Clonal Markers in the Study of the Origin and Growth of Human Atherosclerotic Lesions

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SUMMARY The X-linked enzyme, glucose-6-phosphate dehydrogenase (G-6-PD) was used as a cellular marker to study the clonal characteristics of human atherosclerotic lesions from females heterozygous for G-6-PD isoenzymes. Portions of uninvolved aortic wall contained both isoenzyme types (A and B), and their isoenzyme patterns were used to establish criteria for polyclonal lesions. Portions of uterine leiomyomas contained predominantly one isoenzyme type (either all A or all B) and their isoenzyme patterns were used to establish criteria for monoclonal lesions. These techniques were used to address three questions concerning atherogenesis. First, evidence for the monoclonal origin of fibrous-capped plaques was provided by the findings that small plaques had G-6-PD isoenzyme distributions similar to those of leiomyomas; that in large plaques with multiple portions assayed for G-6-PD, a large proportion (25 of 26, 96%) of plaques had monoclonal characteristics; and that multiple monoclonal portions were present in the same plaque. Second, the role of the fatty streak as a precursor of fibrous plaques was supported by the demonstration that a proportion (11 of 66, 16.7%) of fatty streaks contained isoenzyme patterns intermediate between those of polyclonal uninvolved aortic wall and monoclonal leiomyomas. Increased cellularity of fatty streaks correlated with increased deviation of isoenzyme pattern toward monoclonality. Third, the assay of portions of both small and large plaques provided no evidence for clonal selection as plaques increase in size.

INCREASING evidence points to the proliferation of smooth muscle cells as the principal pathogenetic mechanism in the production of atherosclerotic lesions in man. The fibrous-capped plaque, the lesion characteristic of atherosclerosis, has been shown to consist of a monoclonal cell population. The enzyme, glucose-6-phosphate dehydrogenase (G-6-PD), has been used as a cell marker in these studies by virtue of its presence on the X-chromosome and the phenomenon of X-chromosome inactivation as proposed by Lyon. This phenomenon involves the random inactivation of one of the two X-chromosomes within cells during embryogenesis in the mammalian female. The inactivation is thought to be permanent, so that all descendant cells from a single cell will express the genes found on the same X-chromosome. G-6-PD can be used as a cellular marker in human beings because in the United States there exists a population of females (American blacks) with a high prevalence of heterozygosity for electrophoretically separable isoenzymes of G-6-PD. These two enzymes, designated A and B, have relative electrophoretic mobilities of 110 and 100, respectively. In the female heterozygous for these isoenzymes, normal tissue is thought to consist of a mosaic of minute patches of single clones of cells and, when assayed for G-6-PD, the tissue will express both isoenzyme types. In contrast, a monoclonal cell population will express only a single isoenzyme type. Using G-6-PD as a cellular marker, the monoclonal nature of a variety of human tumors, including uterine leiomyomas, has been established.

We have used the densitometric determination of the relative amount of each G-6-PD isoenzyme present to detect lesions with polyclonal and monoclonal characteristics. This has allowed the identification of lesions with clonal characteristics intermediate between monoclonal and polyclonal cell populations. The purpose of this study is to report the application of G-6-PD cell markers to the investigation of clonal characteristics of the cell populations involved in the origin and growth of atherosclerotic lesions. Several problems in the field of atherogenesis will be addressed: (1) The idea of a monoclonal origin of fibrous-capped plaques has been reexamined by the comparison of clonal characteristics of fibrous-capped plaques with those of uterine leiomyomas, lesions of known monoclonal origin. (2) Two different theories have been proposed to explain the monoclonal characteristic of the fibrous-capped plaque. The monoclonal characteristics within fibrous-capped plaques might arise following mutation and benign neoplastic growth, as proposed by Benditt, or might arise by the selection of a single clone of cells in response to chronic injury. The examination of the clonal characteristics of multiple samples from plaques of varying size makes feasible the study of possible clonal selection during the growth of plaques. (3) The fatty streak is thought to be a forerunner of the fibrous-capped plaque, yet there is little direct evidence for this supposition. If the fatty streak is a precursor of the fibrous-capped plaque, then the fatty streak might be expected either to consist of a monoclonal cell population...
or to have clonal characteristics intermediate between the monoclonal fibrous-capped plaque and the polyclonal uninvolved aortic wall.

**Methods**

**Collection and Storage of Tissue**

Aortas and leiomyomas of the uterus from black females were collected from the autopsy and surgical pathology services of The Johns Hopkins Hospital and the Office of the Medical Examiner for the State of Maryland. Institutional and Department of Health, Education and Welfare rules for protection of human subjects were observed in performing these studies. The tissue was collected as soon as possible after death or surgery, wiped clean of adhering blood, placed intimal side up on a piece of cardboard in the case of the aorta, frozen in liquid nitrogen, sealed in a plastic bag, and stored at −70°C. A small piece of uninvolved aortic wall or uterus was stored separately to screen for G-6-PD heterozygosity. Of approximately 250 black females whose tissues were assayed, 86 were determined to have both A and B isoenzymes of G-6-PD. The population of black females in this study ranged in age from 4 to 94 years and in only a small minority of cases was the cause of death atherosclerotic disease. We have no reason to believe that the basic atherosclerotic disease process in black females is any different from other race or sex groups.

**Identification and Dissection of Lesions**

After thawing the aorta and keeping it cool on a bed of ice, the atherosclerotic lesions were dissected off the aortic surface. Before dissection was performed, a clear plastic sheet was laid over the aorta and outlines of the aorta, various landmarks on the aorta such as ostia of major arteries, and the lesions to be dissected were traced onto the sheet. The surface area of the lesions was calculated later by comparison of the weights of the overlay outlining the lesion with the weight of an overlay of known surface area. The plaques and fatty streaks then were identified for dissection. For the purposes of this study, a lesion was classified as a fatty streak if it (1) was flat or very slightly raised, (2) was white or pearly in color, and (3) contained lipid visible either grossly or under the dissecting microscope. A lesion was considered to be a fibrous-capped plaque if it (1) was raised, (2) was white or pearly in color, and (3) contained a cap of dense connective tissue on the intimal side of the lesion which was visible grossly or under the dissecting microscope.

Dissection of intimal lesions was carried out first grossly and then under a 15-25 power dissecting microscope with a disposable no. 7 scalpel blade. For fatty streaks and small fibrous-capped plaques (< 50 mm² surface area), the lesion was isolated by removal of layers of tissue on the medial side of the lesion. For large fibrous-capped plaques (> 50 mm² surface area), multiple portions were obtained from each plaque and the locations of the portions were noted on the transparent overlay. Again, layers of tissue were dissected off the medial aspect to ensure removal of contaminating media. Each portion or lesion following dissection had the dimensions of a cube with sides of 2-3 mm. Similar sized portions of uterine leiomyomas were removed from an area of tumor containing no normal uterine tissue. Eight to 12 portions of uninvolved aortic wall containing both media and intima were taken from each aorta. In the cases in which normal uterine tissue was available, portions of normal uterine wall were handled in a similar fashion. The surface areas of these control samples were the same as those of the portions from lesions.

**Preparation and Examination of Histology**

The portions of intimal lesions were then bisected. Half was assayed for isoenzyme activity by cellulose acetate electrophoresis and the other half fixed for histology in 10% buffered formalin (pH 7.0). Four-micron sections were cut and stained with hematoxylin and eosin, aldehyde fuchsin, and elastica-van Gieson stains after the tissue was embedded in paraffin. The sections were then examined by light microscopy. Any evidence of contamination by underlying media resulted in the exclusion of that portion from the study. Contamination of the uterine leiomyomas by uninvolved uterus was ruled out by a similar procedure.

The histological sections of fatty streaks were reexamined to assess the morphological characteristics of the lesions semiquantitatively. All identifying information on the slides was concealed to avoid bias from prior knowledge of electrophoresis results. Two observers (T.A.P. and J.M.D.) then graded the fatty streaks on scales of 1 to 4 according to the extent of dense extracellular fibrous tissue in the fatty streak on the one hand and according to the degree of cellularity in the lesion on the other. In assessing the amount of fibrous tissue, the fatty streak was graded 1 (no fibrous tissue), 2 (less than 50% of the fatty streak was fibrous tissue), 3 (more than 50% consisted of fibrous tissue), or 4 (fatty streak consisted totally of fibrous tissue). In assessing cellularity, a fatty streak was graded 1 (contained few or no cells), 2 (consisted of intermediate numbers of cells and predominantly acellular tissue), 3 (contained numerous cells with acellular areas still predominating), or 4 (pronounced cellularity). The degree to which the isoenzyme pattern (percentage of total isoenzyme in the B band) of each fatty streak differed from the mean of the aorta from which it came was plotted for each graded group, to estimate trends within these lesions of deviation of isoenzyme values away from those of uninvolved wall and toward those of monoclonal lesions.

**Cellulose Acetate Electrophoresis**

The materials and methods used in the cellulose acetate electrophoresis have been presented in detail previously. A modified buffer consisting of 0.120 M Tris-0.046 M borate-0.001 M ethylenediaminetetraacetic acid (Sigma), pH 8.7, was used in this study.

Immediately after electrophoresis, the cellulose acetate plates were placed for 10 seconds in a solution containing 2 mg of nicotinamide adenine dinucleotide phosphate, 5 mg of glucose-6-phosphate, 1 mg of MTT tetrazolium, and 0.3 mg of phenazine methosulfate (Sigma) in 5 ml of...
0.1 M Tris buffer, pH 8.0, and developed in total darkness to proper intensity. Eight samples were applied to each cellulose acetate plate, with two sections of uninvolved aortic wall (AB isoenzyme) and one B blood control, prepared by the method of Sparkes et al.\(^13\) included on every plate. A typical cellulose acetate plate is seen in Figure 1.

The relative amount of enzyme activity in each isoenzyme band was determined by using a densitometer (Helena Laboratories) with integration of the area under the densitometric curve. The result of each electrophoresis was expressed as the percentage of total enzyme activity to be found in the B isoenzyme band (\% B isoenzyme).

**Statistical Analysis**

Analysis of variance using one-way classification of samples of unequal size was performed to determine the sources of variation in samples of uninvolved aortic wall.\(^14\)

Analysis of electrophoresis results provided two definitions of monoclonality. In Definition I, that used in previous publications,\(^5\)\(^-\)\(^7\) the mean percentage of total enzyme activity in the B isoenzyme band of the 8-12 portions of uninvolved aortic wall for each aorta was calculated. Around that mean were placed \(\pm 3\) standard deviation (SD) confidence limits. Those portions for which isoenzyme values fell within these confidence limits were considered polyclonal. Portions of intimal lesions for which the percentage of total isoenzyme in the B band fell outside these confidence limits were considered monoclonal by Definition I. These confidence limits are conservative in the definition of monoclonal lesions, since they should include virtually all (99.7\%) samples of uninvolved aortic wall, assuming a normal distribution of isoenzyme values within each aorta. In Definition II, lesions were considered monoclonal if they met the criteria for Definition I and if in addition the isoenzyme values fell within the 95\% confidence limits of values for monoclonal lesions. This definition utilizes the fact that leiomyomas generally are accepted as truly monoclonal.\(^10\)

Confidence limits for leiomyomas were calculated empirically by selection of limits below which 95\% of isoenzyme values for monoclonal A leiomyomas fell and above which 95\% of isoenzyme values for monoclonal B leiomyomas fell. The 95\% rather than the 99.7\% confidence limits were selected in order to be rigorous in the assignment of lesions to the monoclonal group.

Analysis of results in this manner allows the definition of three classes of clonal characteristics: polyclonal, monoclonal by Definition I but not Definition II, and monoclonal by Definition II. Those lesions determined to be monoclonal by Definition I but not Definition II have clonal characteristics significantly different from both uninvolved aortic wall and from known monoclonal lesions and were thus considered to have clonal characteristics intermediate between normal (polyclonal) and monoclonal tissue. A lesion was defined as monoclonal by Definition II by virtue of being both significantly different from normal aortic wall and similar to a known monoclonal lesion.

Trends within fatty streaks of deviation of isoenzyme values toward those of monoclonal lesions were analyzed by calculation of the difference of percentage of total isoenzyme in the B isoenzyme band between the fatty streak and the mean of the aorta from which it came. For each group graded according to its fibrous or cellular characteristics, the mean absolute value of the differences was calculated and \(\pm 95\%\) confidence limits were placed around these means, using Student’s \(t\)-test.\(^14\)

**Results**

**Isoenzyme Patterns in Uninvolved Aorta and Uterine Leiomyomas**

Plotting the percentage of total enzyme activity in the B isoenzyme band for each of the 751 individual portions assayed yields a bell-shaped distribution (Fig. 2A). These 751 portions were taken from 86 individual aortas. Within each aorta, the individual values of the 8-12 samples clustered around the mean for that aorta. The means of % B isoenzyme varied considerably from aorta to aorta (Fig. 2B) such that the analysis of variance showed the variation of mean values between aortas to be significantly greater than the variation of individual values within a single aorta (Table 1). For this reason, the mean and confidence limits for each individual aorta were used to define monoclonality (Definition I) in lesions taken from that aorta.

The mean and median for the distribution of values in Figure 2 was 55% B isoenzyme. This deviation from an expected 50\% may be due to the staining of the enzyme on the cellulose acetate plates at a pH (8.0) at which the B isoenzyme is more active than the A isoenzyme.\(^15\) Also, a second peak or shoulder on the curve was apparent around 70\% B isoenzyme (Fig. 2, A and B) and may be due to the presence in this population of individuals heterozygous for the B and A\(^+\) isoenzymes, the latter variant isoenzyme having lower activity than the A\(^+\) isoenzyme within the cell.\(^19\)
In marked contrast to the central clustering tendency observed in portions of uninvolved aortic wall, portions from uterine leiomyomas, which are monoclonal tumors, manifested distributions which clustered toward the extremes (Fig. 3). The majority of portions (37 of 71, 52.1%) exhibited either entirely A or entirely B isoenzyme. This was in contrast to portions of uninvolved uterine wall which exhibited both (AB) isoenzyme types in every instance. The % B isoenzyme value for all samples fell outside the 99.7% confidence limits derived from the aorta or normal uterus of the woman from which the tumor was taken. Portions from the same leiomyoma were always of the same isoenzyme type, although both isoenzyme types were noted in different leiomyomas from the same uterus. Confidence limits (± 95%) were placed above and below the theoretical means of 0 and 100% B isoenzyme for A and B monoclonal cell populations, respectively. The confidence limits were within 1% of each other whether calculated empirically or by assuming a normal distribution and using ± 2 sd. The confidence limits were 0 to 25.3% B isoenzyme for monoclonal A isoenzyme populations and 89.2 to 100% for monoclonal B isoenzyme populations. Deviation from the theoretical means is explained by the contamination of the leiomyomas by blood cells, blood vessels, or migratory cells such as macrophages. These confidence limits allowed estimation of the isoenzyme patterns which might have been expected from monoclonal lesions (Definition II), using our experimental methods.

**Monoclonal Characteristics of Small Fibrous-Capped Plaques**

The monoclonal characteristics of small, discrete, fibrous-capped plaques were examined by comparison with the confidence limits of the aorta from which they were dissected (Fig. 4A). The distribution of values clustering at the extremes was similar to those of leiomyomas. Of 36 plaques studied, 27 (75%) had isoenzyme values significantly different from uninvolved aortic wall by Definition I (Table 2). Of these 27 plaques, 22 fell within the confidence limits of values for monoclonal leiomyomas (< 25.3% or > 89.2% B isoenzyme). Thus 22 of 36 plaques (61%) had isoenzyme values significantly different from uninvolved aorta and within the confidence limits of monoclonal leiomyomas (Definition II). Only 5 of 27 (19.5%) had clonal characteristics intermediate between polyclonal and monoclonal lesions. Multiple monoclonal plaques were found on a single aorta in seven cases. In four of these seven aortas, monoclonal plaques of both A isoenzyme type and B isoenzyme type were present.

**Monoclonal and Histological Characteristics of Fatty Streaks**

Fatty streaks manifested a distribution greatly different from fibrous-capped plaques (Fig. 4B). The tendency of these lesions to cluster in the center was similar to the pattern in portions of uninvolved wall, i.e., polyclonal tissue. However, the dispersion of values was greater than that of uninvolved wall and 13 of 66 (19.7%) fatty streaks had % B isoenzymes values significantly different from uninvolved aortic wall (Definition I). However, only 2 of these 13 significantly different fatty streaks fell within the confidence limits of known monoclonal lesions (Def-
The histological characteristics of the fatty streaks were examined in the 62 of 66 fatty streaks available for study in order to determine differences in cellularity or amount of fibrous tissue in fatty streaks with different clonal patterns. The degree of deviation of the %B isoenzyme of the fatty streak from the mean of the uninvolved aortic wall was used as a measure of the monoclonal characteristic. \(\text{FIGURE 4 Distribution of G-6-PD isoenzymes in samples of small fibrous-capped plaques (A) and in samples of fatty streaks (B) from aortas of heterozygous black females. The determinations of percentage of total isoenzyme activity in the B isoenzyme band may fall either outside (\(\circ\)) or within (\(\odot\)) the 99.7% confidence limits of %B isoenzyme activity found in the uninvolved aortic wall from the aorta from which the plaque was dissected. Plaques falling outside these confidence limits (\(\bigcirc\)) are considered monoclonal by Definition I.}\)

Table 2 Summary of Monoclonal Characteristics of Lesions Studied

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Uterine leiomyomas</th>
<th>Fibrous-capped plaques</th>
<th>Fatty streaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal by definition I</td>
<td>71 (29A, 42B)</td>
<td>27 (9A, 18B)</td>
<td>13 (1A, 12B)</td>
</tr>
<tr>
<td>% of total</td>
<td>100%</td>
<td>75%</td>
<td>19.7%</td>
</tr>
<tr>
<td>Intermediate</td>
<td>71 (29A, 42B)</td>
<td>22 (9A, 13B)</td>
<td>2 (1A, 1B)</td>
</tr>
<tr>
<td>% of total</td>
<td>100%</td>
<td>61.1%</td>
<td>3.0%</td>
</tr>
</tbody>
</table>

* Monoclonal by definition I but not by definition II.

Table 3 shows the proportion of total portions which had monoclonal characteristics. The trend of increasing monoclonal characteristics was observed in fatty streaks with increasing cellularity (Fig. 5A). However, a trend of increasing monoclonal characteristics was observed in fatty streaks with increasing cellularity (Fig. 5B). Those groups of fatty streaks with greater numbers of cells (group 3 and 4) had significantly greater mean deviations from aortic isoenzyme values than those with fewer cells (groups 1 and 2). The mean of group 4 was greater than the means of both group 1 (\(t = 2.607, P < 0.05\)) and group 2 (\(t = 2.303, P < 0.05\)), and the mean of group 3 was greater than the mean of group 1 (\(t = 2.472, P < 0.05\)). The trend was consistent over all four groups of cellularity. Furthermore, a consistent trend in the proportion of fatty streaks with %B isoenzyme values significantly different from uninvolved wall (Definition I) was demonstrated within the four groups. In the four cellularity groups, the number and percentage of fatty streaks classified as monoclonal by Definition I were: group 1, 1 of 10 (10%); group 2, 3 of 22 (14%); group 3, 4 of 20 (20%); and group 4, 3 of 10 (30%).

Monoclonal Characteristics of Fibrous-Capped Plaques of Increasing Size

Plaques with larger surface areas (>50 mm²) were divided into multiple portions to allow replication of results, the estimation of size of monoclonal cell populations, and the description of patterns of clonal growth if heterogeneous clonal patterns existed. A total of 26 large fibrous-capped plaques were examined over a 10-fold range in surface area (50.5 to 505.7 mm² surface area, mean 150.7 mm²). The number of portions into which each plaque was divided ranged from 2 to 15, with a mean of 6.8 portions per plaque. Of a total of 177 portions examined, 118 (66.7%) had monoclonal characteristics by Definition I (Table 3).

At least one portion assayed was observed to be monoclonal by Definition I in 25 of the 26 plaques (96.2%). In many plaques, all portions assayed had monoclonal characteristics. Examples of such plaques are illustrated in Fig. 6A, which shows two plaques (numbers 6 and 11, Table 3) as they lie next to each other on the same aorta. Plaque 6 (on the left) is made up entirely of portions with monoclonal type A, and plaque 11 on the right consists of portions with monoclonal type B. Both plaques impinging upon ostia of intercostal arteries which are illustrated by the small open circles. The largest plaque made up entirely of monoclonal portions was that of number 24 with a surface area of 292 mm² and surface dimensions of approximately 1 x 3 cm (Fig. 6B). In plaques of increasingly large surface area, no significant trend was noted in the proportion of total portions which had monoclonal characteristics.

A number of plaques contained portions which were not monoclonal or which had monoclonal characteristics with both isoenzyme types. Monoclonal portions of the same isoenzyme type tended to lie next to each other forming clusters or foci of monoclonal portions and sug-
suggested that they came from a single monoclonal cell population. Such foci are illustrated in Figure 6C where the portions on the left consisted of a monoclonal A isoenzyme cell population in contrast to the monoclonal B isoenzyme cell population on the right. Six plaques contained more than a single focus of monoclonal portions, five had two foci and one had four such foci. One-third of all portions from large plaques did not have monoclonal characteristics. These portions tended to lie at the margins of lesions, particularly lateral to the flow of blood, with monoclonal portions found in the plaque's center. Also, portions without monoclonal characteristics in plaques with multiple foci were observed to lie between foci of different isoenzyme type.

Discussion

The clonal nature of cell populations at different stages of atherogenesis was studied with G-6-PD, a cellular marker. The following are pertinent to the discussion of this study's results: (1) the fatty streak as a precursor of the fibrous-capped plaque, (2) the monoclonal nature of atherosclerotic plaques, (3) characteristics of the cellular growth within atherosclerotic lesions, and (4) the concept of monoclonal growth as a process of either mutation or clonal selection.

The clonal characteristics in the majority of fatty streaks that we studied resembled those of uninvolved aortic wall or, as shown by Benditt,17 thickened intima. The polyclonal nature of fatty streaks is in keeping with the hypothesis that these lesions are merely the reaction of normal tissue to injurious stimuli. This hypothesis is suggested by the reversibility of the fatty streak,18 implying that no permanent clonal cellular populations exist.

Furthermore, Haust et al.19 recently compared histological changes in smooth muscle cells from uteri of women with toxemia of pregnancy with those observed in smooth muscle cells in fatty streaks. They interpreted the findings of similar changes in both tissues to mean that fatty streaks arise as a result of adverse stimuli and that arterial wall is not unique in its reaction to such stimuli.

The fatty streak, however, has been assumed for many years to be the forerunner of the fibrous-capped plaque.20-24 This assumption has been based largely on circumstantial evidence. The fatty streak contains the same cellular and extracellular components as the fibrous-capped plaques but in different proportions;20 the smooth muscle cell is the predominant cell type in both lesions.22 For this reason, fatty streaks and fibrous-capped plaques are in many cases difficult to differentiate from one another under the microscope, owing to the presence of apparent transitional forms. Likewise, in experimental animals, the decrease in the number of fatty streaks following catheter-induced intimal injury coincides with an increase in fibrous-capped plaques; the fatty streaks are thus assumed to change into plaques.25 At the same time, evidence has been gathered contesting the role of the fatty streak as a precursor. Schwartz and Mitchell26 noted that, in human aortas, the plaques first occurred in the abdominal aorta, whereas fatty streaks occurred initially in the thoracic aorta. Studies among different racial, geographic, and ethnic groups with different prevalence rates of atherosclerotic diseases showed that the prevalence of fatty streaks in aortas did not differ greatly between groups and did not predict the extent of aortic involvement by fibrous-capped plaques at older ages within the same group.27 Finally, considerable differences between these two lesions have been reported with regard
Such variability has been noted both in the cellular and biochemical constituents of fatty streaks. This also implies that some but not necessarily all fatty streaks can be used as the criterion for the definition of the fatty streak as a site at which chronically injurious or mutagenic stimuli initiate the monoclonal proliferation of smooth muscle cells. The majority of fatty streaks do not have monoclonal characteristics, yet a minority of fatty streaks contain enough cells from their biochemical constituents, particularly fatty acid lipoprotein, fibrin, and residual cholesterol.

The monoclonal characteristic of fibrous-capped plaques can be used as the criterion for the definition of this lesion. The clonal characteristics of alleged precursor lesions might then be compared to those of fibrous-capped plaques to determine the way in which the fatty streak could act as a forerunner. One possibility is that the fatty streak is an early form of the fibrous-capped plaque, i.e., a monoclonal cell population with a gross morphology different from that of the fibrous-capped plaque. From the data presented, this possibility is clearly not acceptable. A second possibility might be that the fatty streak is not the result of a monoclonal cell proliferation, but, once formed, may be a favorable site for this proliferation. Either chronically injurious or mutagenic stimulation may occur within fatty streaks, resulting in a clonal proliferation of smooth muscle cells. This concept implies that some but not necessarily all fatty streaks undergo progression, i.e., that considerable morphological and biochemical variability exists among fatty streaks. Such variability has been noted both in the cellular and lipid constituents of fatty streaks.

The findings presented in the current report support the concept of the fatty streak as a site at which chronically injurious or mutagenic stimuli initiate the monoclonal proliferation of smooth muscle cells. The majority of fatty streaks do not have monoclonal characteristics, yet a minority of fatty streaks contain enough cells from

| Table 3: The Monoclonal Characteristics* of 26 Large Fibrous-Capped Plaques from Heterozygous Black Females |
|---|---|---|---|
| Plaque | Surface area (mm²) | Total no. of portions | No. of monoclonal portions (A, B) | No. of foci† (A, B) |
| 1 | 50.5 | 2 | 1 (B) | 1 (B) |
| 2 | 54.8 | 6 | 5 (4A, 1B) | 2 (1A, 1B) |
| 3 | 60.2 | 4 | 2 (2A) | 1 (1A) |
| 4 | 69.5 | 6 | 6 (6A) | 0 (0) |
| 5 | 71.6 | 7 | 3 (3B) | 1 (1B) |
| 6 | 72.4 | 5 | 5 (5A) | 1 (1A) |
| 7 | 75.8 | 6 | 5 (2A, 3B) | 2 (1A, 1B) |
| 8 | 85.9 | 6 | 6 (6B) | 1 (1B) |
| 9 | 87.4 | 6 | 5 (5A) | 1 (1A) |
| 10 | 96.0 | 10 | 6 (6A) | 1 (1A) |
| 11 | 98.5 | 4 | 4 (4B) | 1 (1B) |
| 12 | 116.7 | 6 | 6 (6B) | 1 (1B) |
| 13 | 117.0 | 9 | 2 (2B) | 2 (2B) |
| 14 | 122.1 | 8 | 7 (5A, 2B) | 2 (1A, 1B) |
| 15 | 152.1 | 7 | 2 (2A) | 1 (1A) |
| 16 | 156.0 | 3 | 3 (3B) | 1 (1B) |
| 17 | 156.6 | 6 | 6 (6A) | 1 (1A) |
| 18 | 158.9 | 2 | 2 (2A) | 1 (1A) |
| 19 | 169.6 | 4 | 3 (1A, 2B) | 2 (1A, 1B) |
| 20 | 185.7 | 8 | 8 (8A) | 1 (1A) |
| 21 | 192.9 | 9 | 9 (9A) | 1 (1A) |
| 22 | 215.2 | 8 | 8 (8A) | 1 (1A) |
| 23 | 239.4 | 12 | 4 (4B) | 1 (1B) |
| 24 | 292.6 | 6 | 6 (6A) | 1 (1A) |
| 25 | 469.4 | 15 | 8 (3A, 5B) | 4 (2A, 2B) |
| 26 | 505.7 | 12 | 2 (2A) | 1 (1A) |
| Total | 177 | 118 (76A, 42B) | | |

* Using definition 1.† A focus is defined as one or more monoclonal portions of the same isoenzyme type lying next to each other and surrounded by polyclonal portions or monoclonal portions of a different isoenzyme type.

Figure 6: A: Two large fibrous-capped plaques (numbers 6 and 11 in Table 2) lying juxtaposed on the aortic surface. The shape, dimensions, and manner in which they were divided into portions are illustrated. The numbers within each portion's boundaries represent the percentage of total isoenzyme activity in the B band. Plaque surface area, the aorta's mean % B isoenzyme, and 99.7% confidence limits around that mean are shown below the illustration. B: Largest fibrous-capped plaque (number 24) consisting entirely of monoclonal portions. Data are displayed in a manner identical to A. C: Large fibrous-capped plaque containing monoclonal portions of both isoenzyme types. Data are displayed in a manner identical to A. Portions on the left end of the plaque were monoclonal A; portion with 100% B isoenzyme was monoclonal B.
monoclonal or oligoclonal cell populations to provide G-6-PD isoenzyme markers significantly different from uninvolved aortic wall. Two isoenzyme distributions are possible within potential precursors. One would be the clustering of some isoenzyme values at the extremes, similar to those of plaques. This distribution might also be attributed to the misclassification of plaques as fatty streaks. A second distribution pattern would be the gradual dispersion of values of lesions away from the uninvolved wall and toward the extremes. This pattern, with many fatty streaks intermediate between monoclonal and polyclonal, is consistent with these lesions being in the process of transformation from fatty streaks into fibrous-capped plaques. It is this latter pattern that was observed in this study.

The histological characteristics associated with the apparent transformation from a fatty streak to a fibrous-capped plaque is an increased cellularity of the fatty streak. The presence of increased fibrous tissue was not associated with any evidence of progression toward fibrous-capped plaques. These findings are interpreted to mean that the basis for conversion of a fatty streak to a fibrous-capped plaque is the proliferation of a monoclonal cell population within the fatty streak. This is in agreement with the findings of Geer et al.\(^\text{30}\) who demonstrated that populations that were likely to develop more severe atherosclerosis had increased cellularity within their fatty streaks.

The present study of the clonal characteristics of fibrous-capped plaques strengthen the hypothesis that these lesions consist of monoclonal cell populations. The first way in which the monoclonal hypothesis was strengthened was by the demonstration of isoenzyme patterns which would be expected of a monoclonal lesion (such as a leiomyoma), using the present methods. Linder and Gartler\(^\text{10}\) observed only a single isoenzyme band in leiomyomas. However, the results of their electrophoresis were read with the naked eye. Our findings of predominantly one isoenzyme type in portions of leiomyomas were the result of quantitative measurements of isoenzyme type, and thus have the advantages of being potentially less biased and of enabling statistical manipulation. Despite the differences in technique, our findings are in close agreement with those of Linder and Gartler. The confidence limits established for monoclonal lesions thus serve as a second criterion for the monoclonal nature of fibrous-capped plaques and take into account the possible contamination of the lesion by erythrocytes and various migratory cells. Despite these even more rigorous criteria, as many as 61% of plaques had the monoclonal characteristic. An additional piece of evidence for the monoclonal hypothesis was the demonstration of a very high proportion (96%) of large plaques containing at least one monoclonal portion. Finally, the study of large plaques allowed manifold duplication of study results. The demonstration of a monoclonal characteristic within several portions encompassing a large surface area makes sampling variations an unlikely explanation for the finding of monoclonality.

The characteristics of growth of fibrous-capped plaques may be examined by observing plaques of varying sizes. Multiple portions of plaques allow the determination of the potential size of the monoclonal populations and description of patterns of clonal growth. The first and most striking finding in this regard is the large size attainable by monoclonal cell populations within plaques. The observation of the lesion with surface dimensions of \(1 \times 3 \text{ cm}\) (Fig. 6B) is undoubtedly a minimum estimate of the potential size of the monoclonal growth. A plaque may also attain large size by the confluent growth of several monoclonal populations. The result of this growth is the finding of multiple foci or clusters of monoclonal portions of the same isoenzyme type within a single plaque. It is easy to visualize the continued growth of the plaques illustrated in Figure 6A resulting in such a lesion. Monoclonal portions were also frequently observed next to ostia of arteries on the aortic surface. Monoclonal portions were also observed predominantly in the center of lesions, particularly along the line of arterial blood flow. The margins of the plaques, particularly those lateral to the arterial blood flow, often consisted of polyclonal cell populations.

The major alternative explanation for the mutational origin of monoclonal characteristics of plaques is that a single clone of cells with a genetic advantage for survival and proliferation may have been selected by repeated injury to the aortic intima. Martin et al.\(^\text{31}\) used cultures of skin fibroblasts, and showed the selection of certain clones and subclones of cells with increased growth potential after serial passages in tissue culture. Thus, cell lines may become monoclonal following long periods of hyperplasia. Thomas et al.\(^\text{32}\) analyzed thick atherosclerotic lesions from human females heterozygous for G-6-PD. Of the small proportion of plaques found to be monoclonal, they reported that monoclonal samples were all A or all B isoenzyme type within the same individual. They concluded that G-6-PD monotypism is secondary to the preferential survival of cell lines with the selection due to genes on the X-chromosome other than G-6-PD.

The hypothesis that fibrous-capped plaques arise by the selection of clones of cells may be studied by examination of plaques of varying size. If selection played a role in the origin of plaques, the plaques of smaller size might be expected to have a smaller proportion of samples with monoclonal characteristics than larger plaques. Small plaques (< 50 mm\(^2\) surface area) in this study showed a higher proportion of portions with monoclonal characteristics than did large plaques. Also, among large plaques, no trend with increasing size was noted, and the larger plaques had no greater proportion of monoclonal portions. We also have no findings to support the contention that G-6-PD monotypism is secondary to the preferential survival of cell lines due to genes on the X-chromosome other than G-6-PD. There were numerous instances in which a single aorta contained plaques of both isoenzyme types or individual plaques consisted of monoclonal portions of both isoenzyme types.

Consideration must be given to the possibility that the process of clonal selection may have been completed prior to formation of the fibrous-capped plaque. The precursor lesion, the fatty streak, should then be examined for evidence of clonal selection or mutation. The findings of...
this study fail to resolve the conflict between theories of selection or mutation in fatty streaks. On one hand, a monoclonal population arising from a mutation might be expected to show increasingly monoclonal characteristics as the clone of cells increases in size, causing an increasing cellularity within the fatty streak. On the other hand, a monoclonal population may be more likely to arise by clonal selection in a fatty streak where the cells are stimulated to proliferate, thus resulting in the association of increased monoclonal characteristics with increased cellularity. The fatty streak rather than the fibrous-capped plaque appears to be the lesion in greatest need of further study in order to resolve the mutation-selection controversy.

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