Proc. Nat. Acad. Sci. USA
Vol. 70, No. 6, pp. 1753–1756, June 1973

Evidence for a Monoclonal Origin of Human Atherosclerotic Plaques

(glucose-6-phosphate dehydrogenase/heterozygotic females/aorta)

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Communicated by Shields Warren, February 23, 1973

ABSTRACT **The main cellular elements of atherosclerotic plaques are smooth muscle cells. Because these plaques differ from their precursors in the underlying artery wall in several ways, we have asked the question: Are human atherosclerotic plaques polyclonal or monoclonal in their origin? The X-linked glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in heterozygotic females has been used to obtain an answer. 30 Plaques of different degrees of complexity and 59 samples of normal aorta and iliac artery walls from four females, 25–79 years old, were investigated. The data show that fibrous caps even of relatively large atheromatous plaques, 0.5 cm or greater in diameter, are composed of cells that produce solely or predominantly one enzyme type, whereas samples of artery wall media and intima as small as 0.1 mm^3 are regularly composed of a mixture of cell types. If plaques were a response to injury akin to a healing wound, a reaction to a growth stimulant, or formed due to an organization of a mural thrombus, they would be expected to be polyclonal. Hence, the results imply that atherosclerotic plaques in human beings arise by another mechanism. The mechanism compatible with the monoclonal nature of atherosclerotic plaques is mutation, and the likely causes are chemical mutagens or viruses.**

Atherosclerosis is a progressive disease of major arteries in human beings. The characteristic lesion of atherosclerosis is the fibrous plaque composed mainly of smooth muscle cells surrounded by a dense collagenous stroma. In advanced lesions it has an underlying layer of lipid debris resembling in its composition the blood lipids. Virchow ascribed the origin of this formation to irritation and subsequent inflammation of the lining of the vessel wall. Rokitansky believed that organization by connective tissues of a mural thrombus, built originally of fibrin, leads to plaque formation. The evidence that cholesterol and/or other blood lipids are altered in some persons with atherosclerosis and coronary artery disease has led to the notion that lipid penetration into the artery wall is the source of the irritant. Chemical, mechanical, and nutritional manipulations have been used in animals in an effort to reproduce lesions like those of atherosclerosis in man; none of these experimental lesions yields wholly satisfactory copies of lesions of the human disease.

Spontaneous atherosclerosis occurs in chickens and, as we have found, produces lesions that strikingly resemble those of man (1, 2). Comparison of the naturally occurring atherosclerotic plaques of human beings and birds with the response of the mammalian aorta to irritation by a suture (3) or in more recent experiments by mechanical injury with a balloon catheter, led us to consider an alternative to the injury–repair hypothesis based on the following considerations: cells of spontaneous lesions differ from cells of normal artery wall and cells populating a repair site. They differ in size, in composition of associated extracellular material, e.g., a preponderance in fibrous plaques of collagen as opposed to elastin, and in the absence of intercellular junctions. These features suggest two possibilities: either the cells of atherosclerotic plaques derive from a population of cells different from those of the normal arterial media or they are transformed cells. If the latter is so, cells of atherosclerotic plaques, like those of the benign smooth muscle tumors of the uterus, could be expected to be monoclonal.

Linder and Gartler examined the nature of the cell populations in benign uterine smooth muscle tumors by investigating the pair of X-linked glucose-6-phosphate dehydrogenase (EC 1.1.1.49) isoenzymes (4). Use of the isoenzymes in the analysis depends upon the fact that there is random inactivation of one or the other of the two X chromosomes in female cells early in embryonic development. Thus, the mammalian female is a mosaic of two cell types, each type having one or the other of the pair of X chromosomes active. About one-third of black females in the population are heterozygous for glucose-6-phosphate dehydrogenase and exhibit mixtures of the A and B enzymes in tissue extracts as the expression of this cellular mosaicism. Because the two cell populations reproduce true to type throughout somatic growth, it is possible to use them as tracers in various studies of development in embryonic systems and of origins of pathologic new formations (5). We report here that individual atherosclerotic plaques from various regions of the human aorta and common iliac arteries exhibit enzyme patterns consistent with their origin as monoclonal growths.

PATIENTS AND LESIONS

Patient 1 was a 31-year-old woman who died of the renal complications of disseminated lupus erythematosus. The lower abdominal portion of the aorta and the common iliac arteries showed early, extensive, typical atherosclerotic intimal plaques having lucent, pearly-grey fibrous character with obvious patches of yellow lipid. Microscopically the lesions were composed of masses of smooth muscle lying entirely on the intimal side of the internal elastic membrane. Some of the smooth muscle cells and a few cells resembling macrophages were shown to contain lipid by fat stains; focal areas of calcification were present in many of the plaques. Cholesterol crystals were present in some cells, and small amounts were detected in some extracellular locations. *Patient 2* was a 79-year-old woman, dead of an endometrial carcinoma. A segment of upper abdominal aorta, received frozen, had raised atheromatous plaques with thick fibrous caps and underlying

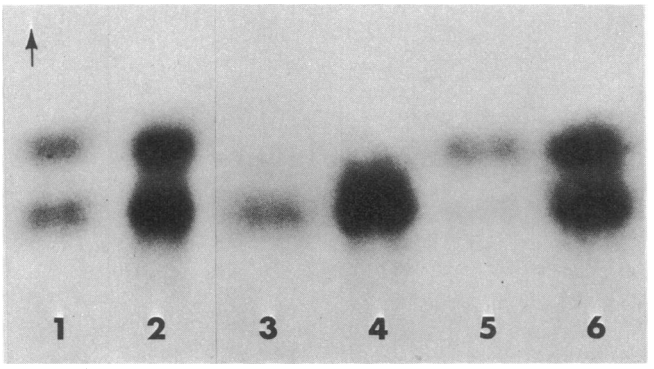

FIG. 1. Zymograms on cellulose acetate of samples from (1) blood; (2) normal tissue; (3–6) atherosclerotic plaques of *Patient 1*.

lipid debris containing cholesterol crystals. Flatter yellowish plaques were present, and more than half of the surface had discrete, grossly visible lesions. *Patient 3* was a 25-year-old woman who died with extensive sarcoidosis. The upper aortic abdominal segment had no grossly discernible atherosclerotic lesions. *Patient 4* was a 62-year-old woman who died of hypertensive cardiovascular disease with left ventricular hypertrophy and had half the surface of her upper abdominal aortic intimal surface studded with typical atheromatous plaques.

METHODS

Aorta and common iliac arteries removed at autopsy 20 hr after death from *Patient 1* were freed of most of the adventitial fat and opened, and gross clots were removed from the endothelial surface. The surface was gently wiped free of any residual blood clots with surgical gauze sponges. Upper and lower segments of the aorta, separated at the level of the diaphragm, were each placed, adventitial side down, on a piece of cardboard. Exposed intimal surfaces were covered with Parafilm to protect them from drying, and the tissues were kept in a covered plastic container placed on ice or in the refrigerator between samplings. Small pieces of blotter, sparingly moistened with water, were placed in the ends of the chamber to prevent gross desiccation of the tissue. Water condensation on the Parafilm covering the vessel surface was removed periodically to prevent its dripping onto the surface. Kept in this fashion, the material yielded useful results for 6 days after death and satisfactory histology after fixation.

Under 3-times magnification with a fine-pointed forceps and iridectomy scissors, samples 1–3 mm^3 in size were removed from various portions of plaque, from grossly plaque-free intima, and from media of the vessels. To the samples in small, round plastic culture dishes, 2–5 μl of membrane buffer (pH 9.1)* was added. Membrane and chamber buffers were those described by Sparkes *et al.* (6), diluted to 10/11 of the described concentration to increase resolution of enzyme bands. Samples were then frozen in the covered dish and kept on dry ice. Enzyme in samples so prepared was stable for more than 6 hr; it was extracted by thawing and refreezing the tissue three times. Once extracted, the enzyme was less stable. Electrophoresis of enzyme on cellulose acetate was performed in a Beckman Microzone apparatus at 400 V for 50 min. Applied sample size ranged from 0.25 to 1.25 μl. Enzyme was developed with the substrate mixture applied to filter paper (6).

Segments of the aorta from *Patients 2, 3,* and *4* were placed on cardboard and frozen immediately at autopsy, packed in dry ice for shipment, and maintained in the frozen state with dry ice during sampling at −15°. Pieces of tissue (0.1–3.0 mm^3) were cut out of the surface with a pointed scalpel blade. In several instances, much larger samples (10–30 mm^3) were removed from the fibrous cap of plaques. Samples were treated otherwise in the same manner as the fresh tissue from *Patient 1*. For quantitative measurements the patterns were scanned in a Beckman Analytrol, modified to take the small cellulose acetate strips.

OBSERVATIONS

Examples of the enzyme patterns from blood, normal tissue, and four individual plaques from *Patient 1* are shown in Fig. 1. These patterns reveal the following: The patient's blood and a sample of media, shown in panels **1** and **2**, respectively, contained equal proportions of A- and B-type enzymes, indicating equal numbers of A- and B-type cells. The plaques sampled for patterns **3** and **4** clearly present enzyme largely of the B type, whereas the tissue enzyme shown in panel **5** is clearly derived mainly from cells having the A-type enzyme. The sample shown in panel **6**, derived from a fourth plaque, is an example of a mixture of nearly equal proportions of enzymes.

81 Samples from 30 plaques and 59 samples from uninvolved intima and media have been assayed. The results are given in Table 1. As a means for comparison of variation in the composition of the aortic wall and for measurement, in some plaque samples, of the relative proportions of enzymes, 59 normal wall samples and 44 plaque samples were scanned photometrically, and the relative areas of the two peaks were determined by planimetry. 19 Normal samples from *Patient 1* yielded a mean value of A = 0.52 ± 0.066 (SD), 7 samples from *Patient 2* had a mean of A = 0.25 ± 0.050, 13 samples from *Patient 3* yielded a mean of A = 0.36 ± 0.065, and 12 samples from *Patient 4* gave a value of A = 0.42 ± 0.075. Enzyme patterns showing alterations equal to or exceeding three-times the standard deviation on either side of the mean were considered different from normal and substantially of

TABLE 1. *Phenotypes of normal vessel wall and fibrous caps of atherosclerotic plaques*

Patient number	Number of sites showing enzymic phenotype							
	Normal				Plaques			
	AB	A	B	Total	AB	A*	B*	Total
1	25	0	2	27	3	4	8	15
2	7	0	0	7	2	1	6	9
3	13	0	0	13	—	—	—	—
4	12	0	0	12	1	3	2	6
Total	57	0	2	59	6	8	16	30

* A plaque was judged to be solely or predominantly of one type, *A* or *B*, when one or more samples yielded an *A* enzyme fraction ⩾3 standard deviations from the mean value for the case. – , no plaques present.

* Buffer contains 124 mM Tris–3.6 mM EDTA (disodium salt)–14 mM boric acid–65 μM NADP.

one population. Four plaques from *Patient 1*, three from *Patient 2*, and four from *Patient 4* were considered solely of one population type (>95% of one enzyme type).

In order to be certain that the apparent diminution or absence of A enzyme in several of the plaques from *Patient 2* was not merely because of its relatively low concentration, samples from three large plaques of 20–30 mm^3 were removed, minced, and treated with $^1/_2$ their volume of membrane buffer, and several applications were made to the cellulose acetate strip. The B-enzyme band was strongly stained, but insignificant amounts of type-A enzyme appeared in two of the samples and none in the third.

A direct comparison was made of tissue enzymes from fibrous caps and media underlying the yellow atheromatous material of two plaques from *Patient 4*. In both instances, the media yielded both A and B enzymes, whereas the plaques clearly contained one enzyme type. Additionally, in these assays tissue sample size was reduced to 0.1–0.2 mm^3 to try further to refine the patch size.

DISCUSSION

The experimental question posed at the outset was: "Do atherosclerotic plaques exhibit the features of an homogeneous, single-cell-derived population, or do they represent a mixed population similar to that of the person's cell mixture?" The answer depends in part upon the size of mosaic patches in the individual and her specialized tissues. Sizes of mosaic patches have been shown to vary among different tissues (7): the epidermis has a higher variability than other tissues; blood and connective tissues appear least variable. Mosaic patterns in vessel walls have not previously been examined. Our data from all four patients presented above indicate that the mosaic patch size in the aorta is small, <0.1 mm^3, and at least as small as that of the myometrium (4). Against this background, the appearance in atherosclerotic plaques of a marked preponderance or sole presence of one or the other cell type appears significant. Statistical evaluation of the data from *Patients 1*, *2*, and *4* was made by the Chi-square test of independence (8); *P* values were obtained for *Patient 1* of <0.0001, for *Patient 2* of <0.001, and for *Patient 4* of <0.005. The tests were performed using Yates' correction, and hence are conservative.

The data seem clear, although it was originally anticipated that blood contamination and leukocyte or macrophage infiltration might frustrate the investigation. To some extent, this background noise is seen in the zymograms of plaque material as trace contamination with the second enzyme. It may have also, in some instances, led to assignment of a plaque to the "mixed" category.

Appearance of a monoclonal enzyme pattern in 2 of the 27 samples taken from presumptively normal artery wall of *Patient 1* is somewhat deviant from the remaining control data. Histologic examination of the sites in both instances revealed slight intimal thickening, but not a definitive plaque. The other data from nonatherosclerotic arterial wall argue strongly against these being mosaic patches. The possibility exists that we were looking at two very small initial foci of plaque formation, a supposition consistent with the widespread early disease in this patient.

One may raise the question whether or not wound healing is initiated from proliferation of a single cell. Several pieces of evidence argue that it is not: it is the general observation that wounds heal by simultaneous proliferation from all margins. Furthermore, in the case of a very small wound in the mammalian aorta we have observed simultaneous mitoses in many cells isolated from each other by the elastic laminae (3). Finally, it has been found that a hypertrophic scar of the skin is polyclonal (Linder, personal communication).

It can be argued that the reason for the single-cell type in the plaque is not its monoclonal origin, but is due to some process selecting from one or the other of the two cell types. Although this is possible, the fact that some plaques are composed of only A cells and others of only B cells argues against the activity of a process of selection involving cells with these enzyme types.

Another concept is reasonable to consider, namely, that during proliferation of cells in response to injury, a single cell develops a selectional advantage, and its progeny thereafter dominate the process. This type of process seems quite compatible with the finding of a monoclonal character of the atherosclerotic plaques. The commonly accepted reason for development of such a selectional advantage is an alteration in a cell genome, i.e., a mutation. The observation that cells of atherosclerotic plaques differ, as pointed out above, from cells of the normal arterial wall in regard to size, to the proteins that they synthesize and deposit locally (i.e., collagen rather than elastin), and the apparent deficiency of cell to cell contacts coupled with their monoclonal character seems to fit with the mutation concept.

Along with the appearance of a monoclonal character of a given plaque, several features consistent with known aspects of atherosclerosis became evident during the study. For example, in *Patient 1* attention was directed to small plaques by appearance in samples of single enzyme types; their presence was verified by histological examination. Apparent confluence of plaques is one of their well-known pathological features. One site originally appeared to be a single large plaque; it yielded two distinct monoclonal patterns, A and B. On closer examination of the plaque, two interdigitated plaques became evident. This also offers an explanation for the plaques of a fully mixed pattern (e.g., sample **6**, Fig. 1): Plaques frequently exhibit layering. This has been ascribed to organization of mural thrombi. Our interpretation of these is that the layers occur in three dimensions, just as we found with interdigitations in two dimensions on the surface.

Certain sites of predilection for plaque formation in atherosclerosis exist. These are near branch orifices and on certain aspects of particular arteries. At least some of these regions now appear to be sites of high turnover of endothelial cells (9), and there is evidence for assuming higher turnover of muscle cells in the underlying wall. While this higher rate of mitotic activity may not by itself be the cause of true plaque formation, anything that increases the rate of proliferation may increase the risk of induction of cell transformation by another agent, such as virus or chemical mutagen. Acceleration of atherosclerosis in the presence of elevated concentrations of blood lipids can be viewed in this way, since it has now been found that elevation of blood cholesterol in young swine by feeding is followed in the arterial wall by increased proliferation of endothelium and smooth muscle cells (10), and we now believe that the mechanical stress of hypertension may do likewise.

In addition to the morphological and functional alterations of cells, the progressive enlargement of atherosclerotic plaques

and their tendency to degenerate and to ulcerate are all compatible with the presence of a transformed cell population.

Although more extensive data are needed, our observations make it reasonable to reconsider the theories of etiology and of pathogenesis of progressive atherosclerosis. The monoclonal character of atherosclerotic plaques indicates that consideration should be given to factors that transform cells, such as chemical mutagens or viruses, or both.

We thank Dr. S. M. Gartler for his helpful and critical comments during the work and Dr. H. Roman for reading and commenting on the manuscript. Dr. Ruth Wong of the Illinois School of Medicine and Dr. Paul Szantos of Cook County Hospital generously provided us with some of the tissues for the investigation. Supported in part by USPHS Grants HE-03174, GM-13543, and the Greenwald Fund.

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