Cellular Composition of Atherosclerotic and Uninvolved Human Aortic Subendothelial Intima

Light-Microscopic Study of Dissociated Aortic Cells

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Alcoholic–alkaline dissociation was used in the study of cellular composition of human aorta. Cells were isolated from an uninvolved intima and intima with different types of atherosclerotic lesions: fatty infiltration, fatty streak, and atherosclerotic plaque. In the isolated suspension we evaluated the ratio of four previously described morphologic forms of cells: stellate, elongated, elongated with side processes, and flat cells of irregular shape. It was demonstrated that the quota of stellate cells in an atherosclerotic lesion considerably exceeds that of the normal intima. For elongated cells the opposite is true. The other two cell forms are represented in the uninvolved and atherosclerotic intima in approximately equal proportions. Alteration of the ratio of different morphologic forms occurs because of the fact that the number of cells belonging to different morphologic forms increases disproportionately in the lesion zone. Specifically, the number of stellate cells is increased much more substantially, compared with elongated cells. (Am J Pathol 1984, 115: 17–24)

SUBENDOTHELIAL CELLS of an adult human aorta have different shapes: round, ovoid, elongated, stellate, and irregular.[1–5] Round and ovoid cells are blood monocytes; most of the elongated cells are smooth muscle cells, and other morphologic forms have an ultrastructure distinct from typical smooth muscle cells and, hypothetically, belong to either modified smooth muscle cells or another cell type.[5]

In this study, we have attempted to quantitate the morphologic content of the cellular population of normal and atherosclerotic human aortic intima using a new approach—alcoholic–alkaline dissociation of a fixed aorta. The alcoholic–alkaline dissociation results in a practically complete dispersion of the aortic extracellular matrix, while the cells prefixed *in situ* are preserved.[6,7] In a suspension of the dissociated cells, one can find all the above-mentioned morphologic forms—ovoid, elongated, stellate, and irregularly shaped cells; ie, the polymorphism characteristic of the *in situ* cellular population is retained in suspension, too.[6,7] Investigation of cell polymorphism in a suspension of dissociated cells has important advantages over other approaches. On ordinary vertical sections of aortic wall, all subendothelial intimal cells look the same, having a spindle-like shape.[2] Polymorphism of the intimal cell population can be revealed in the preparations parallel to the endothelial plane.[2] Preparation of a good *en face* section, on which the shape of all cells is easily distinguishable, is a matter of chance. In contrast to the *en face* preparation, the suspension of dissociated cells gives a full picture of morphologic cell forms in the sample under examination and allows us to evaluate easily and precisely the ratio of these morphologic forms.

Materials and Methods

Nine aortas taken from men 3–6 hours after death were used. Immediately after the removal of the aorta, it was cut longitudinally and fixed with a mixture of 4% formaldehyde and 1% glutaraldehyde dissolved in phosphate-buffered saline (pH 7.4). The fixed material was stored at 4 C for up to 6 months.

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Table 1—Characteristics of the Vessels

Aorta	Area occupied by lesions (%) I	II	III	Type of lesion analyzed	Characteristics of the donor Age	Cause of death
1	0	0	0	0	59	Hypertension, cardiosclerosis
2	80	4	8	I, II, III	52	IHD, thrombosis of coronary arteries
3	24	3	0	0, I	33	IHD, myocardial infarction
4	14	3	2	0, I, II, III	45	Asphyxia with a foreign body
5	91	3	5	I, II, III	60	Myocardial infarction
6	17	0	0	0, I	25	Alcoholic intoxication
7	18	9	4	0, I, II, III	53	IHD
8	81	14	3	I, II	44	IHD, coronary atherosclerosis
9	70	7	20	I	55	IHD, coronary atherosclerosis, cardiosclerosis

0, normal; I, fatty infiltration; II, fatty streak; III, atherosclerotic plaque; IHD, ischemic heart disease.

To identify the type of atherosclerotic lesion we stained the aortas with 1.3% Sudan IV in 40% isopropyl alcohol for 18 hours.

Types of atherosclerotic lesions were classified according to Smith[8] in the following way:

Normal (0): Smooth luminal surface unstained with Sudan IV.

Fatty infiltration (I): Smooth luminal surface with diffusive sudanophilia of light pink or pink color.

Fatty streaks (II): Stripes 2–3 mm wide and 5–30 mm long, slightly elevated over the vessel surface and intensively stained red. Such stripes often coalesced into clusters.

Atherosclerotic plaques (III): Lesions highly elevated over the luminal surface, usually of rounded shape, light yellow or pearl in color and unstained with Sudan IV.

The data on the material used are presented in Table 1, namely, the age of the subject from whom the aorta was taken; cause of death; types of atherosclerotic lesions from which the cells were isolated; relative area occupied in the vessel by each type of lesion. Lesion area was determined by a MOP-3 semiautomatic image analyzer (Reichert-Jung).

From 3 to 8 loci (5 × 5 mm) were cut out of the lesion zones, and those uninvolved in atherosclerosis, the distance between the excised loci being not less than 1 cm. The intima was mechanically separated in each locus. Accuracy of the intima separation from the media was controlled macroscopically and microscopically according to Smith et al.[9] It can clearly be seen in the samples stained for Verhoeff's elastic that separation of the intima from the media went along the internal elastic lamina (Figure 1).

The intima was dissociated by incubation in a mixture of 30% KOH and 96% ethanol (1:1, 1 ml) at 25 C for 2.5–3 hours, as described earlier.[6,7] To evaluate the yield of dissociated cells, we determined the DNA content according to Burton[10] in the initial tissue and in the suspension of isolated and washed cells. The cell yield from the unaffected and atherosclerotic intima was approximately the same and amounted to 97 ± 2% (12 determinations). Prolonged incubation in the alcohol-alkali mixture led to disintegration of a number of cells and appearance of the nuclei devoid of cytoplasm in the suspension. Incubation for 10 hours caused just an insignificant fall in the total "cells plus nuclei" number, while already after 8 hours of incubation there were no whole cells left in the suspension. Proceeding from the fact that during incubation, which normally lasted 2.5–3 hours, the number of nuclei in the suspension obtained both from an atherosclerotic and uninvolved aorta did not exceed 5–10%, we concluded that the suspension subjected to a morphologic analysis represents 90% of cells which constitute a population of the corresponding aortic piece. In our view, the suspension obtained in such a way is adequately representative for a quantitative morphologic analysis.

The obtained suspension of cells (in 0.1 ml of distilled water) was stained by the addition of 1–2 drops of 1% toluidine blue in 1% sodium tetraborate solution. Following 5–10 seconds, a drop of suspension was put on a slide, overlaid by a cover slide, and studied under the microscope. In every preparation,

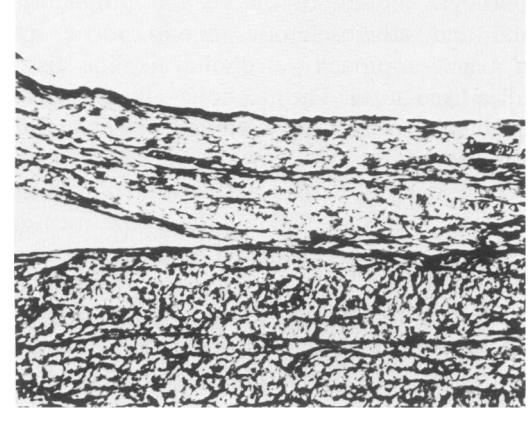

Figure 1—Partially stripped normal intima showing the line of stripping along the elastic lamina. (Verhoeff's stain, × 120)

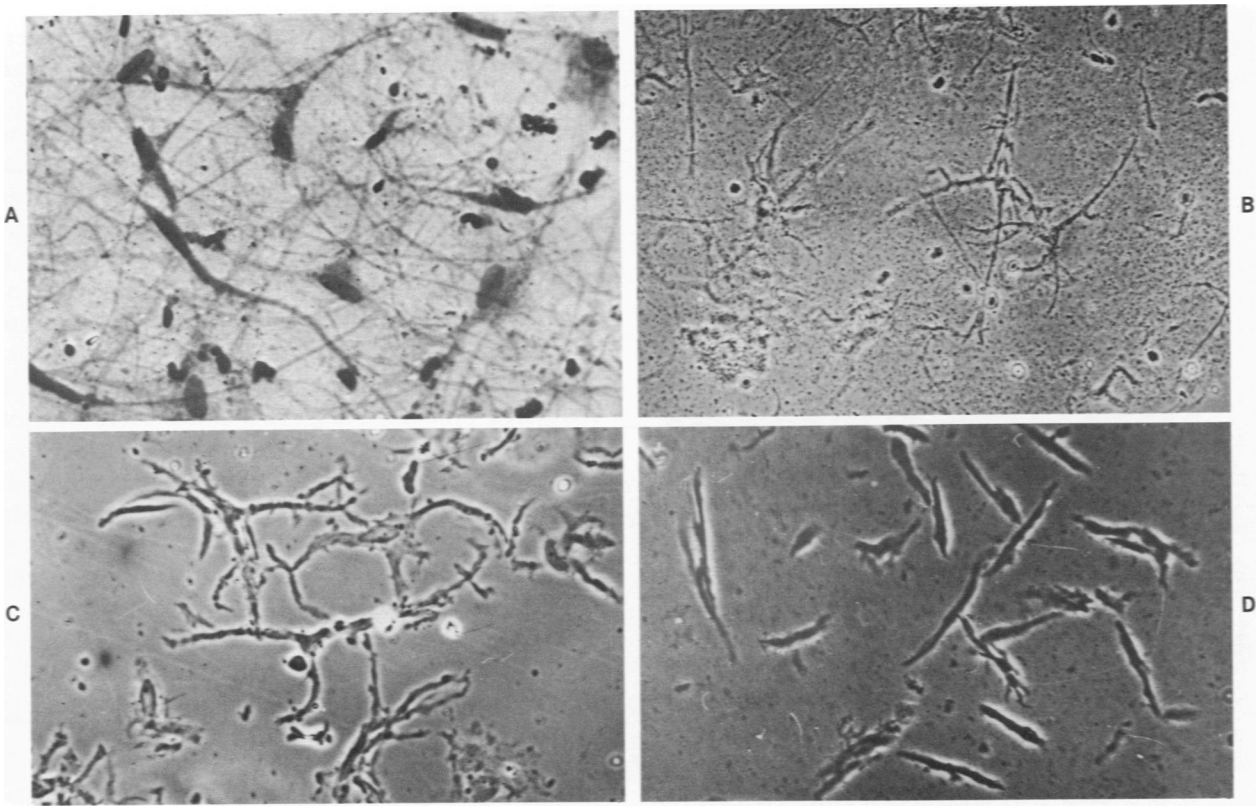

Figure 2—Polymorphism of aortic cells. **A**—An *en face* preparation of the intima. **B**—A piece of intima treated with the alcohol-alkali mixture. **C**—A suspension of intimal cells isolated by alcoholic-alkaline dissociation. **D**—A suspension of medial cells isolated by alcoholic-alkaline dissociation. (**A**, hematoxylin; **B-D**, phase-contrast. **A**, ×225; **B**, ×130; **C** and **D**, ×160)

we estimated a proportion of the previously described morphologic forms: stellate, elongated, elongated with side processes, and flat cells of irregular shape.[6,7] No fewer than 200 cells were counted in every preparation.

En face preparations of aorta were obtained by stratifying the intimal layer of a fixed vessel with microdissectional forceps into thin layers parallel to the endothelial surface. The layers were stained with hematoxylin, dehydrated, cleared in toluene, and mounted in Canada balsam under coverslips.

Significance of differences in the proportion of cell types was evaluated by nonparametric van der Waerden criterion for independent groups.[11]

Results

Classification of Morphologic Forms in a Cell Population of the Intima

Figure 2 shows (A) a general view of the intimal cell population on an *en face* preparation; (B) a micrograph of a small piece of intima at the moment when most of the extracellular matrix was dissociated in the alcohol-alkali mixture, and the cells resembling in shape those on the *en face* preparation have not yet lost contact with each other; and (C and D) a general view of a suspension of dissociated cells. A variety of cell forms, ie, polymorphism of the cell population, is found both in the initial and alcohol-alkali-treated tissue as well as in the suspension of dissociated intimal cells. It should be noted, however, that polymorphism is characteristic of the intimal cell population only, while the population of medial cells is, on the contrary, homogeneous morphologically and composed of thick bipolar cells of elongated shape with an elongated nucleus oriented along the cell body (Figure 2D).

In the suspension of intimal cells, one can find small islets of the endothelium, which are the fragments of vascular endothelial lining (Figures 3A and B), and a certain number of cells of round or ovoid shape, which can be easily identified as monocytes or lymphocytelike cells accumulated under the endothelium and easily distinguishable on histologic preparations of aorta.[5] The bulk of the population of dissociated intimal cells is made by cells with processes of stellate and elongated shape, elongated cells without side processes, and cells of irregular shape, ie, the same morphologic forms which were described in

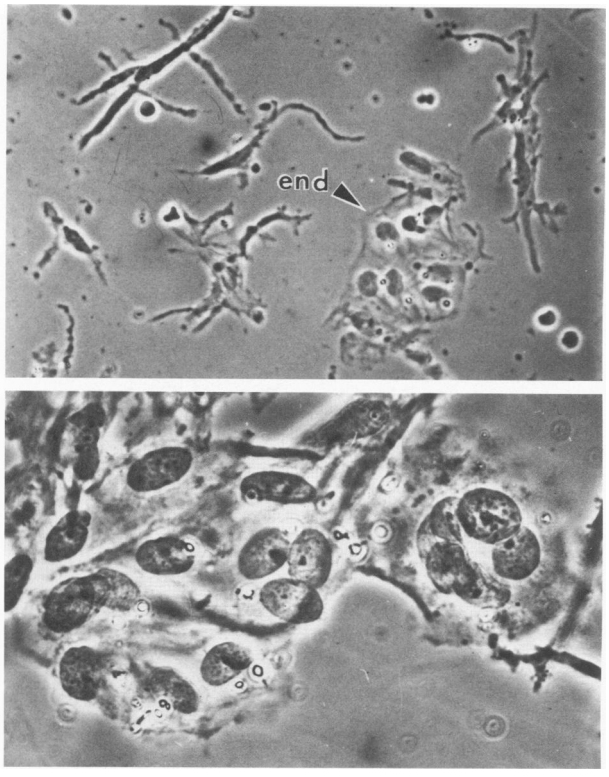

Figure 3—Endothelium in the suspension of intimal cells obtained by alcoholic-alkaline dissociation of the prefixed human aorta. Phase-contrast. **A**—A small region of the endothelial lining (*arrow*). (×160) **B**—A segment of endothelial lining at a higher magnification. (×560)

the study of the *in situ* aortic cellular composition.[5] For the convenience of aortic cell polymorphism description, we have arbitrarily divided the morphologic forms found *in situ* (on *en face* preparations) and their counterparts in the suspension of dissociated cells into four major classes: stellate, elongated with side processes, and flat cells of irregular shape (Figure 4).

Stellate cells have characteristic radial processes (20–100 μ in length), whereof the number varies from 3 to 12 (Figures 4A and B). In most cases the processes exhibit dichotomy. The body of a stellate cell is 10–30 μ in diameter. The nucleus in such a cell is located in the center and has no definite orientation.

Elongated cells without side processes have an elongated body (100–220 μ in length) ending with one or two processes, which are sometimes branching. The rod-shaped nucleus is oriented along the long axis of the cell (Figures 4C and D).

Cells with the same body as the elongated cell but having a varying number of side processes (Figures 4E and F) can be found as well in the intimal population. Their side processes belong to the same type as the processes of stellate cells and end processes of elongated cells.

Flat, irregularly shaped cells (30–70 μ in diameter) have no definite shape. Their nucleus is flattened and has an oval or beanlike shape (Figures 4G and H).

The morphologic forms of cells isolated from the intimal layer of atherosclerotic lesion were the same as in uninvolved intima. In addition, a small number of ovoid cells were detected in the suspension of cells isolated from the lesion zone. Their cytoplasm had a foamy structure. These cells are probably the so-called foam cells of monocytic origin.[5] The stellate cells isolated from the atherosclerotic lesion were distinguished from their counterparts isolated from the unaffected intima by large size and the presence of vacuoles either localized in the processes or occupying the cytoplasm. These vacuoles probably remain in the place of lipid inclusions that are extracted from the cell during alcohol-alkali treatment. Vacuoles of different sizes were also found in other forms of cells isolated from atherosclerotic lesions.

Quantification of Cellular Composition of the Intima

We have examined more than 50 loci taken from 9 aortas, including both atherosclerotic and macroscopically unaffected ones (see Table 1). In most cases we managed to isolate from a locus the four main morphologic forms: stellate, elongated, elongated with side processes and flat cells of irregular shape. As a rule, the number of endothelial cells, lymphocytelike cells, and monocytes did not exceed 5% of the total number of cells in the population. Since more than 95% of the population of any locus taken from a lesion or nonlesion zone was composed of the four above-described morphologic forms, we naturally concentrated our attention on these cells and determined their proportion in each locus.

The relative number of cells of each form in a population of intimal cells is given in Figure 5. One can see that the unaffected and atherosclerotic intimas considerably differ as to the proportion of stellate and elongated cells. Stellate cells are really scarce in the unaffected intima (Figure 5A). We failed to detect them at all in 7 of 15 loci. In other loci their share varied from 1% to 3%.

On the other hand, stellate cells were found in each of 21 loci of the intima with fatty infiltration, and their quota accounted for 11% of the population, which significantly exceeds the normal value.

On the average, the share of stellate cells in the intimal layer of the fatty streak and atherosclerotic plaque exceeded the normal value by 20 times.

Statistically significant differences in the proportion of stellate cells were registered only between the normal intima and all types of lesions, while the

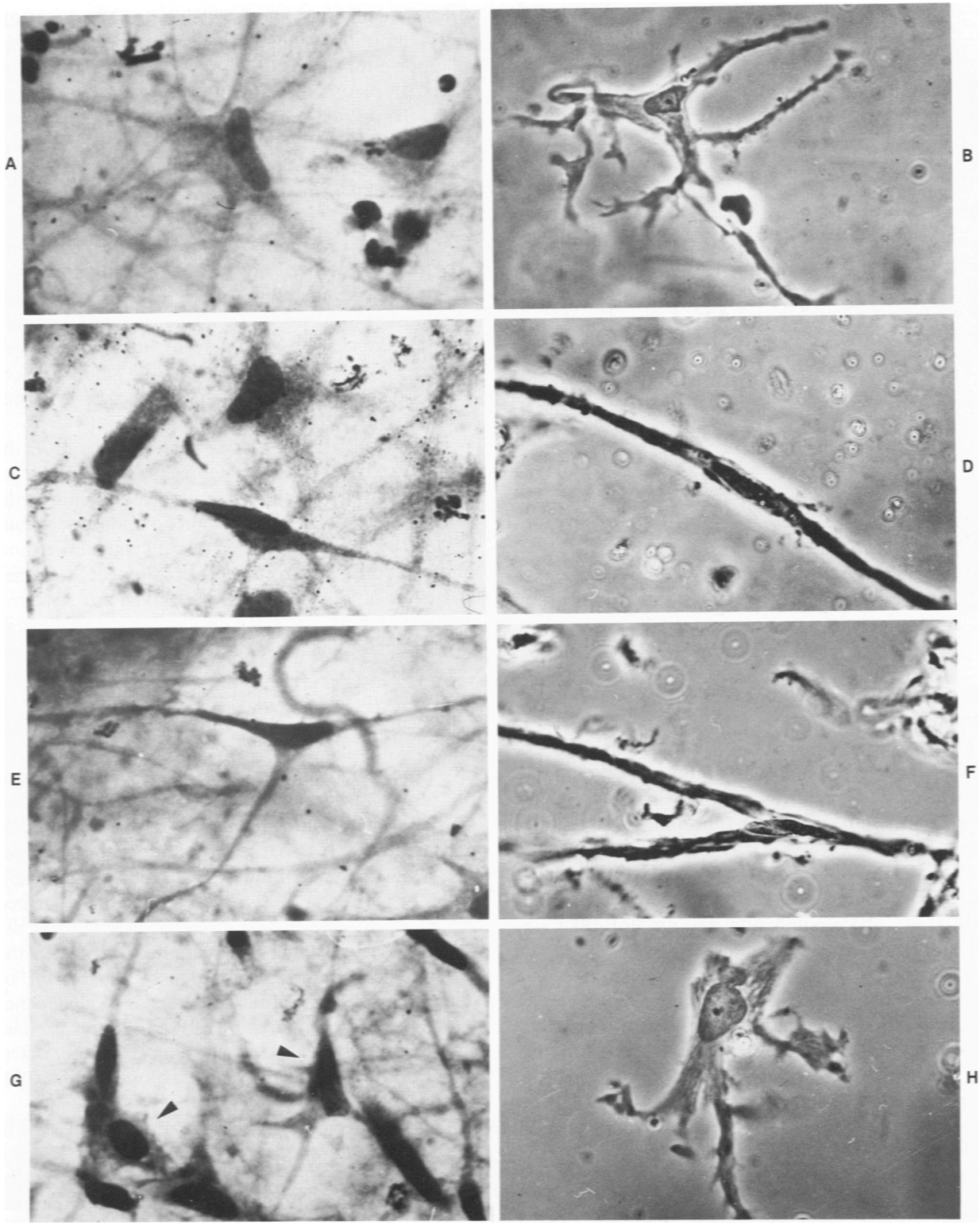

Figure 4—Certain morphologic forms of the cellular population of human aortic intima. *Left*, an *en face* preparation. *Right*, a suspension obtained by alcoholic-alkaline dissociation of the prefixed intima. **A** and **B**—Stellate cells. **C** and **D**—Elongated cells. **E** and **F**—Elongated cells with side processes. **G** and **H**—Cells of irregular shape (*arrow*, in **G**). (**A, C, E, G**, hematoxylin, × 550; **B, D, F, H**, phase-contrast, × 560)

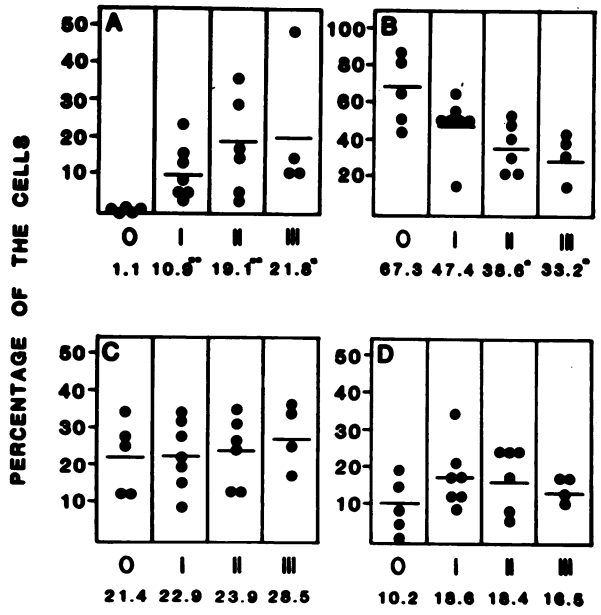

Figure 5—The proportion of main cell forms in uninvolved and atherosclerotic intima of human aorta. Three loci were excised from the analyzed type of lesion in each aorta. A dot in the figure corresponds to the mean value of the cells share in three loci. **A**—Stellate cells. **B**—Elongated cells. **C**—Flat cells of irregular shape. **D**—Elongated cells with side processes. 0, I, II, III, same as in Table 1. The mean value is given under the symbol of lesion type. $*P < 0.05$; $**P < 0.01$.

differences between various types of lesions were nonsignificant.

The share of elongated cells in the lesion zone was smaller than in the unaffected intima. In the plaque elongated cells accounted for 33%, which is two times lower, as compared with the uninvolved intima (Figure 5B).

The content of irregularly shaped and elongated cells with side processes made up approximately one-fifth of the whole population and did not differ significantly from the normal value.

Thus, the difference in cellular composition of the uninvolved and atherosclerotic intima is determined by an alteration in the proportion of the two morphologic forms of cells—the stellate and the elongated. As atherosclerotic lesion becomes pronounced, the share of stellate cells grows, and the share of elongated cells decreases. So the ratios of elongated to stellate cells in the uninvolved intima, fatty infiltration zone, fatty streak, and plaque were 61.2:1; 4.4:1; 2.0:1; 1.5:1, respectively.

In the vessel with both unaffected and atherosclerotic zones, the differences in cellular composition of these zones are rather substantial, which can be seen in Table 2. This table represents the data obtained by the study of aortas 4 and 7 (their characteristics are given in Table 1). These aortas, along with uninvolved areas, had all types of atherosclerotic lesions, namely, fatty infiltration, fatty streaks, and atherosclerotic plaques. From 5 to 8 loci were excised from the lesions of every type and unaffected areas. In every locus the cellular content of the intima was then determined. It turned out that the loci belonging to one lesion type and even the loci of unaffected zones considerably differed as to the proportion of main cell forms, which indicates heterogeneity of the intima (Table 2). On the average, however, the trends discovered while we were comparing the cellular composition of several vessels (see Figure 5) are retained within one aorta containing both normal and affected regions: the share of stellate cells in the lesion zone exceeded the normal value, while the share of elongated cells was, on the contrary, smaller than normal (Table 2).

To find out the reasons for the differences in the ratio of stellate and elongated cells in unaffected intima and atherosclerotic lesions, we determined the total number of cells in the corresponding segments of aorta. The total number of cells and those morphologic forms which could be undoubtedly classified as stellate or elongated cells were taken into account. The cell number was calculated per an area unit (1 sq cm) of unaffected or atherosclerotic intima, respectively. Compared with unaffected intima, there are more cells in an atherosclerotic lesion, the number of cells being increased with the degree of lesion (Figure 6).

With a rise in the total number of cells, the quantity of stellate and elongated cells grows, the number of the former, however, being increased to a considerably greater extent (Figure 6). While the total number of cells increases by 2 times that of the stellate cells grows by more than 10 times, the rise in the elongated cell number being only 2-fold. There is a linear dependence between the total number of cells in the intima and that of elongated cells (coefficient of determination for linear fit, 0.97); and dependence between the total number of cells and that of stellate cells perfectly fits the power curve (coefficient of determination for power fit, 0.88; for exponential fit, 0.84; for linear fit, 0.73).

Thus, the decrease in the share of elongated cells in the cell population of atherosclerotic intima is not a consequence of a fall in the number of these cells. Alteration of the ratio of different morphologic forms occurs because of the fact that the number of cells belonging to different morphologic forms increases disproportionately in the lesion zone. Specifically, the number of stellate cells, compared with elongated cells, is increased much more substantially.

Table 2—Proportion of Main Morphologic Forms of Cells in the Intima of Human Aorta

	Normal				Fatty infiltration				Fatty streak				Plaque			
Aorta	S	E	EP	F	S	E	EP	F	S	E	EP	F	S	E	EP	F
	20	57	15	8	11	57	12	20	4	46	12	38	52	8	3	37
	3	75	7	15	2	69	8	21	36	6	3	55	41	13	5	41
	1	86	5	8	14	42	21	23	36	20	21	23	18	7	2	73
4	2	77	2	19	33	20	22	25	16	30	24	30	37	12	5	46
	3	70	9	18	5	76	5	14	13	20	25	42	44	21	15	20
	24	5	68	3	58	25	14	3	10	35	27	28	46	23	15	16
	0	44	11	45	16	62	13	9					45	24	29	2
Mean	7.6	59.1	16.7	16.6	19.9	50.1	13.6	16.4	19.1	26.2	18.7	36.0	40.4	15.4	10.6	33.6
SEM	3.8	10.4	8.7	5.2	7.4	8.2	2.4	3.1	5.6	5.7	3.8	4.7	4.1	2.7	3.7	8.8
P (versus normal)					NS	NS	NS	NS	NS	NS	NS	<0.05	<0.01	<0.05	NS	NS
	2	67	11	20	22	20	24	34	1	87	3	9	25	27	18	30
	1	97	1	1	1	96	1	2	5	56	13	26	31	21	15	33
	7	45	25	23	2	75	9	14	2	71	8	19	15	46	13	26
	0	63	12	25	4	84	10	2	18	10	48	24	10	32	19	39
7	0	85	2	13	13	72	11	4	12	5	55	28	19	35	26	20
					35	7	48	10	6	32	32	30	6	47	12	35
					35	8	48	9					1	36	3	60
													1	59	13	27
Mean	2.0	71.4	10.2	16.4	16.0	51.7	21.6	10.7	7.3	43.5	26.5	22.7	13.5	37.9	14.9	33.8
SEM	1.3	9.0	4.3	4.4	5.6	14.5	7.3	4.2	2.7	13.6	8.9	3.1	3.9	4.3	2.3	4.3
P (versus normal)					<0.05	NS	NS	NS	NS	NS	NS	NS	<0.05	<0.01	NS	<0.01

S, stellate; E, elongated; EP, elongated with side processes; F, flat cells of irregular shape; NS, not significant.

Discussion

According to the electron microscopic data of Geer,[4] the cells which have an elongated shape in human aortic intima and are located near the media have ultrastructural signs of typical smooth muscle cells, namely, a large number of cytoplasmic myofilaments, numerous pinocytotic vesicles along the plasma membrane, and a limiting basement membrane. This type of cells prevails in uninvolved intima. Near the endothelium Geer found and described the cells of stellate and irregular shape, most of which differed from typical smooth muscle cells. They have a developed granular endoplasmic reticulum and a varying number of filaments, which, in some cells, cannot be distinguished from myofilaments. A lot of pinocytotic vesicles along the plasma membrane were found in stellate cells; some cells had a partial limiting basement membrane. Preliminary results of the study presently being carried out and aiming at examination of the ultrastructure of individual morphologic forms described in this report fully agree with the data of Geer (Krushinsky et al., to be published).

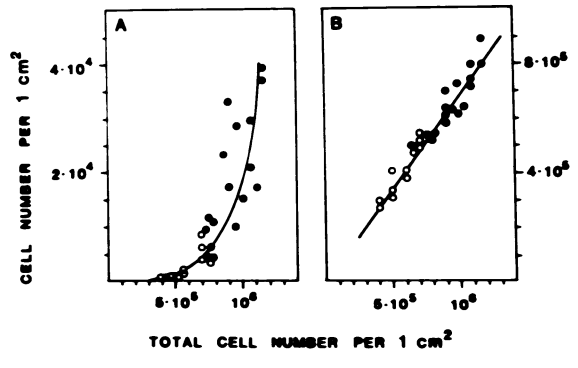

Figure 6—Dependence between the total number of cells in the intima and that of stellate (A) and elongated (B) cells. 1 × 1-cm loci were excised from an unaffected intima (○), fatty streaks (◑), and atherosclerotic plaques (●) of a formalin-fixed aorta. The tissue was dispersed in the alcohol-alkali mixture, and the total number of cells as well as stellate and elongated cells were determined in the obtained suspension by counting in a hemocytometer.

The characteristic shape of the elongated and stellate cells alkali-isolated from the prefixed aorta as well as their distribution in the intima[7] make it possible for us to identify these cells with the corresponding types described by Geer.

The ultrastructure of elongated cells allows us to identify them easily as smooth muscle cells, while the morphologic signs of stellate cells admit, according to Geer, of two possibilities. First, these cells may belong to another cellular type of intima differentiating into smooth muscle; second, stellate cells may be modified smooth muscle cells.

One of the main conclusions that can be drawn from the analysis of the cellular composition of an atherosclerotic and unaffected intima is that the morphologic features of cells are the same in both cases. It is their proportion that changes, without leading to the predominance of one cell type.

Proceeding from the two assumptions concerning the origin of stellate cells, a sharp increase in the

proportion and number of cells of this morphologic form in atherosclerotic intima may be the consequence of two processes. First, it may be due to transition of elongated cells to the stellate with modification accompanying this transshaping. Second, it may result from selective proliferation of stellate cells in the atherosclerotic lesion zone.

Irrespective of whether stellate cells are modified smooth muscle cells or representatives of some other cell type, the increase of their proportion and absolute number is a very noticeable alteration in the cellular composition of atherosclerotic intima. It is impossible to realize the significance of this change for the physiology of atherosclerotic vessel wall without knowing the characteristics of the stellate cell metabolism. Recently we have obtained a primary culture from an uninvolved and atherosclerotic human aorta and studied certain properties of cells in this culture.[12-14] Since the polymorphism discovered in aorta *in situ* is retained in primary culture,[6] we hope that this system will make it possible for us to investigate the physiologic characteristics of stellate cells.

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