The Clonal Characteristics of Human Aortic Intima

Comparison With Fatty Streaks and Normal Media

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The clonal characteristics of normal-appearing but thickened aortic intima were studied by the use of the isoenzymes of glucose-6-phosphate dehydrogenase (G-6-PD) as cellular markers in females heterozygous for this X-linked enzyme. Isoenzyme patterns of 133 samples of intima were compared with those of 237 samples of underlying media and with those of 58 fatty streaks dissected from the same aortas. The proportion of samples of intima and fatty streaks with monoclonal or intermediate characteristics was the same, but both had more monoclonal or intermediate samples than underlying media ($P < 0.05$). However, samples of intima showed a central clustering tendency of isoenzyme values similar to that of underlying media, while values from fatty streaks showed a bimodal distribution suggesting the presence of cell populations in the process of becoming monoclonal. The data suggest that clonal proliferation may begin in normal-appearing intima but that it progresses through a fatty streak stage before proceeding to the monoclonal fibrous plaque. (Am J Pathol 1983, 113:33–40)

CONSIDERABLE EVIDENCE exists for the proliferation of smooth muscle cells as the principal pathogenetic mechanism in the production of atherosclerotic lesions in man.[1-3] Further information on this cellular proliferation has been obtained with the use of the isoenzymes of glucose-6-phosphate dehydrogenase (G-6-PD) as cellular markers, a technique that had previously been used in defining the clonal characteristics of leiomyomas and other human neoplasms.[4,5] The technique depends on the following: that G-6-PD is an X-linked enzyme; that in American black females there is a high prevalence of heterozygosity for the two electrophoretically separable isoenyzmes of G-6-PD which are designated A and B; and that according to the Lyon hypothesis,[6] either the A or the B isoenzymes but not both, should be demonstrated in tissues consisting of a single clone of cells. With the use of this technique, most atherosclerotic fibrous plaques in black females heterozygous for G-6-PD have been shown to contain either the A or the B isoenzyme, allowing them to be defined as monoclonal.[7-9]

Claims have been made that the fatty streak is the precursor of the fibrous plaque, but the evidence is mainly circumstantial.[10-15] Other investigators have disputed this claim and have maintained that the fatty streak is not involved in the atherogenic process.[16-18] We used G-6-PD isoenzyme patterns[8,9] to study this question and found that although most fatty streaks resembled normal arterial wall in their isoenzyme values, a significant minority showed clonal characteristics intermediate between those of normal wall and those of fibrous plaques. Furthermore, we provided evidence that two populations of fatty streaks exist: one develops these intermediate clonal characteristics before 65 years of age, and the other never develops such clonal characteristics even in the very old.[19]

Despite the studies of the clonal characteristics of arterial lesions such as fibrous plaques and fatty streaks, no investigations have been performed on grossly normal-appearing intima. It is important to study the latter because clonal proliferation may occur within the intima even before a recognizable lesion is formed. Theoretically, this clonal prolifera-

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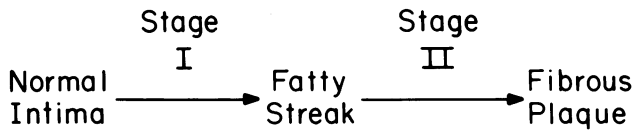

Figure 1—Two potential sites of clonal proliferation during the formation of fatty streaks and fibrous plaques.

tion could occur at one or the other of two possible stages of fibrous plaque development (Figure 1). At Stage I, clonal proliferation might occur prior to the formation of the fatty streak. This suggests that the fatty streak is the result of this clonal proliferation. The fatty streak may or may not be an essential intermediary for the development of atherosclerosis. At Stage II, clonal proliferation may occur following the formation of the fatty streak. This suggests that the fatty streak is the site at which various stimuli cause clonal proliferation to occur, with the resultant formation of the fibrous plaque.

The present study was designed to determine whether clonal proliferation occurs at Stage I, Stage II, or both. The clonal characteristics of thickened but normal-appearing intima and of fatty streaks were determined from aortas of black women heterozygous for G-6-PD. This report describes 1) the feasibility of dissecting normal-appearing intima free from underlying media so that its G-6-PD isoenzymes can be determined; 2) the clonal characteristics of the normal-appearing intima; 3) the clonal characteristics of fatty streaks dissected from the same aortas; 4) a comparison of the G-6-PD isoenzyme patterns in the normal-appearing intima, fatty streaks, and underlying media. These data provide insight into which of the stages in Figure 1 best describes the role of clonal proliferation in the production of human atherosclerotic lesions.

Materials and Methods

Aortas from black females were collected from the Autopsy Service of The Johns Hopkins Hospital and the Office of the Medical Examiner of the State of Maryland. The tissue was collected as soon as possible after death, wiped clean of adhering blood, placed intimal side up on a piece of cardboard, sealed in a plastic bag, and stored at −70 C. We stored a small piece of full-thickness aortic wall separately to screen for G-6-PD heterozygosity.

Those aortas found to be heterozygous for G-6-PD isoenzymes were thawed and kept cool on a bed of ice in preparation for dissection. Before dissection was performed, a clear plastic sheet was laid over the aorta, and outlines of the aorta and lesions to be dissected were traced onto the sheet. The various tissues were then identified for dissection. For purposes of this study, a *fatty streak* was considered to be a flat, or very slightly raised, yellow-colored lesion which contained lipid visible either grossly or under the dissecting microscope. Isolated lesions only were assayed. *Intima* was the layer of tissue which could be separated from the underlying media and which contained neither lipid nor focal fibrous thickening under the dissecting microscope. *Media* was the tissue which was situated immediately below the fatty streak or intima.

Dissection of intima and fatty streaks was carried out with the naked eye and then under a dissecting microscope (15–25 power) with the use of a disposable No. 11 scalpel blade. To obtain specimens of intima, 5 × 5-mm blocks that were free of obvious lesions were dissected by isolating both the media and its intima from the rest of the aorta. The intima and media were separated by dissecting up a corner of the intima, grabbing the free edge with needle-tipped forceps, and pulling gently to strip the intima off the underlying media. Remaining media was then dissected from the isolated intima. Eight or more samples were taken from a number of areas on the aorta. For fatty streaks, the lesion and its underlying media were isolated from surrounding tissue, and the fatty streak was stripped from the underlying media. Excess layers of tissue were dissected from the medial aspect of the isolated fatty streaks. All isolated fatty streaks that could be dissected from each aorta were included in the study.

The portions of intima and fatty streaks were then bisected. One half was fixed for histologic examination in 10% buffered formalin (pH 7.0). Four-micron sections were cut and stained with hematoxylin and eosin after embedding the tissue in paraffin. The sections were examined by light microscopy; evidence of contamination by the underlying media resulted in exclusion of that portion from the study.

The other half of the intima or fatty streak was then assayed for G-6-PD isoenzyme activity with the use of cellulose acetate electrophoresis. The materials and methods used in the procedure have been presented in detail previously.[9] Twelve samples were applied to each cellulose acetate membrane. Each sample of intima or fatty streak was alternated on the membrane with the sample of media dissected from the area immediately beneath it. After specifically staining for G-6-PD activity,[9] the separated bands were developed to a proper intensity. The relative amount of enzyme activity in each isoenzyme band was determined at 520 nm with the use of a high-reso-

lution densitometer (Helena Laboratories), with integration of the area under the densitometric curve. The result of each electrophoresis was expressed as the percentage of total isoenzyme activity found in the B isoenzyme band (% B isoenzyme).

Isoenzyme patterns were defined as follows.[9] Samples were defined as monoclonal if the following two criteria were met: first, that the isoenzyme values fell outside the ± 3 SD (99.7%) confidence limits of the samples of normal media from the same aorta; second, that the isoenzyme values fell *within* 2 SD confidence limits for samples of a known monoclonal lesion, the uterine leiomyoma (0–25.3% B isoenzyme for monoclonal A lesions, 89.2–100% B isoenzyme for monoclonal B lesions). The lesions were regarded as having clonal characteristics intermediate between monoclonal and polyclonal if only the first of these criteria was met.

We used the chi-square test[20] to determine whether the proportion of samples with monoclonal or intermediate characteristics was different from one type of tissue to another. We performed analysis of variance using one-way classification of samples of unequal size to determine whether the variance of isoenzyme values between types of tissue (media, intima, fatty streak) was greater than that within each type of tissue.[20] The relationship between the samples of intima or fatty streak and the media underlying them was studied by plotting the % B isoenzyme of the intima or fatty streaks against the isoenzyme value of the underlying sample of media. Correlation coefficients and the slopes of the resulting linear relationships were calculated and compared.[20]

Results

Tissue was studied from 13 aortas from black females heterozygous for isoenzymes of G-6-PD (Table 1). The ages of the subjects in the study ranged from 11 to 62 years. Most subjects were relatively young, and none of them was known to have died from atherosclerotic disease. At least 8 samples of normal-appearing intima were dissected from each case, with as many as 16 samples analyzed. The number of fatty streaks from each case ranged from 0 to 15 lesions. The number of fatty streaks available for analysis increased with age, with few fatty streaks present in women in their third decade, but with increasing numbers available in women in their fifth and sixth decades.

The ability to dissect aortic intima free of all underlying media was demonstrated by examination of the histologic sections. Of the 145 samples of intima initially studied, only 12 (8.3%) were found to be contaminated by underlying media. The usual histologic appearance of the samples of intima studied is seen in Figure 2. The intima is thickened and contains moderate numbers of cells of undetermined origin. No foam cells were seen, nor were there large collections of smooth muscle cells. An elastic membrane can be vaguely discerned at the bottom; this forms the boundary of the underlying media, none of which is present in the specimen.

The isoenzyme values for samples of media clus-

Table 1—Age and Number of Samples of Media, Intima, and Fatty Streaks Studied From 13 Black Females Heterozygous for G-6-PD Isoenzymes

Case	Age (yr)	Number of samples studied		
		Media	Intima	Fatty streak
1	11	11	3	0
2	21	12	6	0
3	24	16	15	0
4	25	12	12	0
5	25	24	8	4
6	40	16	12	3
7	41	16	11	4
8	46	16	8	6
9	49	8	16	5
10	53	25	12	8
11	54	20	8	4
12	59	25	12	9
13	62	36	10	15
Total		237	133	58

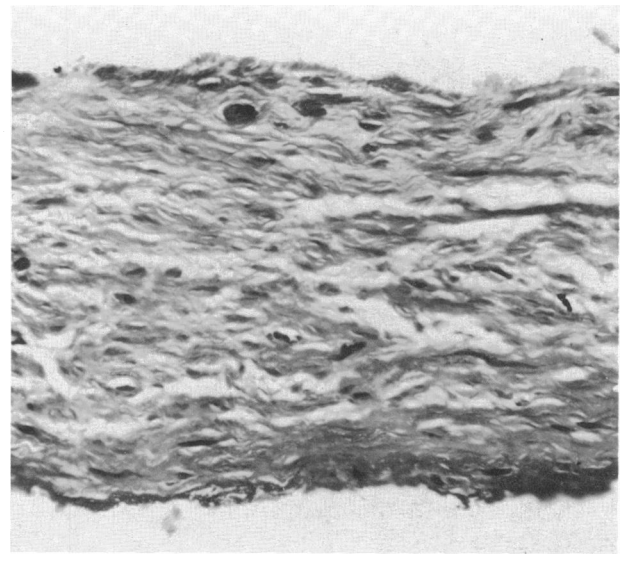

Figure 2—High-power view of thickened intima dissected free from underlying media. Occasional cells of undetermined type are seen. At the bottom an elastic membrane can just be discerned, but no underlying media is present. The aorta from which this sample was obtained had been frozen and thawed. This accounts for the relatively poor quality of the picture. (H&E, ×550) (With a photographic reduction of 14%)

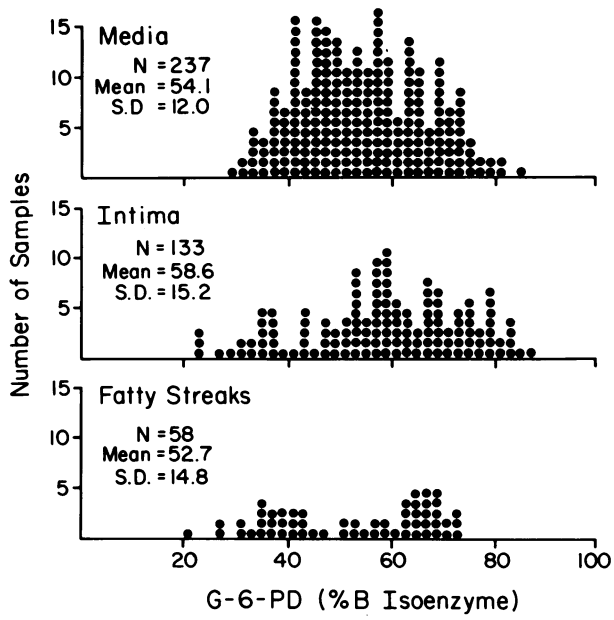

Figure 3—Isoenzyme values for samples of media, thickened intima, and fatty streaks. The number of samples, mean isoenzyme values, and standard deviation for samples of each type are included.

tered around a central value of 54% B isoenzyme, with a distribution like that of a bell-shaped curve (Figure 3). Isoenzyme values for intima also showed a central clustering tendency and a mean value of 59% B isoenzyme. However, the distribution of samples of intima appeared to have greater variation than did samples of media, with a larger standard deviation (15.2% B isoenzyme versus 12.0% B isoenzyme) and a wider range of isoenzyme values (23% B to 87% B isoenzyme versus 29% B to 85% B isoenzyme). The distribution of isoenzyme values of fatty streaks (Figure 3) was markedly different from that of the intima or that of the media. Although the mean value of samples of fatty streaks (53% B isoenzyme) was similar to the means of the other two tissues, there was no central clustering tendency. Rather, a bimodal distribution was observed, with values clustering around 35% and 65% B isoenzyme.

No samples of media showed monoclonal or intermediate clonal characteristics (Table 2). Despite the difference in the distribution of isoenzyme values between intima and fatty streaks, the percentages of samples with monoclonal or intermediate clonal characteristics were similar. Only 2 of 133 samples of intima (1.5%) and 1 of 58 samples of fatty streaks (1.7%) met the criteria for monoclonality. The percentages of intima or fatty streaks having intermediate characteristics were also similar (9.0% samples of intima, 5.2% of fatty streaks). The proportions of samples of intima or fatty streaks which had either intermediate or monoclonal characteristics were both significantly different from the proportion of samples of media with these clonal characteristics ($P < 0.001$) but were not different from each other.

We performed analysis of variance to determine whether significant differences existed between mean % B G-6-PD isoenzyme values for each of the three

Table 2—Number and Percentage of Samples With Monoclonal and Intermediate Clonal Characteristics for Samples of Media, Intima, and Fatty Streaks From 13 Black Females Heterozygous for G-6-PD

	Number of samples with monoclonal (MC) or intermediate (Int) clonal characteristics					
	Media		Intima		Fatty streak	
Case	MC	Int	MC	Int	MC	Int
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	3	0	0
4	0	0	0	0	0	0
5	0	0	0	1	0	0
6	0	0	0	0	0	0
7	0	0	0	1	0	0
8	0	0	1	0	1	0
9	0	0	0	1	0	0
10	0	0	0	0	0	0
11	0	0	0	3	0	3
12	0	0	0	3	0	0
13	0	0	1	0	0	0
Total	0	0	2	12	1	3
% of total	0	0	1.5*	9.0†	1.7‡	5.2†

* $0.10 > P > 0.05$ for difference in percentage with monoclonal characteristics between samples of intima and media.

† $P < 0.001$ for difference in percentage with intermediate characteristics between samples of either fatty streaks or intima and those of media.

‡ $P < 0.05$ for difference in percentage with monoclonal characteristics between samples of fatty streaks and media.

Table 3—Mean % B G-6PD Isoenzyme Values (± 1 SD) for Samples of Media, Intima, and Fatty Streaks From 13 Black Females Heterozygous for G-6-PD and Results of Analysis of Variance

Case	Mean % B G-6PD (± 1 SD)			F test	P*
	Media	Intima	Fatty streaks		
1	51.7 (7.3)	52.5 (5.9)	–	0.02	0.900
2	58.9 (5.1)	63.3 (3.8)	–	3.37	0.085
3	40.1 (5.2)	47.6 (9.3)	–	7.88	0.009
4	73.8 (3.9)	79.2 (3.6)	–	12.40	0.002
5	70.7 (5.3)	76.7 (6.7)	67.0 (7.4)	4.68	0.016
6	66.0 (6.5)	67.3 (8.6)	62.3 (3.8)	0.21	0.655
7	46.2 (6.3)	47.1 (11.1)	48.0 (5.0)	0.09	0.911
8	39.7 (5.4)	30.5 (5.9)	29.6 (6.1)	10.70	<0.001
9	56.5 (7.9)	63.1 (8.0)	62.2 (6.4)	1.99	0.157
10	56.2 (6.1)	57.6 (8.5)	61.5 (6.5)	1.85	0.170
11	44.0 (5.0)	57.2 (7.7)	62.8 (11.5)	19.30	<0.001
12	59.9 (4.4)	68.7 (4.6)	66.9 (3.5)	20.30	<0.001
13	46.1 (6.0)	41.5 (10.1)	38.4 (4.2)	8.15	<0.001

* Probability from the *F* test that the variance of values between types of tissues was equal to the variance of values within a type of tissue.

types of tissue (Table 3). In 8 of the 13 aortas, the variance of isoenzyme values between samples of the different types of tissue was significantly or marginally significantly greater ($P < 0.10$) than the variance within samples of each tissue. Thus, in Cases 2, 3, and 4, the mean % B G-6-PD in samples of media was significantly different from that of samples of intima (no fatty streaks were present in these cases). In Cases 5, 8, 11, 12, and 13, significant differences existed between the three types of tissues. This is illustrated further in Figure 4, showing the isoenzyme values for the three types of tissue from Case 13. The samples of media cluster in the central portion of the distribution, with samples of intima and fatty streaks having in general lower % B G-6-PD values. Thus, significant differences between intima, fatty streaks, and media often existed.

To better describe the relationship between either the fatty streak or the intima and the media lying beneath them, we plotted the % B isoenzyme values for each tissue sample and the sample of media directly underlying it. The isoenzyme values for the samples of intima closely resembled those of the underlying media (Figure 5). The points clustered around the line that represented equality between the two samples. Consequently, the slope of the regression line for these samples essentially represented unity (0.993), and a high correlation coefficient was observed ($r = 0.817$, $P < 0.0005$).

A similar analysis was performed for fatty streaks and the media underlying them (Figure 6). Points tended to cluster above the line representing unity when the isoenzyme value in media was greater than 54% B and below that line when the isoenzyme value in media was less than 54% B. Thus, the correlation coefficient was less for this analysis ($r = 0.667$) than

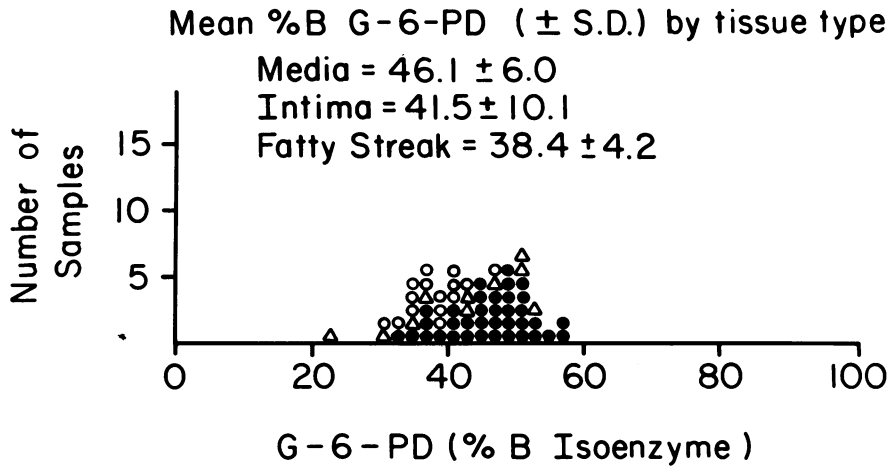

Figure 4—Isoenzyme values for samples of media (●), thickened intima (△), and fatty streaks (○) from Case 13. Samples of media cluster in the center of the distribution: samples of intima and fatty streaks have lower values, as indicated by the mean values of % B G-6-PD.

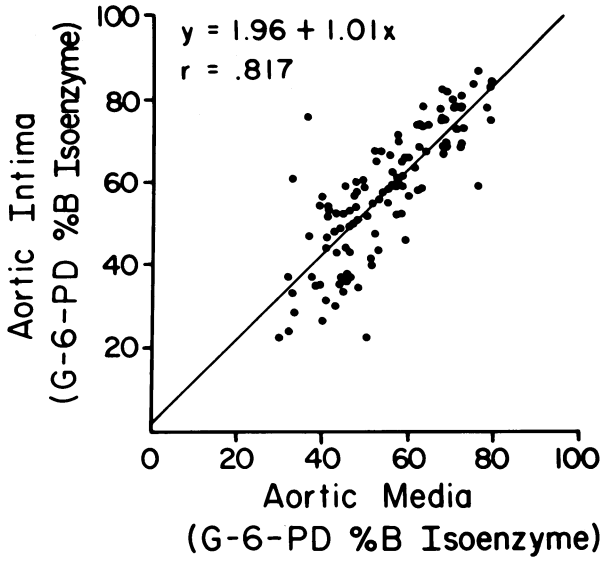

Figure 5—The correlation of % B isoenzyme values for samples of aortic intima with % B isoenzyme values for the samples of aortic media lying directly beneath the intimal sample. A strong correlation between the two types of tissue was observed ($r = 0.817$, $P < 0.0005$).

it was for the analysis of intima and media. However, the correlation coefficient was still significantly different from zero ($P < 0.005$).

Discussion

The role of intimal thickening in atherogenesis is poorly defined. The intima in the aorta of an infant consists usually of a single layer of endothelium lying directly upon the internal elastic lamina.[21] With aging, there is a uniform accumulation of smooth muscle cells and extracellular connective tissue matrix. These smooth muscle cells are thought to originate from the underlying media and may undergo proliferation in response to a variety of stimuli.[1,2] Movat et al[22] noted that this intimal thickening occurred in all individuals up to the age of 30 and that considerable variation in the degree of intimal thickening was observed in older persons. In older persons, the thickening is uniformly present in coronary arteries as well[23] and is considered to be part of the normal aging process. However, other authors have suggested that the process of intimal thickening is a precursor of atherogenesis. Wilens[24] noted that intimal thickening occurred only in the arteries which also develop atherosclerosis. He also noted that intimal thickening progressed with age, was greater in men than women, and was increased in the presence of hypertension. Intimal thickening has also been noted to be more pronounced in the distal than in the proximal part of the aorta, and is very similar in distribution to that of atherosclerosis.[22]

The sequence of events by which normal arterial intima progresses to become involved in the atherosclerotic process is difficult to assess in man, because the lesions can be studied only once, rather than serially. The use of clonal markers offers the opportunity to identify precursor lesions in which one or a few clones of cells are becoming predominant. Since the majority of fibrous plaques show monoclonal characteristics, this monoclonal proliferation can be regarded as the hallmark of the disease and may be used in identifying lesions that are becoming fibrous plaques.

Despite extensive studies of the clonal characteristics of fatty streaks and fibrous plaques,[7-9] no prior study of the clonal characteristics of the intima has been reported. For the main part, this lack has been due to the presumed difficulty in isolating intima in the absence of fatty streaks or fibrous plaques. This study demonstrates that normal-appearing aortic intima can be dissected free from underlying media so that its clonal characteristics can be studied. It must be emphasized that histologic examination discloses that the intima, although appearing normal on its endothelial aspect, is in fact thickened and consists of variable numbers of cells and increased connective tissue. Therefore, the findings of this study

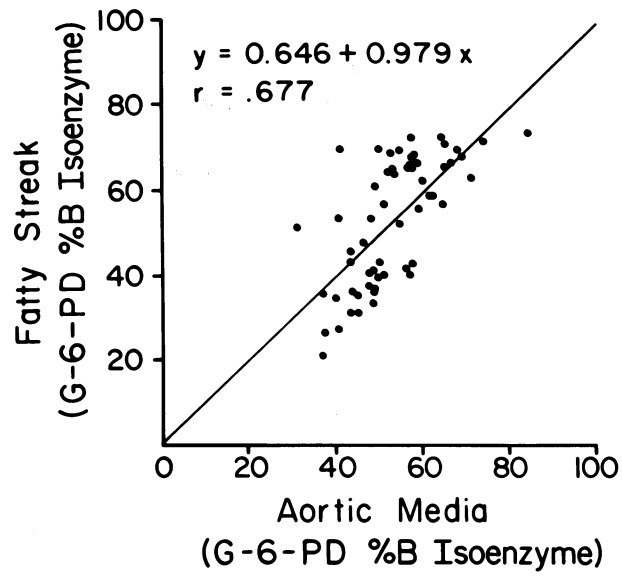

Figure 6—The correlation of % B isoenzyme values for samples of fatty streaks with % B isoenzyme values for the samples of aortic media lying directly beneath the fatty streaks. A significant correlation between the two types of tissues was observed ($r = 0.677$, $P < 0.005$).

refer to thickened but grossly normal-appearing intima.

The results of this study can be discussed in terms of the site of clonal proliferation in atherogenesis (Figure 1). Four possible alternatives are present. First, clonal proliferation may not occur at either stage. This seems unlikely, because the number of monoclonal or intermediate samples is significantly increased in both normal intima and fatty streaks, suggesting the presence of clonal proliferation in these tissues. Second, clonal proliferation may occur in Stage II but not in Stage I. Again, the data from this study suggest that monoclonal cellular populations are present within arterial intima prior to the formation of fatty streaks. Third, clonal proliferation may begin in Stage I but not in Stage II. If this were true, the isoenzyme characteristics of both normal intima and fatty streaks would be identical. Indeed, the proportions of normal intima and fatty streaks that have monoclonal or intermediate characteristics are similar. However, the distribution of isoenzyme values in normal intima and fatty streaks is quite different, with a central tendency of isoenzyme values in normal-appearing intima and a lack of such tendency in fatty streaks. The presence of a bimodal distribution of isoenzyme values in fatty streaks is interpreted as a progression of the process of clonal proliferation to a stage intermediate between those of the central clustering media and intima and those of the fibrous plaque, which tend to cluster toward the extremes of isoenzyme values.[7-9]

Thus, the findings appear most supportive of the fourth alternative, that clonal proliferation occurs at *both* Stage I and Stage II. This suggests that there is a continuum toward monoclonality, with monoclonal proliferation occurring even prior to fatty streak formation but occurring within fatty streaks to allow progression to form the monoclonal fibrous plaque.

These data must also be discussed in terms of our previous findings that two subpopulations of fatty streaks may actually exist.[19] One subpopulation appeared to be developing clonal characteristics intermediate between those of fibrous plaques and normal media, whereas the other had polyclonal characteristics similar to those of normal underlying media. The proportion of fatty streaks with intermediate or monoclonal characteristics appeared to decrease in women older than 65 years of age, suggesting that those remaining fatty streaks would never convert to fibrous plaques and that all the fatty streaks that were going to convert to fibrous plaques had already done so.

The findings of the present study are quite consistent with the notion that two subpopulations of intimal lesions may exist. Almost identical proportions of samples of intima and fatty streaks were identified as having monoclonal characteristics. The only difference between intima and fatty streaks was in the degree to which isoenzyme values deviated from a central clustering of values. These data may be interpreted as evidence that a subpopulation of intimal cells has been identified that is destined to undergo clonal proliferation. This clonal proliferation begins even within normal-appearing intima and becomes more apparent as the subpopulation evolves first into a fatty streak and later into a fibrous plaque. It would be important to identify the characteristics of these subpopulations in order to understand the factors important in the initiation and promotion of this clonal proliferation.

Finally, these data might be discussed with regard to the competing theories proposed to explain the presence of monoclonal characteristics within atherosclerotic plaques. Some authors have suggested that this clonal proliferation begins with a mutational event.[25] Others have suggested that recurrent cycles of cell proliferation and death select one or a few clones with the greatest proliferative advantage.[5,26] If monoclonal characteristics were to arise frequently in intima that was diffusely thickened, this would suggest that clonal selection has occurred within large areas of intima. The continued thickening of the intima during fibrous plaque formation would then allow further clonal selection to occur. This mechanism has been suggested by Thomas et al.,[26] who studied lesions of varying thicknesses and found that the thicker the lesion, the greater the likelihood that monoclonal characteristics would be observed. However, it was not clear whether the authors regarded the monoclonal thick lesions as fibrous plaques or the polyclonal thin lesions as intima or fatty streaks. Portions of intima and fatty streaks from the present study were of similar thickness, yet quantitatively had different isoenzyme patterns. Thus, it is difficult to equate a thick intimal lesion with one that is monoclonal.

The data from the present study appear to be evidence for clonal selection within samples of intima. The analysis of variance and comparison of mean isoenzyme values between intima and media suggest that significant differences exist between these two types of tissue. It is not clear whether these differences are due to a difference in type or whether there has been a selection of cells with one or the other X-chromosome during the process of intimal thicken-

ing. However, these differences were again too small (5–10% B isoenzyme) to account for the large differences observed between fibrous plaques and underlying media. These results can be compared with those observed in human scars. Both thickened intima and cutaneous scars are assumed to result from cellular proliferation related to mechanisms other than mutation. Thus, the finding of only a small proportion of samples of thickened intima with monoclonal characteristics with the majority of samples exhibiting central clustering of isoenzyme values is interpreted as consistent with a minor degree of clonal selection similar to that observed in human cutaneous scars.[27] In cutaneous scars, there is a significant difference in isoenzyme characteristics between normal skin and scar, suggesting that clonal selection has occurred. However, the average magnitude of that selection is small, less than 6% B isoenzyme. This is far too small to explain the large difference observed between fibrous plaques and underlying media. The magnitude of differences observed between intima and media was similarly small. However, it must be considered that the long period of further proliferation which may be required for the formation of a fibrous plaque could conceivably result in an intimal lesion with monoclonal characteristics.

In conclusion, this study demonstrated the feasibility of isolating intima suitable for the analysis of its clonal characteristics. A small minority of both intima and fatty streaks met established criteria for monoclonality. While the general distribution of isoenzyme values of intima resembled that of underlying media, a greater deviation of these values was present among fatty streaks, suggesting that they may frequently have contained cells undergoing clonal proliferation. These results provide a framework for more extensive mapping of the clonal characteristics of arterial intima and the lesions developing in it.

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