Implications of the Monoclonal Character of Human Atherosclerotic Plaques

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Evidence for the monoclonal nature of human atherosclerotic plaques is reviewed. Eighty percent of discrete raised atherosclerotic plaques are of single phenotype. Interpretations alternative to single-cell origin, based on patch size, selection due to linked genes, or repetitive sampling do not seem to explain the apparent monoclonality. Search for carriers in serum of mutagens, such as may be present in cigarette smoke, show them to be the lipoproteins, and the presence and possible role of intrinsic mutagens, e.g., cholesterol-α-oxide, are presented. The possible role for other factors implied by the monoclonal hypothesis, e.g., the mechanism by which estrogen therapy may increase coronary attacks, is discussed. (Am J Pathol 86:693–702, 1977)

THE FEATURES OF THE LESIONS of human atherosclerosis have been reviewed in this symposium by Dr. Heptinstall.[1,2] In this presentation, therefore, I wish only to call attention, for the sake of emphasis, to certain features that seem of special importance.

The developed lesions of atherosclerosis seen in vessels of human beings at autopsy take the form of a smooth-surfaced mass raised above the level of surrounding nonatherosclerotic vascular intima. Such "raised" lesions are, on histologic examination, composed mainly of a variant of smooth muscle cell embedded in dense extracellular connective tissue. Selective stains identify collagen as the main connective tissue fibrillar constituent and elastin usually as a minor or variable constituent. Along with some kinds of cell debris, glycosaminoglycans are present in varying amounts in the extracellular matrix. These histologic findings have been confirmed by electron microscopy.[3-5]

Lipid stains and electron microscopic examination show that the smooth muscle cells may have very little fat in them, except in the deeper layers of the plaques. Lipid present in superficial layers of the plaques frequently appears to be in foamy cells, presumably macrophages, lacking any of the features of smooth muscle cells.

In some plaques, lipid is present, and substantial amounts of cholesterol can be found both in cells of the deeper layers of the plaque and in the

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atheromatous debris underlying the fibrous cap of the plaque. Smooth muscle cells in the deeper layers of plaques, adjacent to the atheromatous debris, frequently exhibit pathologic fatty changes, indicative of cell injury, and cells in the deepest layers appear frequently to be dying and disintegrating.

All of this mass of the plaque lies in the intima beneath the endothelium and entirely on the luminal side of the internal elastica of the artery. In the undistended vessel the masses are usually sharply raised from the adjacent intima. Some features of the cellular mass and associated extracellular matrix appear to set it off from the underlying media: cells of plaques have a different form than cells of normal media. The main extracellular material produced by plaque cells is collagen, whereas elastin is a major item in the aortic media. The arrangement of cells of the plaque lacks the order found in the arterial media, and numbers of intercellular junctions appear to be reduced.[5]

The impressive feature of the human lesions is the presence of an excessive and apparently useless mass of cells resembling in many respects—but differing from, in the subtle ways indicated—the cells and structures comprising the media of arteries. It seems reasonable, even now, to emphasize the cellular nature of human atherosclerotic plaques because the lipid insudation concept has for so many years dominated research in atherosclerosis. The emphasis on blood lipids and cholesterol has led to many important observations on the structure and physiology of the plasma lipoproteins. However, a simple cause–effect relationship between levels of blood lipoproteins or cholesterol has not been found.

Our goal is the answer to the question, What is the nature and origin of *human* atherosclerosis? Since the lesion has a mass of cells as its major feature, we can rephrase the question as follows: What is the nature of the cellular proliferation involved in the "new formation," the atherosclerotic plaque? Specifically, we can ask, Is it of multicellular or is it of monoclonal origin?" The importance of this distinction lies in the fact that many neoplasms have been found to be of monoclonal origin. On the other hand, many common cell proliferations seen with embryogenesis, maintenance, and repair seem to be multicellular in character. It becomes immediately apparent, when one asks the question in the form indicated, that upon the answer depends the direction of our search for causal factors in the disease. Phrased this way, the question takes on a new significance since there is now a basis for obtaining an answer and the methods involved are applicable to human tissues and lesions.

The method of analysis applicable to study of proliferated masses of cells and for distinguishing the origin of these from one or from many

precursor cells has been discussed in this symposium by Dr. Gartler.[6] I review it here only briefly: According to the concept of Mary Lyon,[7] all human females are mosaics, composed of two phenotypically distinct cell types. This is due to the fact that early in embryonic development there is a random inactivation in each somatic cell of one or the other of the two X-chromosomes. Once inactivation has occurred, each cell reproduces true to type and all daughter cells of a particular cell exhibit the activity of the single, same X-chromosomal genes. The stability of this state has been shown by cultivating single cells from connective tissue of human mosaic donors.[8] Given a stable mosiac population, it is possible to ask questions in regard to cell population origins in embryogenesis and whether a pathologic new formation is derived from one or from many cells.

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is a polymorphic, X-linked gene product. Of particular interest among the large number of variants now known to exist are two, the common B form and the A form migrating more rapidly in an electric field. The A form of G6PD is present in a substantial proportion of the black population. About 40% of black females are heterozygous, and their tissues are comprised of two cell types each producing one or the other enzyme form.[9] This property of female somatic cell populations has been used to assess the origin of cell populations in smooth muscle tumors of the uterus.[6]

We applied this use of mosaic features to the analysis of discrete raised lesions of atherosclerosis on material obtained at autopsy from human females and found that 24 of 30 plaques were of one enzyme type; whereas only 2 of 59 samples of artery wall, with no discernible plaques, were of one enzyme type.[10] Our current data combined with that of Dr. Heptinstall's group [1] indicate that more than 75% of more than 100 raised fibrous plaques are clearly monotypic, whereas fewer than 1% of more than 1500 samples of normal artery wall exhibit single isoenzyme pattern.

The fact that the bulk of raised atherosclerotic lesions is made up of one cell type, as indicated by isoenzyme pattern, does not immediately yield the strong inference that lesions of atherosclerosis are monoclonal. Such monotypic cell masses could arise in one of several ways: a) If the G6PD enzyme variant itself, or if some gene linked by location on the X-chromosome, imparted to cells a selective growth advantage (or disadvantage), then we could get a cell population of one enzyme type due to stimulus to cell multiplication of any kind, e.g., local injury or growth-promoting factors. b) If the pattern of cell mixture in the artery wall were such that clusters of cells of one type (not necessarily clones) were present and of sufficient size, then a population of one cell type might result from localized proliferative stimuli. c) A third possibility has been raised by

Thomas *et al.* that during cycles of cell death and cell multiplication repetitive sampling of population could lead to a drift toward a monotypic population.[11]

The question whether one or the other of the G6PD variants gives a selective advantage to the cell types is easily disposed of in the following manner: in all cases where large enough numbers of plaques have been sampled, individual plaques of each of both types have been found in the same vessels, even adjacent to one another. Thus, neither G6PD isoenzyme type nor linked genes appear in these cases to provide some subset of the total cell population with a major selective advantage. The problem of whether there may be some instances where genes linked to the G6PD locus can provide a selective advantage is a more subtle one and requires further analysis. However, in the majority of cases, the proliferative advantage must arise in other ways.

In order to see if patches of clusters of cells of the same enzyme type in the normal artery wall were of sufficient size to provide a basis for the apparent monoclonality of the cells comprising a plaque, we have examined the patterns of cell populations in artery walls in an effort to obtain the size of single enzyme-containing clusters in the following way. Sample size was reduced to the smallest one yielding readable enzyme patterns. Small samples of the order of 0.03 cu mm in volume gave measurable enzyme ratios. These small samples regularly show a mixed enzyme pattern. Examination of the statistical variation of the results indicated that we were not approaching the size of the clusters of one cell type. However, when we examined larger samples to see if their variability was limited to that of methodologic error, we found a variation larger than that of the methods error. A systematic map of the pattern of cell population variation in the artery wall revealed that the pattern of the variation showed grouping of cell mixtures with a much lower variance, of the order of the error variance.[12] The range of variation between clusters was observed to be some five to seven times that of the error variance as indicated by the percentage of A cells. This clustering may be a manifestation of confined growth of a group of cells recruited during early embryonic development from the mesenchyme to form the arterial media, or it may be a random phenomenon, or both. It should be emphasized that in this analysis, and even with the intimal thickening associated with aging, single samples in the size range of 0.1 to 0.3 cu mm or less appear with one enzyme pattern less than 1% of the time in more than 1200 samples. Irrespective of the interpretation of how these groupings arise, the fact of their presence becomes important in the analysis of the limiting patch size.

An analysis of the sources of variation indicates that there should be three factors contributing to the variance: a) a variance contribution due to the draw from the embryonic pool, b) a variance contribution due to true clonal proliferation plus accidental clustering of cells of the same type, and c) a contribution from methodologic error. Analysis of variance has shown that almost the entire variance is accounted for by the contribution from the factor we have labeled *embryonic*; this and the methods error leave only a very small fraction of the total variance attributable to the individual patches of monotypic character. From these data, we have arrived at an estimate of the size of clusters of cells of a single type of the order of 10^{-4} cu mm and composed of perhaps 10 cells. The important point to be derived from this analysis is as follows: any injury that allows access of nutrients or growth stimulants in order to produce coherent monotypic cell proliferation would have to operate on a very small group of cells, of the order of 10, in a space of the order of 10^{-4} cu mm. It seems highly unlikely that such injury could produce so high a proportion of one cell type.

With regard to the question of a selection based on population drift, we have examined patch size in the nonatherosclerotic aortic wall over an age span of 1 week to 91 years. There does not appear to be any substantial change in the character of the cell mixture over this age range. This is so in spite of the fact that with age the intima becomes progressively thicker. This thickening is most certainly due to death and replacement of cells resulting from the mechanical, chemical, and microbial insults that afflict artery walls during life.

The presence of mixed cell populations observed in some plaques could originate from one or more of the following sources: some plaques could originate from a few cells as suggested for the virus-originated condylomata.[6] Plaques could, and likely do, originate in intima thickened first in the aging process, so that samples encompass the two lesions intermixed. There could be contamination of plaques of monoclonal origin by cells from the blood, e.g., as seen in inflammation associated with tumors. Plaques are preferential sites for initiation of thrombus formation, and thrombi are organized by cells migrating and proliferating from adjacent arterial wall. It seems likely that one or more of these processes operate to produce the mixed character of the cell population found in some plaques. All of these "noise" factors make it even more surprising that a significant proportion of the population of plaques appear to be monoclonal.

If we take these facts all together, the reasons seem strong for believing that raised lesions are monoclonal. As stated earlier, a simple lipid insudation modal is no longer tenable. Furthermore, the mural thrombus

model as the cause of discrete atherosclerotic plaques in human beings seems similarly unsatisfactory as an explanation for the origin of discrete raised lesions of atherosclerosis. This is not to say that simple injury and repair or insudation do not occur in artery walls. As Elspeth Smith has shown,[13] insudation does occur; and as Ross and co-workers have reemphasized, mural platelet aggregates are formed and do incite organization.[14]

If we accept the idea that the majority of atherosclerotic plaques originate, like benign tumors, as monoclonal growths, then we are led to formulate the pathogenesis of the plaques and their consequences as follows. We can visualize three parts in the total process: a) *initiation*, b) *promotion* or *progression*, and c) *complication*. In this schema the initiation of plaque formation may then involve some factors causing a cell to gain a selective growth advantage over its neighbors. Such a change is thought usually to be by mutation. The agents that cause such change are chemical mutagens, radiation, and some viruses. The mutagens (or premutagens) may be derived from the environment. On the other hand, it is also possible that endogenous chemicals, possibly derived from cholesterol, could act as mutagen. Alterations in the genome produced by the agents listed is not expressed necessarily, unless some local environmental conditions provide the opportunity for their expression. This seems likely to be true for minimal viable alterations occurring in the cell's genome.

The kinds of conditions that permit expression of a selective advantage are to be found among conditions that stimulate cell proliferation. Injurious factors—chemical, nutritional, or mechanical—that cause cell death and result in local proliferation of cells can permit expression of the particular advantage a cell has acquired. The kinds of advantage a somatic cell might gain in the initiation process could be resistance to some injurious agent or escape from the need for some nutrient or growth factor.

Complications of atherosclerotic plaques in human beings appear to be necrosis and thrombosis, the latter encouraged by the death of artery wall cells and leading to platelet sticking in a region of turbulent flow. The important subject of causes of necrosis in plaques is large, and I will not discuss it further. I would like to indicate that cells with some form of selective advantages may have, when conditions change, reduced viability.

With any formulation regarding the etiology and pathogenesis of a disease, one asks whether there are instances that can be tested, or does it offer explanation of facts already available. I wish now to examine some implications that this formulation provides that none of the other hypoth-

eses introduced so far have made and to examine these in relation to several established "risk" factors for atherosclerosis.

Cigarette smoking is considered an important risk factor for clinical manifestations of atherosclerosis. Burning of cigarettes produces aryl hydrocarbons and some of these, such as benzo[a]pyrene, are well-known premutagens. It is easy to conceive of the hydrocarbons from the smoke affecting the lung; can they also affect the arteries? The question is whether chemical substances from the smoke get into the blood and then to the artery wall. Evidence that the premutagens get into the blood is available. The aryl hydrocarbon hydroxylase, an enzyme system induced by polycyclic aryl hydrocarbons, is elevated enormously in the placentas of women who are heavy smokers and to a lesser degree in women of intermediate smoking habits, when compared with enzyme levels in nonsmokers.[15] Clearly, the route must be from the lung via the blood to the placenta.

The next question to ask is, Where in the serum are these injurious substances carried? We have recently begun studies of the role of the lipoproteins of the serum as *carriers* of mutagens or potential mutagenic agents. The first experiments have been to see where substances such as 3-methylcholanthrene and benzo[a]pyrene are taken up in human serum and to compare their behavior with that cholesterol. The results are striking and show, as might be expected, that the aryl hydrocarbons are taken up and carried by the same fractions in the plasma as the cholesterol, namely, the lipoproteins.[12]

It has now been observed in several laboratories that low density lipoproteins, among the serum proteins, are preferentially taken up by smooth muscle cells derived from human and primate artery walls when cultivated *in vitro*. In addition to that, we now have evidence that there is in the artery wall the enzyme system—aryle hydrocarbon hydroxylase—that converts substances such as benzo[a]pyrene and a 3-methylcholanthrene from a premutagen to a mutagen. The enzyme system is present in human artery walls and like the enzyme system of liver, peripheral white blood cells, skin, and fibroblasts is inducible.[16] Thus we begin to see that several of the ingredients required for production of mutations in artery wall cells are present in the human system. The facts presented now sketch the outlines of how one *risk* factor—cigarette smoking—may be operating to induce and/or to enhance the occurrence of atherosclerotic lesions.

Having seen one way in which we can relate cholesterol and lipoproteins to an exogenous source of agents that may be inducing atherosclerosis, I wish to turn to another. It has seemed possible that choles-

terol, while not itself injurious, might be the source of some injurious agent. There is already evidence that there is such a substance, cholesterol-α-oxide.[17] This material has been found to be associated with effects of ultraviolet irradiation on the skin. It is a known inducer of tumors in animals. Of great interest for those searching for the causes of atherosclerosis is the fact that cholesterol-α-oxide may be found in substantial concentrations in the serums of people prone to get atherosclerosis, including those with Type II hypercholesterolemia and hypertension.[17]

Methods are available for detecting and measuring amounts of this cholesterol derivative and other substances that have similar injurious properties. How these substances are formed and degraded needs to be investigated. We need to know what enzymes are involved. Once we know this, we can take a new look at genetic factors that may lie at the root of the accelerated atherosclerosis seen in some people. In several studies, such as that of the Framingham Study, some persons with low blood lipid levels exhibit coronary disease and strokes and some persons with high blood lipids and cholesterol do not. Clearly, genetic as well as environmental factors play a role in the origins of atherosclerosis, since only some individuals exposed to the same environmental and nutritional conditions get exaggerated forms of the disease.

Before closing, I wish to return to the results of the recently completed Coronary Drug Project.[18] This large and expensive study was well designed and fastidiously executed. The results were disappointing to the originators. Perhaps one of the most resounding disappointments was that with estrogen therapy. The original hope was that treatment with natural estrogen would lower blood lipids and prevent recurrent myocardial infarctions. This belief was supported by some interpretations of the effects of estrogen on atherosclerosis in chickens. Dr. Moss and I, in repeating the work of the Katz group,[19] treated one set of birds with estrogen. We found that half the animals died after several months, some of a virus lymphoma that chickens are prone to develop.[20] In addition, we noted in the survivors that the atherosclerotic plaques were worse. This was similar to what Pick *et al.* had noted. Furthermore, we found in one of the animals that there were typical C-type virus particles multiplying in the cells of the plaque and elsewhere in the artery wall. This observation seems specially important when we recall that in the Coronary Drug Project the administration of estrogen was associated *not* with a decreased but with an increased death rate from new myocardial infarcts. Furthermore, there was seen an increase in the incidence of cancer in the treated group. The effects of estrogens in eliciting latent viruses and inducing expression of lymphomas in mice have now been observed.[21] Could this be

the mechanism involved in the excess mortality found in the estrogen portion of the Coronary Drug Project?

This new grouping of facts indicates the fact that we must look at the inner surface of arteries in the same way that we view the inside of the bronchi, the inner lining of the gastrointestinal tract, and the surface of the skin. All three of these are more or less closely, and in different ways, in contact with the environment. Most of the material absorbed from the gastrointestinal tract, much of the gaseous material inhaled, and some of the material applied to the skin get into the blood. In this way the vascular wall becomes exposed to the environment. Coronary heart disease and other forms of atherosclerosis are clearly related to some factors in the environment. The concept proposed here provides us with a new set of guides in our search for the relationship of environmental and genetic factors to the manifestations of atherosclerosis.

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