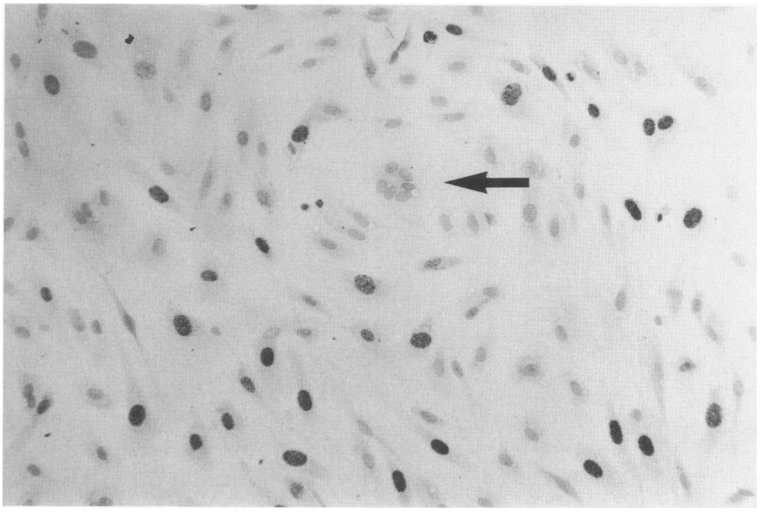

Figure 4. *No [^{3}H]thymidine uptake in the multinuclei of giant cells (arrow). Most of the incorporated nuclei are mononucleated cells at primary culture (case 4, ×360).*

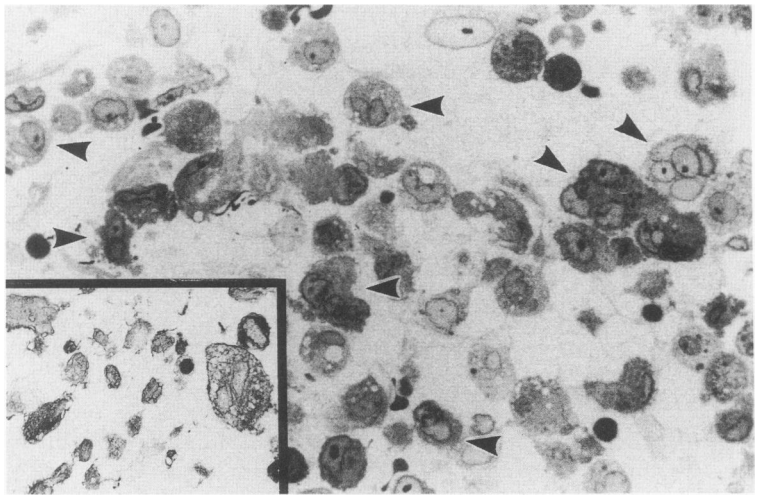

Figure 5. *Demonstration of multinucleated endothelial cells in precultural cell pellet. Many various-sized and possibly multinucleated cells are seen (arrowhead; Toluidine blue staining of epon embedded thick section from 57-year-old man ×550). Inset: Positive vWF in the multinucleated cells by PAP method (72-year-old woman. Paraffin-embedded section ×400). Darkly stained small cells are either erythrocyte or lymphocyte.*

scattered throughout aortas that showed age-related intimal thickening (Figure 6B). The giant endothelial cells were extremely large, sometimes ten times as large as the surrounding typical small ones. The cell surface was smooth, but the cell border was often indistinct when outlined by silver nitrate treatment. Advanced atherosclerotic lesions, such as fibrous plaques and calcified lesions, were almost invariably covered by large and giant endothelial cells among which, in contrast to intimal thickened regions, small colonies of typical endothelial cells were sometimes noted (Figure 6C). Subendothelial denudation with a lack of endothelial covering was occasionally observed on the latter lesions. Colonies consisting of large variant cells were not found in aortas from infants or children or in inferior venae cavae from subjects of any age; these tissues were always covered with small uniform cells (Figure 6D).

Although colonies of large cells were observed by scanning electron microscope, it was not evident whether they contained multiple nuclei. The *in vivo* presence of

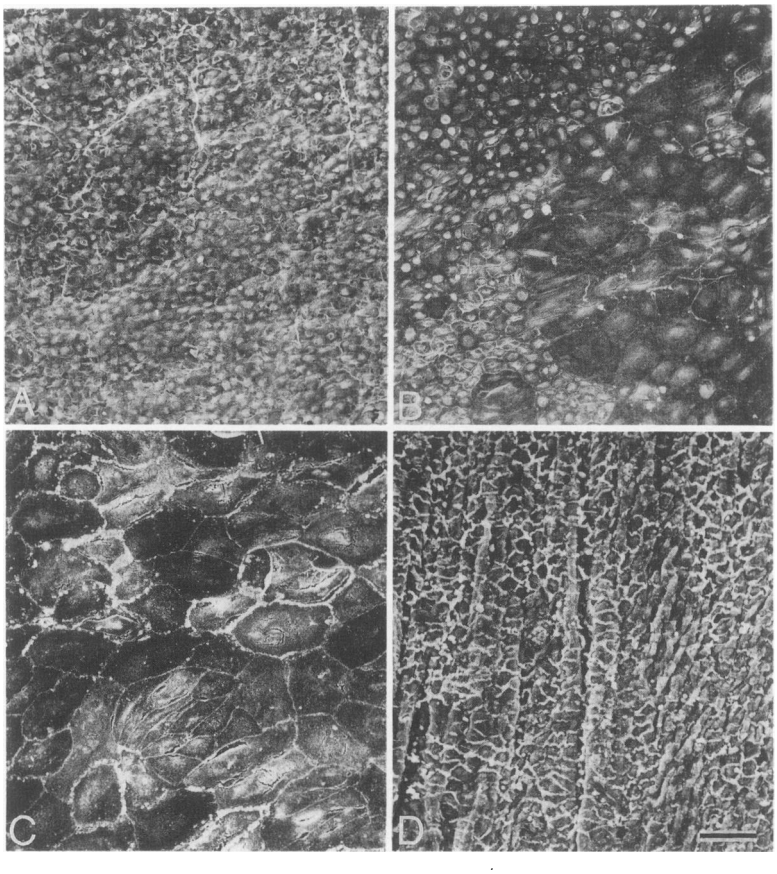

Figure 6. *Representative pictures of endothelium covering in vivo aorta and vein observed by scanning electron microscopy.* **A:** *Inner surface of aorta from a 5-year-old boy is thoroughly covered by typical endothelium.* **B:** *Typical endothelial cells (left half) and colony of giant endothelial cells (right half) are seen in intima-thickened aorta from a 69-year-old man.* **C:** *Calcified atherosclerotic aorta from a 74-year-old woman is covered by giant endothelial cells.* **D:** *Surface of inferior vena cava from a 41-year-old man is covered by small typical endothelial cells. —indicates 50 μm in length and all pictures are printed at the same magnification.*

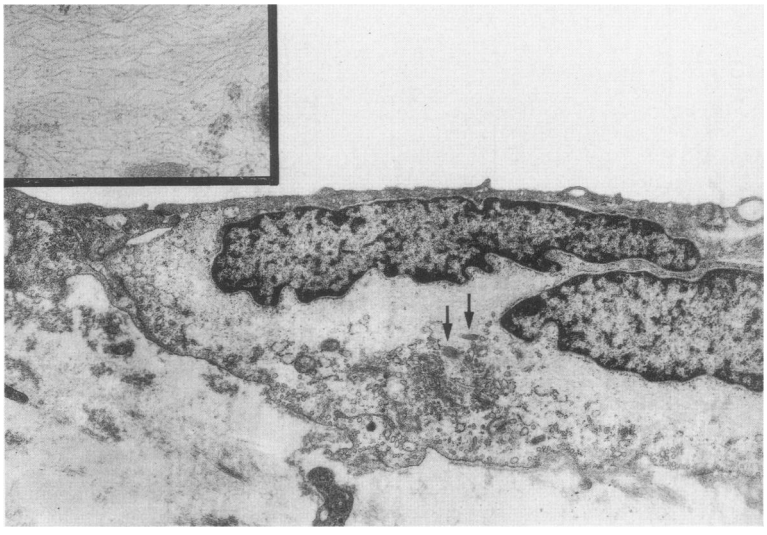

Figure 7. *Binucleated endothelial cells in in vivo aorta observed by transmission electron microscopy (33-year-old woman). The cell contains Weibel-Palade bodies (arrows, ×7800). Inset: Numerous intermediate filaments and Weibel-Palade body at lower margin (inset ×30,000).*

multinucleated variant endothelial cells was confirmed by transmission electron microscopy. Adjacent to the tissues fixed for scanning electron microscopy, serial thick tissue sections were made for transmission electron microscopy. Multinucleated cells were sometimes seen in the specimens taken from aortas with intimal thickening or those with atherosclerotic lesions (Figure 7). In such cells, intermediate filaments were occasionally well developed around the nuclei. It was evident that they belonged to the endothelium because Weibel-Palade bodies specific for endothelium were clearly observed (Figure 7).

Relationship Between AI or Aging and Variant Endothelium

The AI of the aorta was calculated according to Gore and Tajada's method[13] after the endothelial cells were harvested. Severely complicated cases with calcification and ulceration were not included because either the number of harvested cells was too small or the cells were contaminated with lipid-laden foam cells. AI ranged from 0.01 in a 14-year-old boy to 21.17 in an 81-year-old man. Although no atherosclerotic lesions were detected in the case of a 2-month-old baby, the AI was placed at 0.01 for convenience of statistical analysis.

To place a minimal AI value of 0.01 on the base line of graphic representation, conventional AI values were multiplied by 100 and converted into logarithms (Table 1). Thereafter, modified values of log (AI × 100) were simply referred to as AI. The number of concomitant variant endothelial cells from the primary culture was significantly correlated with the severity of atherosclerosis (Figure 8; $P < 0.01$ at +, ++, and +++ against ±, and $P < 0.05$ at +++ against +, Student's *t*-test).

The relationship between aging and the number of variant endothelial cells was not as significant as between AI and the number of these cells. This might be the result of selective cultures from older persons with less severe atherosclerotic lesions. For this reason, cases of ± grade were composed of a vast range of persons, from a 0.2-month-old girl to a 70-year-old man, and cases of + grade from elderly persons. Therefore, all three groups from +, ++, and +++ grades were significant against the ± group, whereas no significance was found between +, ++, and +++ (Figure 9; $P < 0.05$, Student's *t*-test).

Multinucleated variant endothelial cells were also seen in primary cultures of inferior venae cavae, but their number was low throughout all age groups attempted. These cells were not correlated with either atherosclerosis or aging. Neither grading for the number of variant endothelial cells nor statistical analysis was done because the ratio of the variant endothelial cells was too small.

Discussion

Recent advances in endothelial-cell culture techniques have facilitated *in vitro* investigations of morphologic and functional properties in human aortic endothelium[2,7,14] and venous endothelium.[15] Both isolation of ECGF[10] and use of heparin contributed greatly to the recent success in endothelial-cell cultures from adult aortas. Despite such advances, however, the precise characteristics are not yet fully understood. In the past, most endothelial-cell cultures used human umbilical veins and animal aortas. Because the umbilical vein is a fetal component and animal aortas were usually obtained from slaughtered younger animals, the cultured endothelial cells were monotonous and typical in population. For this reason, the shape of the endothelial cell has generally been considered to be round or polygonal in conventional culture conditions.[7,16–18]

A few studies on the shape of endothelial cells have been reported. The presence of multinucleated giant cells

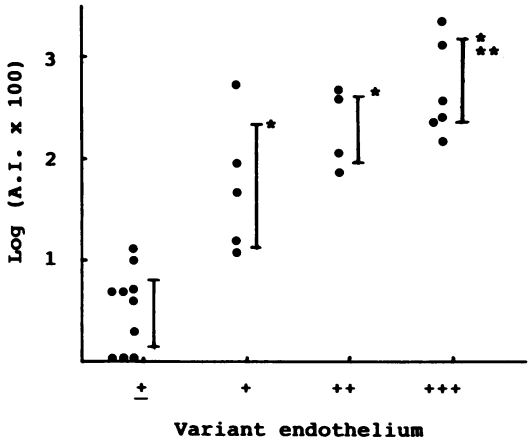

Figure 8. *Scattergram of AI and the degree of variant endothelial cells. The number of concomitant variant endothelial cells is graded as follows: ±: 0% to 5%, +: 5% to 10%, ++: 10% to 15%, +++: 15% to 30%. * means significance against ± grade at P < 0.01 and ** against + grade at P < 0.05 (Student's t-test).*

was demonstrated by Efskind in a Häutshen preparation in 1941,[3] and was further reported by Sinapius[4] and McGovern[5] in the 1950s. However, no further studies were reported until Repin et al[6] in 1984 reconfirmed the heterogeneity of endothelium in the human aorta, mainly on the basis of scanning electron microscopic observations. Successful cultures of endothelial cells were reported from the human iliac artery by Glassberg et al,[7] from human aortas by Fryer et al,[19] and also by Hoshi et al.[8] These authors found no morphologic differences between the umbilical vein endothelium and the atherosclerotic aortas. The presence of multinucleated giant cells in cultured endothelial cells from human aortas was first, but only briefly, reported by Repin et al,[6] then by Antonov et al,[1] which was followed by our report.[2]

Other subtypes morphologically or functionally different from conventional typical endothelial cells have been reported by some investigators. These were ring-forming endothelial cells,[20,21] variant endothelial cells producing much fibronectin as a transducer of signals for migration and neovascularization,[22] and phenotypical endothelial cells with increased prostacyclin production.[14] Recently several factors were revealed as affecting the morphologic changes of endothelial cells. Shear stress was one of the major factors for cell elongation in aortic stenosis.[23] Extracellular matrices affected the cell size and further modulated cell proliferation and migration.[24] Extracellular matrices were also responsible for angiogenic transformation of endothelial cells,[25] which was modulated by transforming growth factor beta (TGF-B).[26] The presence of multinucleated endothelial cells, however, was not mentioned in any of these investigations.

The question of whether the multinucleated cells were primarily present in the aorta or secondarily formed during culture manipulation was clearly resolved by the fact that the harvested and centrifuged cell pellets from the atherosclerotic aorta consisted of large endothelial cells of variable sizes with possible multinuclei. It is evident that the multinucleated giant cells were endothelial cells because they were positive for vWF, a specific marker for endothelial cells.[27] In addition, Weibel-Palade bodies,[28] rod-shaped structures specific for endothelium, were seen in endothelium both *in vivo* and *in vitro* by electron microscopic observation. The *in vivo* presence of multinucleated endothelial cells was also demonstrated by both scanning and transmission electron microscopy.

In infants, the aortic lumen was lined by a sheet of small polygonal endothelial cells, whereas adult aortas were lined by endothelial cells of various sizes. Multinucleated endothelial cells were almost invariably noted in aortas with atherosclerotic lesions, and also in age-related intimal-thickened aortas. The presence of colonies made up of giant cells was a surprise, giving rise to questions regarding their mechanism of formation. No incorporation of [^{3}H]thymidine was detected in nuclei of the cultured multinucleated endothelial cells, which ruled out the possibility of intracytoplasmic nuclear multiplication without cell division. Ingber et al[29] reported that DNA synthesis rates were always closely correlated with the size of bovine calf adrenal capillary endothelial cells in projected cell areas in which the cell size became greater. Nichols et al[30] reported that polyploidy cells in multi-passaged human umbilical endothelial cells, rather than those in human renal artery endothelial cells, reflected the end of *in vivo* developmental lifespan. Although interesting, these report do not bear directly on our results because our [^{3}H]thymidine uptake experiment was performed at a low cell density seeding whereby [^{3}H]thymidine was incorporated at ran-

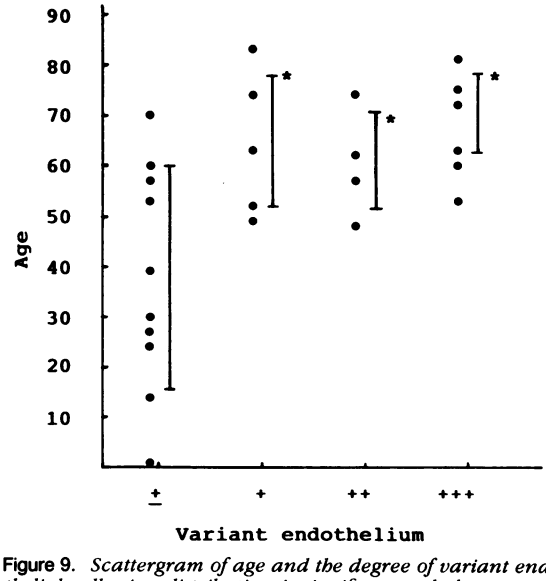

Figure 9. *Scattergram of age and the degree of variant endothelial cells. Age distribution is significant only between the ± group and the other three groups at P < 0.05 (Student's t-test).*

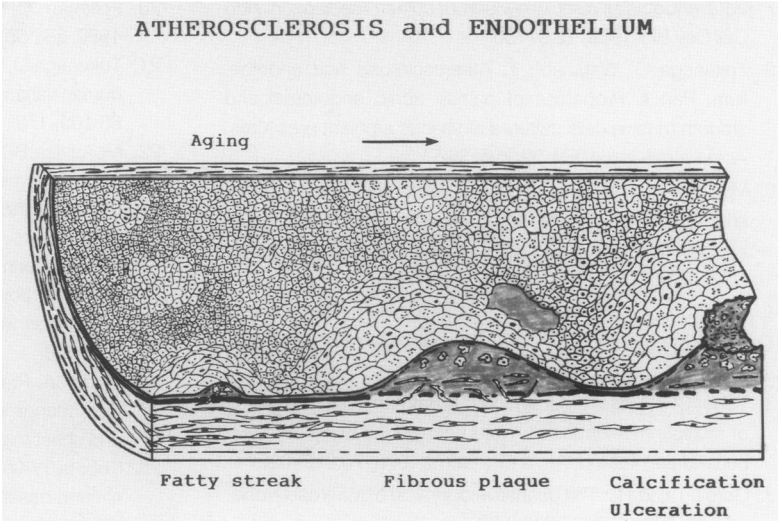

Figure 10. *Schematic illustration of aortic endothelium. Endothelial cells are small at the beginning of life. The size increases and cells become multinucleated during the course of atherosclerotic development and aging. Noncovered area indicates denudation of subendothelial intima.*

dom in the proliferating cells. And the multinucleated variant endothelial cells had no mitotic activity as shown by the lack of incorporation of [^{3}H]thymidine.

Although the ratio of concomitant variant endothelial cells at primary culture was well correlated with the AI of aortas from which the endothelial cells were harvested, the relationship between the ratio of variant endothelial cells and aging was not so closely correlated and was significant only at the grade of variant endothelium between ± and the other grades of variant endothelial cells (Figure 9). One of the reasons for such a poor correlation would be the result of selective cultures from eldery persons with less severe atherosclerotic lesions. Antonov et al[1] reported that the number of multinucleated endothelial cells at primary culture from atherosclerotic aortas was significantly higher than that from normal aortas, and similarly found no positive relationship to donor age. These authors also found no multinucleated endothelial cells in primary cultures from human umbilical vein and infant aortas. In contrast, we found that multinucleated endothelial cells were already present, to some extent, in the aorta of a 14-year-old boy.

Apart from the morphologic studies, functional investigation was not performed, except for vWF expression. In multinucleated endothelial cells, vWF was fully expressed, and even hyperexpressed, by immunofluorescence observation when these cells were compared with the adjacent typical endothelial cells. Morphogenesis and the role of variant endothelial cells in atherosclerosis remain unclear. Our results, however, suggest a cyclic effect of these processes. Whether they were formed primarily or secondarily by some unknown mechanism, they would trigger a further development of atherosclerotic lesions. The development of such lesions would then induce further formation of multinucleated variant endothelial cells.

The endothelial cells that cover the inner surface of the aorta are initially small, polygonal, and single layered, but large and multinucleated giant cells emerge in correlation with atherosclerotic severity and aging. Atherosclerosis and age-related morphologic alteration occurs in human aortic endothelium. A schematic aortogram correlating these three parameters is shown in Figure 10. The mechanism responsible for genesis of multinucleated endothelial cells and their functional role are presently under investigation.

References

1. Antonov AS, Nikolaeva MA, Klueva TS, Romanov YuA, Babaev VR, Bystrevskaya VB, Perov NA, Repin VS, Smirnov VN: Primary culture of endothelial cells from atherosclerotic human aorta. Part 1. Identification, morphological and ultrastructural characteristics of two endothelial cell subpopulations. Atherosclerosis 1986, 59:1–19
2. Tokunaga O, Watanabe T: Atherosclerosis and endothelium. Part I. A simple method of endothelial cell culture from human atherosclerotic aorta. Acta Pathol Jpn 1987, 37:527–536
3. Efskind L: Die Veränderungen im Gefässepithel bei Arteriosklerose. Acta Pathol Microbiol Scand 1941, 18:259–276
4. Sinapius D: Über das Aortenendothel. Virchows Arch 1952, 322:662–694
5. McGovern VJ: Reaction to injury of vascular endothelium with special reference to the problem of thrombosis. J Pathol Bacteriol 1955, 69:283–293
6. Repin VS, Dolgov VV, Zaikina OE, Novikov ID, Antonov AS, Nikolaeva MA, Smirnov VN: Heterogeneity of endothelium in human aorta. A quantitative analysis by scanning electron microscopy. Atherosclerosis 1984, 50:35–52
7. Glassberg MK, Bern MM, Coughlin CC, Haudenschild LW, Hoyer LW, Antoniades HN, Zetter BR: Cultured endothelial cells derived from the human iliac arteries. In Vitro Cell Dev Biol 1982, 18:859–866
8. Hoshi H, McKeehan WL: Isolation, growth requirements, cloning, prostacyclin production and life-span of human

adult endothelial cells in low serum culture medium. In Vitro Cell Dev Biol 1986, 22:51–56

9. Tokunaga O, Watanabe T: Atherosclerosis and endothelium. Part II. Properties of human aortic endothelial and smooth muscle cells cultured at various ambient pressures. Acta Pathol Jpn, 1989, 39:356–362
10. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R: An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization. Proc Natl Acad Sci USA 1979, 76:5674–5678
11. Thornton SC, Mueller SN, Levine EM: Human endothelial cells: Use of heparin in cloning and long-term serial cultivation. Science 1983, 222:623–625
12. Taylor CR, Russell R, Chandor S: An Immunohistologic study of multiple myeloma and related conditions using immunoperoxidase method. Am J Clin Pathol 1978, 70:612–622
13. Gore I, Tajada C: The quantitive appraisal of atherosclerosis. Am J Pathol 1957, 33:875–882
14. Hahn GL, Polgar PR: Prostaglandin production in phenotypically distinct cultured bovine pulmonary artery endothelium. Atherosclerosis 1984, 51:143–150
15. Levin RI, Jaffe EA, Weksler BB, Tavk-Goldman K: Nitroglycerin stimulates synthesis of prostacyclin by cultured human endothelial cells. J Clin Invest 1981, 67:762–769
16. Maruyama Y: The human endothelial cell in tissue culture. Z Zellforsch 1963, 60:69–79
17. Folkman J, Haudenschild CC, Zetter BR: Long-term culture of capillary endothelial cells. Proc Natl Acad Sci USA 1979, 76:5217–5221
18. Davison PM, Bensch K, Karasek MA: Isolation and growth of endothelial cells from the microvessels of the newborn human foreskin in cell culture. Lab Invest 1980, 75:316–321
19. Fryer DG, Birnbaum G, Luttrell CN: Human endothelium in cell culture. J Atheroscler Res 1966, 6:151–163
20. Folkman J, Haudenschild C: Angiogenesis in vitro. Nature 1980, 288:551–556
21. Tokunaga O, Morimatsu M, Nakashima T: Ring formation by human variant endothelial cells in vitro. Br J Exp Pathol 1984, 65:165–170
22. McAuslan BR, Hannan GN, Reilly W, Stewart FHC: Variant endothelial cells. Fibronectin as a transducer of signals for migration and neovascularization. J Cell Physiol 1980, 104: 177–186
23. Zand T, Nunnari J, Hoffman AH, Savilonis B, MacWilliams B, Majno G, Joris I: Endothelial adaptation in aortic stenosis. Correlation with flow parameters. Am J Pathol 1988, 133: 407–418
24. Madri JA, Pratt BM, Yannariello-Brown J: Matrix-driven cell size change modulates aortic endothelial cell proliferation and sheet migration. Am J Pathol 1988, 132:18–27
25. Kubota Y, Kleinman HK, Martin GR, Lawley TJ: Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structure. J Cell Biol 1988, 107:1589–1598
26. Madri JA, Pratt BM, Tucker AM: Phenotypic modulation of endothelial cells by transforming growth factor-B depends upon the composition and organization of the extreacellular matrix. J Cell Biol 1988, 106:1375–1384
27. Jaffe EA: Endothelial cells and the biology of factor VIII. N Engl J Med 1977, 296:377–383
28. Weibel P, Palade GE: New cytoplasmic components in arterial endothelia. J Cell Biol 1964, 23:101–112
29. Ingber DE, Madri JA, Folkman J: Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. In Vitro Cell Dev Biol 1987, 23:387–394
30. Nichols WW, Buynak EB, Bradt C, Hill R, Aronson M, Jarrell BE, Mueller SN, Levine EM: Cytogenetic evaluation of human endothelial cell cultures. J Cell Physiol 1987, 132:453–462