Intimal Cells and Atherosclerosis

Relationship Between the Number of Intimal Cells and Major Manifestations of Atherosclerosis in the Human Aorta

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The subendothelial intima of human aorta is populated by cells of various shapes. Round and ovoid cells which are lymphocyte- and monocyte-like hematogenous cells account for less than 5% of the cell population. The bulk of the intimal population (over 95%) is made up of cells that can be described as elongated, stellate, elongated with side processes, and irregularly shaped. To identify these morphologic forms, the authors have used target electron microscopy. It has been established that elongated cells devoid of side processes possess all the ultrastructural features of differentiated smooth muscle cells: a developed contractile apparatus in the form of microfilament bundles with dense bodies occupying most of the cytoplasm, basal membrane surrounding the whole of the cell, and micropinocytotic vesicles along the plasma membrane. The other morphologic forms have an ultrastructure that allows us to identify them as so-called modified smooth muscle cells. They differ from the typical smooth muscle cells in that they have fewer contractile structures and a more developed biosynthetic apparatus. Some of stellate and irregular shaped cells are utterly devoid of contractile structures. To quantitate the number of cells of different morphologic forms, the authors used alcoholic-alkaline dissociation of prefixed intima. It was established that the intimal population is multiplied at the site of an atherosclerotic lesion, the number of stellate cells being increased much more substantially, compared with other morphologic cell forms. It was found that an increase in the number of stellate cells is related to such sequelae of atherosclerosis in aorta as intimal thickening, deposition of lipids, and an increased amount of collagen. There was a high positive correlation between the alteration in the stellate cell number occurring in the intima and the above-mentioned parameters (correlation coefficients were 0.732, 0.800 and 0.953, respectively). The correlations between these indexes and the total number of intimal cells or the number of cells belonging to each of the other morphologic forms were not so high. A multivariate analysis gave similar results. Thus, it may be suggested that stellate cells are the principal cell type involved in the disease. This report discusses the origin of stellate and other intimal cells and their role in atherogenesis. (Am J Pathol 1986, 125:402–415)

THE SUBENDOTHELIAL INTIMA of the human aorta is populated by cells of different shapes. A small portion of the intimal population is made up of cells of a hematogenous origin: lymphocytelike cells of round shape and cells of variable, usually ovoid, shape morphologically indistinguishable from monocytes.[1] The bulk of the population (over 95%) is represented by other morphologic forms: bipolar elongated cells; cells of elongated or stellate shape with a variable number of long, thin, branching processes (from 3 to 12 or more); and irregularly shaped cells.[2]

We have recently reported that the number of cells in the cell population of an atherosclerotic lesion is increased and the proportion of main morphologic cell forms is substantially altered: specifically, the share of stellate cells in the plaque is higher than in grossly normal intima by an order of magnitude.[2,3] This shift is accounted for by the fact that in atherosclerosis the

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Table 1—Characteristics of Autopsy Material

Aorta	Number of samples analyzed NO	FS	PL	Total	Age (years)	Sex	Postmortem interval (hours)
1	25	18	6	49	55	M	1.7
2	5	7	6	18	54	M	2.0
3	5	6	2	13	46	M	2.5
4	11	11	6	28	51	M	2.3
5	9	0	1	10	60	M	2.3
6	4	1	2	7	54	M	3.0
7	5	7	2	14	38	M	2.7
8	4	2	2	8	34	M	2.7
9	2	3	0	5	50	F	3.0
10	0	2	1	3	54	M	1.5
11	0	1	3	4	61	M	2.5
12	0	0	1	1	50	M	4.0
13	5	3	1	9	47	M	0.6
14	1	0	2	3	45	M	5.5
15	0	1	3	4	57	F	1.1
16	2	1	1	4	59	M	2.2
17	2	4	0	6	45	M	2.5
18	2	2	0	4	46	M	2.7
19	2	0	0	2	44	F	2.7
20	1	0	0	1	52	M	3.0
21	0	0	1	1	55	M	1.5
22	0	0	1	1	53	M	0.6
23	2	0	1	2	50	M	3.0
24	2	0	0	2	42	M	3.0
25	2	0	1	3	58	M	2.0
26	1	0	1	2	26	M	2.3
27	2	0	0	2	24	M	2.7
28	2	0	1	3	67	M	2.5
29	1	0	1	2	45	M	2.3
30	1	0	1	2	60	F	2.7
31	2	0	1	3	65	M	2.5
32	2	0	1	3	47	M	2.3

NO, normal; FS, fatty streak; PL, atherosclerotic plaque.

number of stellate cells increases much more than other cell forms.

In the present study, which continues this research, we attempted to establish a relationship between the alterations in the number of intimal cells during atherosclerosis and major manifestations of an atherosclerotic lesion in aorta such as intimal thickening, increased amount of collagen, and accumulation of lipids. The second task was to identify on the basis of the ultrastructural criteria the previously described morphologic forms constituting the intimal population: elongated, stellate, elongated with side processes, and irregularly shaped.

Materials and Methods

Tissue

The study was carried out on 32 aortas taken from subjects who suddenly died of myocardial infarction. Age and sex of cadaver donors, periods of time which passed after death before the aorta was taken out, and other characteristics of the autopsy material are given in Table 1.

Immediately after the aorta was taken out, it was cleaned free of surrounding tissues. The adventitia was removed, and the vessel was cut lengthwise. The samples selected for electron microscopy were fixed for 24 hours at 4 C in 2.5% glutaraldehyde dissolved in pH 7.4 phosphate-buffered saline (PBS).

Nonfixed aortas were used for combined measurements of collagen, lipids, thickness of intima, and cell number in one sample. Grossly normal regions, fatty streaks, and atherosclerotic plaques were determined on the inner surface of nonfixed aortas as described elsewhere.[2] Only uncomplicated plaques were used for the study. In each arota, we selected several loci (8 × 8 mm) localized in a grossly normal region, at the site of fatty streak congestions or on atherosclerotic plaques, the interlocus separation being not less than 1 cm. Each locus was excised, the intima was mechanically separated from the media with forceps on approximately two-thirds of the locus' surface and a strip 2 mm in width was cut out in the center (Figure 1). The strip was fixed at 4 C for 16–24 hours in a mixture of 4% formaldehyde and 2.5% glutaraldehyde dissolved in PBS. Subsequently, this piece of tissue was used to control by microscopy the accuracy of separation of the

intima from the media and measurement of the intimal thickness. Examination of 218 pieces revealed a faulty separation of the intima from the media in 25 of them, and the loci from which they had been taken were discarded.

In the remaining part of the locus, two 0.125-sq cm disks were cut out of the separated intima on each side of the excised strip (Figure 1). In small plaques, we managed to excise only one disk on each side of the strip. One pair of contralateral disks was used to measure lipids and another to measure collagen.

Light Microscopy

Fixed pieces of tissue were dehydrated in graded ethanol (70°, 96°, 96°, 100°, 100°) and chloroform (2 changes) and embedded in Paraplast (Polysciences, Inc., Warrington, Pa). Five-micron-thick sections were prepared on a Reichert-Jung microtome, deparaffinized, and stained for collagen according to van Gieson and elastic according to Verhoeff. The internal elastic lamina served as a morphologic border between the intima and media.[2] The detachment of the intima from the media was considered faulty if it occurred above or beneath this line. The thickness of the intima was measured with the use of an ocular micrometer.

Target Electron Microscopy

To compare the shape and ultrastructure of aortic intimal cells, we used the material fixed in 2.5% glutaraldehyde and a series of special procedures.

1. A vessel wall sample was laminated with microdissectional forceps into thin films parallel to the endothelial plane. We then treated all the samples separately, preserving the sequence of their distribution in the vessel wall.
2. Each sample was stained with Karacchi hematoxylin for 20 minutes at room temperature. The stain was then washed off with distilled water, and the samples were postfixed with 1% OsO_4 in PBS at 4 C for 30 minutes.
3. The samples were dehydrated in a graded series of ethanol and embedded in Epon 812-Araldite (Polysciences) according to the standard procedure. Each sample was then smoothed out on an Epon block and polymerized for 2–3 days at 60 C.
4. From each block, serial sections (7–10 μ) were cut with a glass knife in an LKB-IV Ultratome. The sections were placed on slides in a drop of water and smoothed out on a hot plate at 90 C.
5. After drying, the sections were examined under a light microscope for determination of the cell shape. The cells chosen for examination were enclosed in a frame with a marker attached to the objective. Unframed parts of the section were removed with a razor blade under a stereomicroscope.

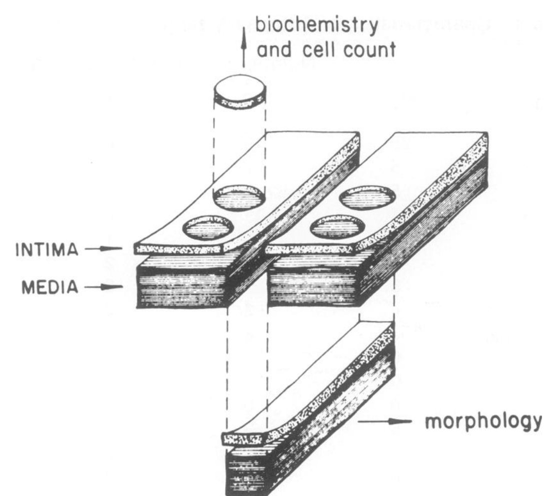

Figure 1—Preparation of intimal samples for determination of the cell count, thickness of intima, and content of lipids and collagen.

6. Areas of interest were photographed, and a capsule with a ready-made Epon 812 mixture was placed over each section. Then the sections, together with the slide, were subjected to polymerization for 2 hours at 95 C.
7. After polymerization, the blocks were separated from the slide by heating it over the flame of a spirit lamp.
8. Ultrathin sections were prepared in an LKB-IV Ultratome, stained with uranyl acetate and lead cytrate and examined in a JEOL-100CX electron microscope at a beam voltage of 80 kv.

Lipid Extraction and Determination

Lipids were extracted from intact intimal disks by treatment with a 2-ml chloroform–methanol mixture (2:1) three times at 2 C for 2 hours. To register losses, during the first extraction 10,000 dpm 4-^{14}C-cholesterol (sp. act. 57 mCi/mmol; Amersham International, Amersham, U.K.) was added to the extraction medium. It was established in special experiments that this procedure affords a practically complete extraction of lipids. Additional treatment of the tissue or homogenate with a chloroform–methanol mixture did not lead to the emergence of significant amounts of lipids in the extract (Table 2).

Three milliliters of chloroform and 3 ml water were added to the combined supernatant, and it was centrifuged at 200g for 5 minutes. The upper water–methanol phase was aspirated, and the lower one was washed

Table 2—Extraction of Lipids From Aortic Intima Samples

Extraction	Amount of extracted lipid (μg/mg wet weight)			
	Phospholipids	Cholesterol	Triglyceridges	Cholesteryl esters
Normal				
1	5.82 ± 0.37	0.96 ± 0.10	0.74 ± 0.06	1.22 ± 0.11
2	1.12 ± 0.21	0.27 ± 0.05	0.12 ± 0.01	0.45 ± 0.05
3	0.15 ± 0.05	0.11 ± 0.01	ND	ND
4	ND	ND	ND	ND
5	ND	ND	ND	ND
6 + Hmg	ND	ND	ND	ND
7 + Hmg	ND	ND	ND	ND
Fatty streak				
1	6.94 ± 0.65	1.61 ± 0.12	1.25 ± 0.11	4.99 ± 0.45
2	1.92 ± 0.11	0.57 ± 0.05	0.25 ± 0.03	1.03 ± 0.25
3	0.24 ± 0.07	0.10 ± 0.02	ND	0.34 ± 0.04
4	ND	ND	ND	ND
5	ND	ND	ND	ND
6 + Hmg	ND	ND	ND	ND
7 + Hmg	ND	ND	ND	ND
Plaque				
1	6.73 ± 0.66	2.12 ± 0.12	1.44 ± 0.16	4.99 ± 0.12
2	2.17 ± 0.26	1.30 ± 0.19	0.40 ± 0.03	1.58 ± 0.20
3	0.20 ± 0.06	0.19 ± 0.04	ND	0.34 ± 0.10
4	ND	ND	ND	ND
5	ND	ND	ND	ND
6 + Hmg	0.14 ± 0.02	ND	ND	0.14 ± 0.03
7 + Hmg	ND	ND	ND	ND

Each value represents the mean of three determinations ± SEM
ND, not determinable (< 0.01 μg).
First five extractions were carried out as described in Materials and Methods. Sixth and seventh extractions were carried out after grinding the remaining tissue samples by pestle in a porcelain mortar with cover glasses and subsequent homogenization (Hmg) in a Dounce glass homogenizer.

twice with 3 ml water, evaporated, and dissolved in 25–50 μl chloroform.

Phospholipids were determined on lipid phosphorus according to the colorimetric method of Vaskovsky et al.[4]

Triglycerides, cholesterol, and cholesteryl esters were separated by thin-layer chromatography on silica gel plates, and the amounts of lipids were measured by scanning densitometry as described elsewhere.[5]

Alcoholic-Alkaline Dissociation and Cell Count

After extraction of lipids, two intimal disks were fixed in a mixture of formaldehyde–glutaraldehyde. Then, the fixed tissue was dissociated by incubation in 500 μl of a 30% KOH–96% ethanol mixture (1:1) at 37 C for 1.5–2.5 hours as described earlier.[3]

The suspension of dissociated cells was examined in a phase-contrast microscope. In every preparation, we estimated a proportion of the previously described morphologic forms[2,3,6]: 1) stellate cells with a small body, rounded nucleus and long, thin, branching processes, the number of which, as a rule, exceeds three (Figure 2G); 2) elongated bipolar cells with the nucleus stretched along the body (Figure 2E); 3) elongated cells with one, or rarely, two long side branches thinning toward the periphery (Figure 2F); and 4) flat cells of irregular shape having two to three short, thick processes (Figure 2H). No less than 400 cells were counted in every preparation.

The total number of dissociated cells in a suspension was estimated with a hemocytometer.

Collagen Determination

Two disks of the intimal tissue meant for collagen determination were fixed in a mixture of formaldehyde–glutaraldehyde and then dissociated in 500 μl KOH–ethanol mixture as described above. A half of the resulting suspension was used to determine the number of cells and ratio of morphologic forms; 12 N HCl was added to the remaining 250 μl to a final concentration of 6 N, after which it was sealed in glass ampules and hydrolyzed at 106 C for 20 hours. The hydrolysate was evaporated at 100 C. A dry residue was dissolved in 2 ml distilled water, and the level of hydroxyproline was determined in the solution by the colorimetric method of Stegeman and Stalder.[7] To register losses, 10,000 dpm ^{14}C-hydroxyproline (sp. act. 11 mCi/mmol; Amersham International, Amersham, U.K.) was added

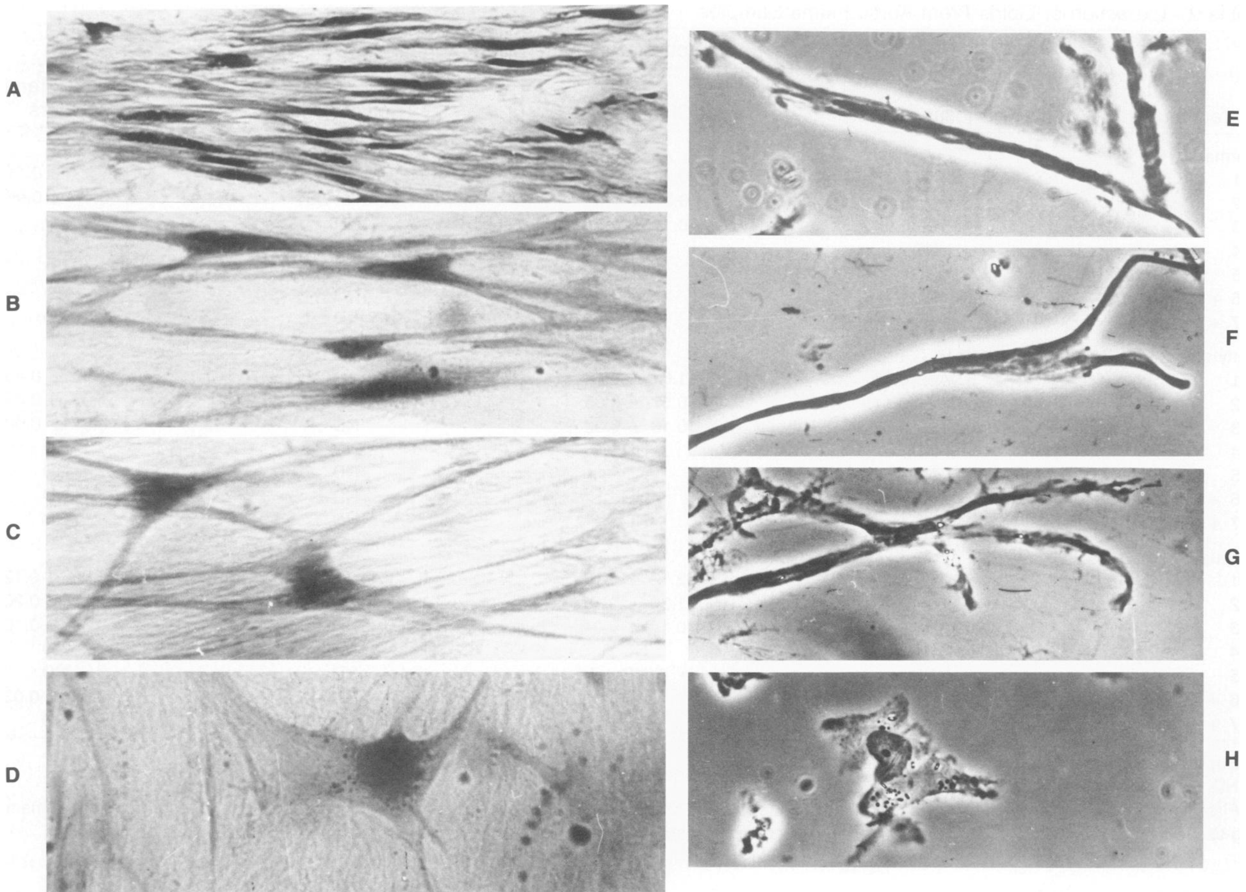

Figure 2—Different morphologic forms of subendothelial cells of human aorta. **A** and **E**—Elongated bipolar cells. **B** and **F**—Elongated cells with side processes. **C** and **G**—Stellate cells. **D** and **H**—Irregularly shaped cells. (**A–D**, *en face* section [hematoxylin]; **E–H**, suspension of alcohol–alkali-isolated cells [phase contrast], ×400)

to the sample prior to hydrolysis. The yield of hydroxyproline was 84–98%. The amount of collagen in the sample was calculated using a factor of 7.2 to convert hydroxyproline to collagen values.

It was established in a special series of experiments that tissue fixation and alcoholic–alkaline dissociation had no effect on hydroxyproline determination in the hydrolysate. They were performed as follows. Aortic tissue was cut with scissors into small pieces (approximately 2 × 2 mm), and the minced tissue was divided into three portions of equal weight. One portion was left without fixation, and the other two were fixed with formalin–glutaraldehyde as described elsewhere. One of the fixed portions was subjected to alcoholic–alkaline dissociation. Then all the three were hydrolyzed, and hydroxyproline was determined in the hydrolysate. It was established that the samples showed no difference with respect to the hydroxyproline content.

Statistical Analysis

To assess the significance of differences between the number of cells in unaffected intima and intima with various types of atherosclerotic lesions, we used the one-way ANOVA and the Welch and Brown–Forsythe tests, which do not suggest the equality of dispersions. The choice of these tests was determined by the fact that the range, normal–fatty streak–plaque, was marked by a substantial increase in variance of the cell number. In a case like this, the standard *F* test is unacceptable because of considerable error.[8] The calculations were carried out according to the BMDP 7D program from a BMDP 77 package.[8]

For crude estimation of the relationship between the number of cells belonging to different morphologic forms and collagen content, accumulation of lipids, and thickness of intima, we have used the correlation coefficient value. Significance of the correlation coefficient difference from zero and differences between the coefficients were estimated with the use of the Fisher z-transformation[9]: $z = 0.5 \ln[(1 + r)/(1 - r)]$.

A more detailed examination was performed with the use of multiple regression analysis (see Appendix). The calculations were carried out according to programs

BMDP 1R and 9R from the BMDP package.[10,11] The results of this analysis corroborated the major conclusions obtained by the correlation coefficient estimation.

Results

Identification of Intimal Cells

For identification of intimal cells differing in shape we settled on electron microscopy. However, transmission electron microscopy revealed certain disturbances in the ultrastructure of cells dissociated by the alcohol-alkali mixture: the intracellular membrane components disintegrated completely, whereas nuclear chromatin looked normal (not illustrated). Thus, the alcoholic-alkaline technique destroys internal cellular structures, making the isolated vascular cells more suitable for analysis by light rather than electron microscopy.

We thought that this problem could be solved by studying the ultrastructure of various intimal cells *in situ*, ie, without isolating them from the tissue. As was previously pointed out, the cell forms discovered in a suspension of dissociated intimal cells have exact counterparts in the *in situ* vessel wall. Cells that are stellate, elongated, elongated with side processes, or irregularly shaped can be found in suspension and in the vessel (Figure 2). Therefore, we decided to use target electron microscopy to elucidate the ultrastructure of diverse morphologic forms of intimal cells. For this purpose, we developed a special procedure described under "Materials and Methods." The cardinal feature of this method is that thick sections for identification of the cell shape by light microscopy and subsequent ultrathin sections for an ultrastructural examination were prepared parallel to the plane of the endothelial lining. This was essential because most of the intimal arterial cells are very much flattened and oriented parallel to the endothelial plane. Therefore, on vertical sections all cells look alike and spindle-shaped. At the same time, on the sections cut strictly parallel to the vascular luminal surface, intimal cells exhibit variable shape. Among them, one can tentatively distinguish elongated cells with and without side processes, stellate cells, and flattened cells of irregular shape (Figure 2).

Elongated cells without side processes have a more or less lengthened body and contain a rod-shaped nucleus oriented along the longitudinal axis of the cell. As a rule, these cells are arranged parallel to each other, forming bundles and layers with cellular elements packed as densely as in the media (Figure 2A and E). In addition, in the intima one can find cells with a similarly shaped body but having a variable number of side filamentous processes (Figure 2B and F). When these cells occur in groups, they are completely detached and oriented parallel to each other. Stellate cells are distinguished by characteristic radial processes varying in number from 3 to 12. In most cases, the processes are characterized by dichotomy. A rounded or bean-shaped nucleus is localized in the center and devoid of any definite orientation toward the cell body (Figure 2C and G). Flattened cells of irregular shape have no definite outline. Their nucleus is flattened and has an oval or beanlike shape (Figure 2D and H). The cells of the last two types, as well as elongated cells with side processes, are freely spaced in the surrounding connective tissue without contacting the adjoining cells.

The material used for the study of differently shaped cells by target electron microscopy comprised 27 samples taken from 12 aortas (Aortas 21–32, see Table 1) including 10 samples from atherosclerotic lesions. We examined a total of 220 cells: 70 stellate, 73 elongated 67 elongated with side processes, and 10 irregularly shaped.

Comparison of the cell shape visible under a light microscope to the ultrastructure revealed that only elongated cells without side processes possess the ultrastructural features of differentiated smooth muscle cells. The nucleus of these cells is rod-shaped and oriented along the cell's longitudinal axis. Nearly the whole of the cytoplasm is occupied by longitudinally oriented microfilament bundles with clearly discernible "dense bodies." A small number of rough endoplasmic reticulum (RER) profiles and other cellular organelles are concentrated in a narrow perinuclear zone of the cytoplasm. Each cell is surrounded by a well-marked basal lamina represented by an amorphous substance of medium electron density (Figure 3A).

Cells of which the shape differs from the elongated without processes have the ultrastructural signs distinguishing them from typical differentiated smooth muscle cells (Figure 3B and C). Thus, elongated cells with side processes and most of stellate cells and flattened irregularly shaped cells are characterized by a sharp increase in the number of RER profiles. Filament bundles with associated dense bodies are confined to the periphery of the cells. Like differentiated smooth muscle cells, RER-rich cells have a basal lamina which partially surrounds them. Some of the cells have a reduced poorly visible contour of basal lamina with loci of disintegration.

A small part of stellate and flattened irregularly shaped cells have no ultrastructural signs of contractile cells. Microfilament bundles with dense bodies are absent in their cytoplasm, and the basal lamina is indistinctly marked or absent on a large part of the cell surface. The nucleus of these cells is rounded or bean-shaped and has numerous invaginations. In the cyto-

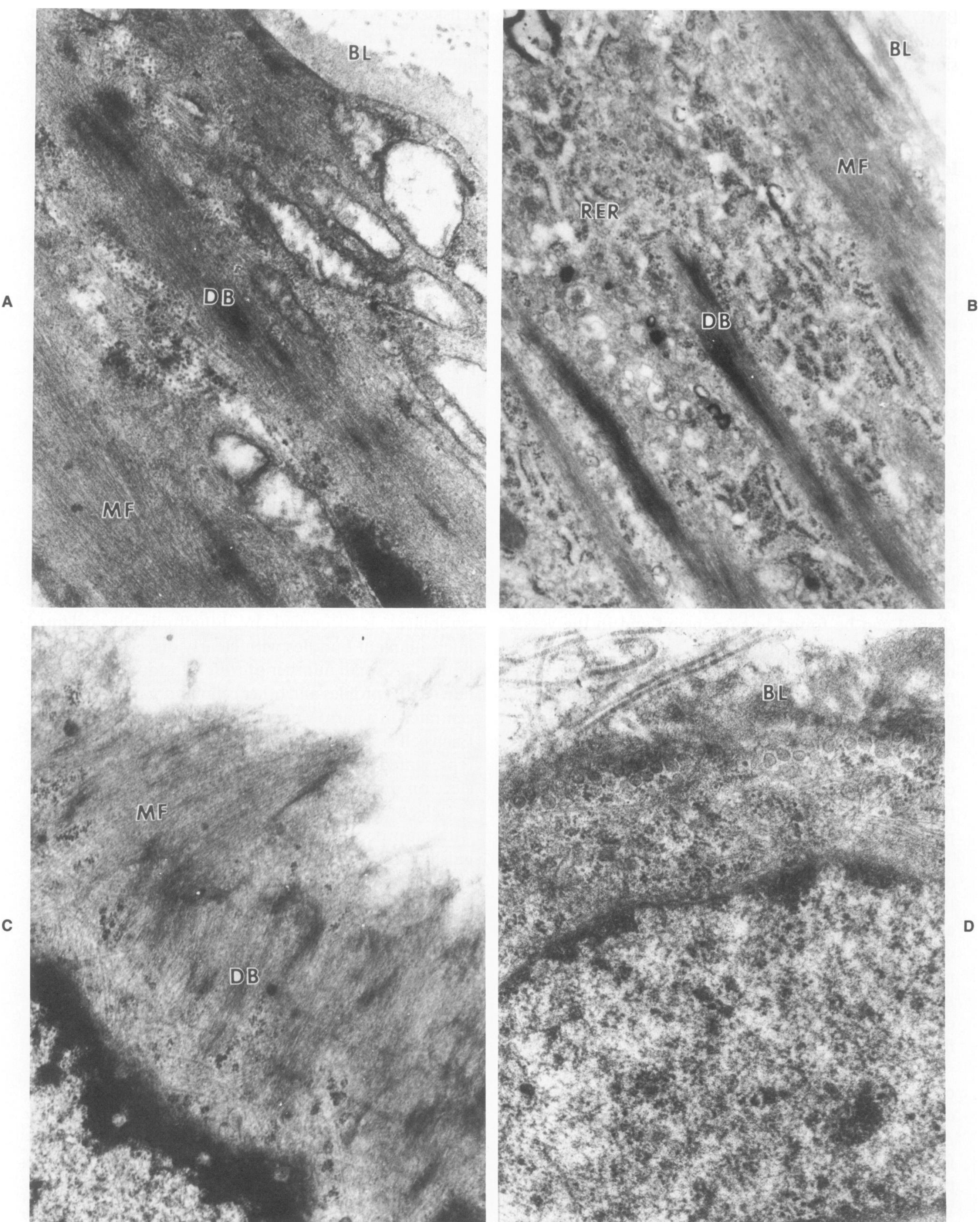
BL
DB
MF
A
BL
MF
RER
DB
B
MF
DB
C
BL
D

Table 3—Cell Number in Grossly Normal Intima and in Atherosclerotic Lesions (Aorta 1)

	Cell number per 0.5 sq cm			Significance*	
	Normal	Fatty streak	Plaque	P_W	P_{B-F}
	1763 ± 204	9520 ± 1508	68396 ± 12336	<0.001	0.002
	115102 ± 6859	142745 ± 9323	229052 ± 43979	0.022	0.004
	17151 ± 907	28506 ± 2757	67366 ± 12332	0.001	0.006
	74944 ± 4117	76449 ± 4609	133958 ± 16422	0.017	0.005

* The null hypothesis for each cell type was that the cell numbers in unaffected intima and atherosclerotic lesions are equal.
P_W, *P* value based on Welch statistics.
P_{B-F}, *P* value based on Brown–Forsythe statistics.

plasm, one can find mostly the membrane of smooth and rough ER, free ribosomes, and microfilaments without regular orientation (Figure 3D).

Thus, only the cells of elongated shape without side processes are differentiated smooth muscle cells. Intimal cells with processes as well as flattened cells of irregular shape differ from the elongated cells without processes by a reduced contractile apparatus and characteristic increase in the content of biosynthetic organelles.

The revealed patterns in the relationship between the shape and ultrastructure of intimal cells were characteristic both of an unaffected intima and atherosclerotic lesions. The cells of all morphologic forms in fatty atherosclerotic lesions were distinguished by a great number of lipid inclusions. Furthermore, all the morphologic forms in an atherosclerotic plaque were substantially larger in size as compared with their counterparts in unaffected intima.

Correlations

As was reported earlier, the number of cells in the intimal population of an atherosclerotic lesion zone is increased by twofold on average.[2,3]

The number of cells belonging to various morphologic forms is multiplied to a different degree: namely, stellate cells, by approximately 40-fold; elongated bipolar cells and irregularly shaped cells, approximately 2-fold; and elongated cells with side processes, 4-fold (Table 3). As a result, quantitative alterations in the cellular composition of the intimal population naturally occur in lesion zones.[2]

We decided to find out whether there was a relationship between the alterations in the cellular composition and well-known atherosclerotic disorders such as thickening of intima and accumulation of lipids and collagen. For this purpose, the thickness of intima, the collagen content, and the amount of lipids of different classes were measured in the aorta. These parameters were juxtaposed with the total number of intimal cells and the number of cells belonging to the four major morphologic forms: stellate, elongated, elongated with side processes, and irregularly shaped. For this study, we have chosen Aorta 1 with fatty streaks and atherosclerotic plaques as well as grossly normal areas.

Table 4 shows the correlation coefficient between the number of cells of the four main morphologic forms, as well as the total cell number, and intimal thickness, collagen content, total lipids, phospholipids, triglycerides, total cholesterol, free cholesterol, and cholesteryl esters, which were determined in 49 samples taken from Aorta 1. Juxtaposition of all the above-mentioned parameters with the stellate cell number yielded the highest correlation coefficients. The highest correlation was found between the number of stellate cells and cholesteryl ester content as well as the total lipid content. Correlation coefficients between the stellate cell

Figure 3—Electron micrographs of subendothelial cells of human aorta having a different ultrastructure. **A**—Contractile smooth muscle cell (a bipolar elongated cell without side processes). Microfilament bundles (*MF*) with dense bodies (*DB*) predominate in the cytoplasm. Basal membrane (*BM*) is well marked. **B**—Cell with the signs of synthetic activity (an elongated cell with side processes). Contractile structures are preserved in the form of separate microfilament bundles with dense bodies. Characteristics predomination of rough endoplastic reticulum (*RER*) is seen in the cytoplasm. Basal membrane is loose. **C**—Cell with the signs of synthetic activity (a stellate cell). The micrograph shows a region with a preserved contractile apparatus. Basal membrane is not seen. **D**—Cell of stellate shape with the signs of synthetic activity devoid of contractile structures. One can see separate microfilaments. Basal membrane is retained. (×32,000)

Table 4—Correlation Between Cell Number and Collagen Content, Lipid Accumulation, and Intimal Thickness (Aorta 1)

					Total Cell Number
Intimal Thickness	0.732	0.504*	0.687	0.456*	0.585*
Collagen	0.800	0.521*	0.722	0.446*	0.612*
Total lipid	0.953	0.665*	0.796*	0.747*	0.806*
Phospholipids	0.896	0.571*	0.703*	0.651*	0.717*
Triglycerides	0.937	0.667*	0.782*	0.704*	0.795*
Free cholesterol	0.865	0.526*	0.700*	0.634*	0.681*
Cholesteryl esters	0.958	0.725*	0.843*	0.763*	0.819*
Total cholesterol	0.942	0.672*	0.800*	0.733*	0.808*

All correlation coefficients differ significantly from zero ($P < 0.01$)
* Significant difference from the value of correlation coefficient for stellate cells ($P < 0.05$).

number and collagen content and thickness of intima were somewhat lower but still exceeded the correlation coefficients between these indexes and the number of cells belonging to other morphologic forms, as well as the total cell number. The correlation between the parameters characterizing an atherosclerotic lesion and the number of elongated cells with side processes was always a bit lower, compared with stellate cells. However, their correlation was better than that obtained for elongated and irregularly shaped cells, which differed from each other insignificantly.

These results suggest that stellate cells are more intimately related to the major manifestations of atherosclerosis than other morphologic forms. Because this conclusion seems rather important to us, we decided to substantiate it with a more detailed statistical analysis of the data. Multidimensional analysis also brought us to the conclusion that the stellate cell is the principal cell involved in the disease (see Appendix).

Thus, we discovered a positive correlation between an increased number of stellate cells in the intima and such manifestations of atherosclerosis as intimal thickening and accumulation of lipids and collagen.

Having established such a correlation in Aorta 1, we, naturally, decided to verify this finding by the experiments on other aortas and examined three more vessels (Aortas 2–4). In these cases, too, we obtained rather a high correlation between the number of stellate cells in the intima and the content of collagen, total lipids, and thickness of intima (Table 3). And again, just as in Aorta 1, the correlation coefficient for stellate cells was higher than for the other morphologic forms, the lowest correlations being obtained for the cells of irregular shape (in some cases, there was no significant correlation at all).

We calculated the correlation coefficients between the stellate cell number and "atherosclerotic" indexes from the combined data obtained on several aortas (Aortas 1–4). The coefficients of linear regressions were substantially different for different aortas (Figure 4). As a result, for the whole group collected from several aortas, the approximation by a single linear function proves to be less precise (Table 5). As mentioned above, however, high positive correlation was obtained for each individual aorta. The most dramatic result was obtained when we attempted to find a correlation on the basis of measurements performed on limited data combined from 8 or 16 aortas (1 to 14 samples from each aorta). In this case, no correlation was found between the number of stellate cells and the content of collagen, lipids, or thickness of intima (Table 5; Aortas 5–12 and 5–20). Thus, it is advisable for this type of experiment to increase the number of samples from one aorta, while

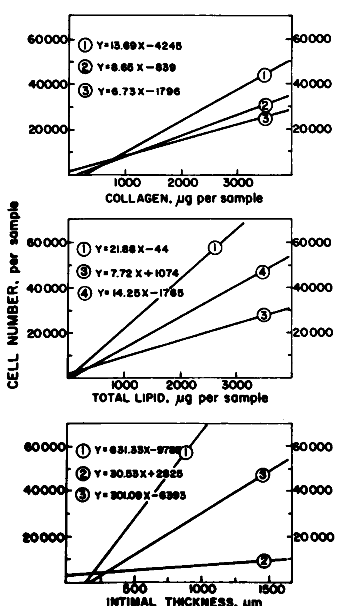

Figure 4—Linear regressions of lipid content and collagen content as well as the thickness of intima on the number of stellate cells. Linear regression were obtained for the respective parameters determined in the samples taken from Aorta 1, 2, 3, or 4. To the left, regression equations for each aorta are shown.

Table 5—Correlations Between Cell Number and Collagen Content, Lipid Accumulation, and Intimal Thickness in Different Aortas

Aorta	Total number of samples				
Collagen					
1	48	0.80*	0.52*	0.72*	0.45*
2	18	0.75*	0.54*	0.51*	0.37
3	13	0.92*	0.65*	0.60*	0.47
1+2+3	79	0.84*	0.44*	0.41*	0.33*
5–20	83	0.03	0.09	0.14	−0.15
Total lipid					
1	49	0.95*	0.66*	0.80*	0.75*
3	13	0.82*	0.60*	0.48	0.35
4	28	0.91*	0.68*	0.72*	0.08
1+3+4	90	0.84*	0.14	0.45*	0.30
5–12	57	−0.14	−0.13	−0.03	−0.24
Intimal thickness					
1	49	0.73*	0.50*	0.64*	0.46
2	18	0.57*	0.51*	0.46	0.52*
3	13	0.85*	0.71*	0.47	0.41
1+2+3	80	0.26*	0.38*	0.60*	0.42*
5–20	85	0.05	0.09	0.23	−0.13

* Correlation coefficients significantly different from zero ($P < 0.05$). The table shows the correlation coefficients between the parameters determined in the samples from each aorta taken separately (Aorta 1, 2, 3, or 4) as well as in the samples obtained from different aortas (Groups 1+2+3, 1+3+4, 5–12, and 5–20). The number of samples derived from each aorta and other characteristics are given in Table 1.

the analysis of limited data from a large number of vessels seems inexpedient because of significant individual differences between aortas. Individual differences in the measured parameters between aortas are so great that it leads to a fall in the correlation.

Discussion

The following major conclusion may be drawn from this study. An altered cellular composition of the intima during atherosclerosis manifesting itself primarily in a sharply increased number of stellate cells, which was found in our previous studies, is closely related to the main morphologic and biochemical changes accompanying atherosclerosis. Our data on the relationship between the increased number of stellate cells and intimal thickening, deposition of lipids, and accumulation of collagen can be supplemented by the data of Schönfelder, who discovered a positive and very close relationship between the number of these cells and glycosaminoglycan content in the intima.[12] He found out that with age a subpopulation of cells having mainly stellate shape (he called them Langhans' cells) and amount of glycosaminoglycans grow parallel in the intima of human aorta. Schönfelder also revealed this association in many diseases including atherosclerosis.

Taking into account our data and Schönfelder's findings, we cannot ignore the increase in the number of stellate cells during atherosclerosis or regard it as a minor attribute of the atherosclerotic process. Most atherosclerotic changes are undoubtedly related to the vital functions of intimal cells. Specifically, most of the connective tissue components which make up the extracellular matrix of a plaque are synthesized by arterial cells. Therefore, such manifestations of atherosclerosis as an increased amount of collagen and glycosaminoglycans in the intima and the resulting thickening have a cellular origin. If one takes into account the close relationship between an increase in stellate cells and accumulation of collagen, glycosaminoglycans, and intimal thickening, Schönfelder's assumption that stellate cells are the main producers of glycosaminoglycans in the artery wall seems very interesting.[12] As far as the most vivid atherosclerotic manifestation, ie, deposition of lipids, is concerned, stellate cells may play an active role in this process as well. It is well known that in normal intima lipids are present mainly outside cells in the form of drops oriented along connective tissue fibers.[13,14] However, in early atherosclerotic lesions lipids accumulate mainly inside cells.[5,15] Anichkov attributed much importance to the role of stellate cells in this process, assuming that they grow in number at early stages of intimal lipidosis and accumulate lipids, which are condensed in the cytoplasm in the form of small drops.[16] The results of our current study agree with these views: we have found that the cells overloaded with intracellular lipids are most frequently found exactly among the stellate cells (Andreeva et al, to be published).

In this report, we are focusing on a close association between the number of stellate cells and major manifestations of atherosclerosis as well as on the facts which

point to a decisive role of these cells in atherosclerotic events. We are far from thinking, however, that an increase in the number of stellate cells is responsible for all disorders occurring in the vessel wall. On the contrary, we admit that certain biochemical changes characteristic of atherosclerosis, eg, deposition of lipids, may selectively stimulate proliferation of stellate cells, because, as was shown, accumulation of lipids occurs mainly in these cells. It cannot be ruled out that the accumulated intracellular lipids actually stimulate cell proliferation. So the deposition of lipids in the intima may lead to an increased number of stellate cells, which explains the discovered close association between these two processes.

The third hypothesis that we are ready to admit is that one common cause responsible for changes in the cellular composition and biochemical manifestations of atherosclerosis brings about simultaneous alterations in the cell population and chemical components of affected intima. We hope that intensive exploration of functional characteristics of arterial wall cells, which is currently under way in many laboratories, will reveal causalities between the cellular and biochemical aspects of atherosclerosis.

Another issue arising from the assumption that stellate cells play an active role in atherosclerotic lesion formation is the origin of these and other intimal cell forms. Trying to solve this problem, we have investigated the ultrastructure of the main cell forms of the intimal population. The data obtained in this study by target electron microscopy and the conclusion about a relationship between the shape and ultrastructure of intimal cells on the whole agree with the observations of other researchers.[1,17]

Twenty years ago Geer noted that "smooth muscle cells in the intima . . . were fusiform in shape" and "were identified by the presence of large numbers of cytoplasmic myofilaments, numerous pinocytotic vesicles along the plasma membrane, and a limiting basement membrane."[17] In addition to smooth muscle cells, he observed in human aortic intima cells of variable, usually stellate, shape. The description of these cells' ultrastructure by Geer completely coincides with our observations, the results of which can be briefly summed up as follows: stellate cells have substantially fewer contractile structures, compared with smooth muscle cells, and have a more developed synthetic apparatus. In addition, we found some cells of stellate or irregular shape fully devoid of the smooth muscle features. Earlier, Geer had also pointed out that some of the stellate cells are characterized by "total absence of a limiting basement membrane . . . and only occasional pinocytotic vesicles along the plasma membrane."[17] The results of our study coincide with earlier observations of Geer, who did not set himself a special task to perform target electron-microscopic examinations of the ultrastructure of cells having a different shape.

Proceeding from our own data and those of Geer, we think that the elongated cells without side processes are typical differentiated smooth muscle cells. As far as the other morphologic forms are concerned, we, following Geer, can conclude that "on the basis of morphology it is likely that these cells represent either cells differentiating into smooth muscle or smooth muscle cells dedifferentiating."[17] Now, these cells are mostly termed "modified smooth muscle cells." If there is an association between these and smooth muscle cells, the elongated cells with side processes apparently represent an intermediate element between the extreme forms. Cells of irregular shape have the same ultrastructure as the stellate but, as we found out, they are significantly less related or even completely unrelated to the biochemical manifestations of atherosclerosis. Schönfelder revealed that in some diseases, for example, bacterial-toxic lesions of the aorta, processes of stellate cells shorten and completely disappear, which transforms them into irregularly shaped cells.[12] Taking into account the similarity in the ultrastructure of stellate and irregularly shaped cells as well as Schönfelder's observations, one may assume that the cells of irregular shape are degenerating stellate cells.

Stellate cells, the most impressive cellular element of the intima by appearance, have long been the focus of researchers' attention. Since the discovery of stellate cells by Langhans in 1866,[18] they have been given all sorts of names: myointimal cells,[19] multipotent and multifunctional mesenchymal cells,[20] intermediate smooth muscle cells,[21] intermediate cells,[22] myoendothelial cells,[23] modified smooth muscle cells,[1,24] cambial cells of the subendothelium,[25] intimacytes,[26] Langhans' cells,[9,27] unidentified cells,[28] primitive cells,[21] poorly differentiated cells.[29]

There have been different opinions as to the nature of these cells. Langhans, who was the first to describe subendothelial cells, regarded them as fibroblasts or fibrocytes.[18] For a long time, the researchers who employed light-microscopic techniques were basically of the same opinion. Nearly a hundred years later, the subendothelial cells were classified as fibroblasts,[30] fibrocytes, histiocytes, and monocytoid cells.[31] At the same time, there appeared a hypothesis that these cells originated from endothelial cells, which, after a "transformation," occupied the space beneath the endothelial lining and started performing functions unusual for typical endothelium.[23,32-34] It was also assumed that the subendothelial cells different from the smooth muscle were the cells that had recently undergone division and differentiated into either smooth muscle or endothelial cells.[21,28] Some workers considered them mesenchymal cells, intermediate between fibroblasts and smooth mus-

cle cells.[27] According to still another hypothesis, stellate cells originate from the pluripotent fibroblasts or they themselves are pluripotent cells that can be transformed into fibroblasts, endothelial cells, and smooth muscle cells, depending on the organ's requirements or corresponding stimuli.[33] Still and O'Neal regarded these cells not as resting fibrocytes, but as the cells which, if needed, are transformed into stem cells of cellular defense, endothelial cells, fibroblasts, and macrophages.[15] Shchelkunov numbered them among nondifferentiated elements of the organismic mesenchymal reserve.[35]

Application of electron microscopy to morphologic studies threw new light on the old problem.

The presence of a number of filaments resembling myofilaments in the cytoplasm of stellate cells as well as the basal membrane adjoining a part of the plasma membrane was regarded by many researchers as decisive evidence in favor of the earlier assumption that these cells have a smooth muscle origin.[36] Since the discovery of certain ultrastructural features of smooth muscle in stellate cells,[37,39] there appeared a widespread tendency to term the subendothelial cells different from the smooth muscle "modified smooth muscle cells," while substantial diversity in the appearance of these cells was attributed to different degrees of differentiation of the one cell type.[1] At present, the concept of smooth muscle origin of subendothelial cells predominates.[1,40] Smooth muscle cells of the media which migrate into the intima and undergo modification are generally thought to be the precursors of these cells.[41,42]

It was earlier pointed out by Geer that "most stellate cells differed sufficiently from typical smooth muscle to conclude that they represented another cell type in the intima."[17] Now this possibility is practically out of discussion, though there are apparently no grounds to rule it out completely. The presence of some morphologic features of smooth muscle in subendothelial stellate cells does not contradict the earlier hypothesis that these cells are pluripotent poorly differentiated mesenchymal cells which, if necessary, can differentiate into fibroblasts, macrophages, endothelial cells, or smooth muscle cells.[15,16,23] Certain cells of an adult organism, eg, pericytes, may serve as an example of pluripotent poorly differentiated cells.

Pericytes are found along capillaries as well as the smallest precapillary arterioles and postcapillary veins.[43] These cells possess long and thin branching processes and often have a stellate shape. They form a girdle around the outside of the endothelial tube of small vessels, being in direct contact with the endothelium. Further from the capillary bed, as arterioles become larger, pericytes around the endothelium are replaced by smooth muscle cells. Now it is thought that the latter are most probably formed of pericytes.[44] Unlike smooth muscle cells, pericytes are characterized by a more diffusive localization of organelles; neither filaments nor dense bodies are revealed in their cytoplasm. Each pericyte, however, is surrounded by a basal membrane.[43] Hypothetically, pericytes may differentiate not only into smooth muscle cells but also into osteoblasts, lipocytes, mast cells, and so forth.[44] In some diseases, specifically hemangiomas, pericytes acquire certain ultrastructural features of the endothelium, smooth muscle cells, or fibroblasts, due to which fibroblast-, endothelium- and smooth muscle-like pericytes are found in the lesion zone.[44]

The ultrastructural peculiarities, a characteristic stellate shape, and localization in proximity to the endothelium are the signs of aortic stellate cells which are suggestive of their association with pericytes. Only special studies can give an answer to the question of whether there is a relationship between stellate cells of the aorta and pericytes. However, the idea that poorly differentiated mesenchymal cells with properties similar to those of pericytes may exist in the vessel wall of an adult was put forward long ago. Beneath the vascular endothelium, Shchelkunov[35] distinguished cells forming the cambial layer of the intima, which produces all tissue elements both outside, toward the media, and inside, toward the endothelium. He thought that this layer was a residual mesenchyma adjoining the endothelium during embryogenesis, of which the external part turned into connective tissue and smooth muscle vascular elements as a result of differentiation. According to Shchelkunov, after a certain period of time the endothelial cells lining the interior of the vascular bed die. They are replaced by younger cells of the subendothelial layer. He maintains that all hyperplastic processes in the intima are related to vital functions of these cells. They are responsible for vigorous new growth of tissue in arteries and various hypertrophies of the vasculature, including atherosclerosis. They actively participate in the age-related transformations of vessels from the moment of their emergence until extreme old age.

We think that the time has come to corroborate and specify speculations about the origin and functions of stellate and other cells inhabiting the intima. Applications of modern methods, such as cell culture and typing of cells with monoclonal antibodies, must finally allow us to elucidate the origin of all vessel wall cells as well as their functions in health and disease.

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Appendix

The relationship between the individual atherosclerotic indexes and the number of cells belonging to different morphologic forms was assessed using multiple linear regression analysis. To test the adequacy of the linear model, we also used some other models (y = a + bx, y = a + blnx, lny = a + bx, lny = a + blnx). The quality of the model approximation was evaluated by determination of coefficient *R*, which, in the case of a simple linear regression, is equal to the squared correlation coefficient for dependent and independent variables. In all cases, linear approximation y = a + bx was either optimal or insignificantly different from the optimum, which was a sufficient reason for using a linear model.

The main objective of the analysis was not to obtain a regression equation proper, but to determine the best predictors. This was accomplished with the help of two methods. First, for all atherosclerotic indexes we performed multiple linear regression by the number of cells of the four morphologic forms and estimated significance of the regression coefficients. In all cases, only the stellate cell number was significantly different from zero ($P < 0.002$), whereas the other coefficients were not significant ($P > 0.05$), *P* for these coefficients being mostly over 0.1 The calculations were performed according to program BMDP 1R.[10] Secondly, we considered all possible subsets of predictors (ie, the number of cells belonging to each type and the number of cells of two, three, and all four types) and chose a combination producing the best approximation. These calculations were carried out according to program BMDP 9R.[11] Table 6 shows three optimal variants of predictor combinations. For evaluation of the approximation quality, we used Mallow's C_p coefficient to be able to compare regression by a different number of variables:

$$C_p = \frac{RSS}{S^2} - (N - 2p'),$$

where RSS is the residual sum of squares for the analyzed subset of predictors, S^2 is the residual mean square based on regression with the use of all independent variables, p' is the number of variables in the analyzed subset (including the intercept), and N is the total number of observations.

In all cases but one (approximation of cholesterol content) the number of stellate cells is one of the three best variants for the set of predictors with respect to the C_p value (Table 6). The highest quality of approximation for all the predictor sets which do not include the number of stellate cells was 4- to 80-fold lower than for the sets containing this parameter. So these results give enough reasons for us to assume that stellate cells are the principal cellular element involved in atherosclerosis.

Table 6—Choice of the Optimum Set of Predictors for "Atherosclerotic" Index (Aorta 1)

Index	Set of predictors	Quality of approximation		
		ARsq	C_p	$C_p$min
Collagen	St	0.631	1.93	11.92
	St, Ir	0.629	3.15	
	St, Ep, Ir	0.637	3.15	
Phospholipids	St, El	0.827	1.74	75.68
	St, Ep	0.816	4.70	
	St	0.799	8.27	
Triglycerides	St, El	0.876	1.05	89.69
	St	0.872	1.11	
	St, Ir	0.874	1.95	
Free cholesterol	St, El	0.784	2.94	57.95
	St, El, Ir	0.788	3.01	
	St, El, Ep	0.778	4.92	
Cholesteryl esters	St	0.916	2.50	101.13
	St, El	0.915	3.96	
	St, Ep	0.915	4.01	
Intimal Thickness	St, Ir	0.546	3.83	17.17
	St, El, Ir	0.554	3.96	
	St	0.528	4.70	

St, stellate cell number; El, elongated cell number; Ep, the number of elongated cells with side processes; Ir, irregular shaped cell number ARsq, adjusted squared multiple correlation; C_p, C_p Mallow coefficient; C_p min, minimum C_p value in all the predictor sets which do not contain St.