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PROLIFERATION AND THE MONOCLONAL ORIGINS OF ATHEROSCLEROTIC LESIONS

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ABSTRACT

Benditt's observation of the monoclonal origin of the atherosclerotic lesion has been controversial because it appeared to conflict with conventional wisdom. A new method based on a polymerase chain reaction amplification of the DNA of an X-inactivated gene from microdissected tissue confirms that Benditt was correct. However, this monoclonal expansion can also be found in nonatherosclerotic intima and media. These new data suggest that plaque clonality may represent expansion of preexisting patches of cells arising during development of the media. This developmental view does not conflict with other recent evidence that plaque expansion is associated with mutation or viral events. However, if plaques arise from patches, then early developmental mechanisms may be critical to the later evolution of the lesions.

INTRODUCTION

Since Virchow's lectures in the 1850s, conventional wisdom has been that the atherosclerotic lesion is defined by a proliferative response to toxic products that accumulate in the vessel wall as a reaction to insudation of lipid. This view has led to an extensive literature both in vivo and in vitro on the growth factors that modulate smooth muscle replication in culture or in animals in response to traumatic injury (1, 2). The conventional view of replication as a smooth muscle central event in atherosclerosis is problematic for several reasons. First, nu-

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merous studies of smooth muscle proliferation in human atherosclerotic lesions have failed to show evidence of extensive replication (3–8). Second, recent concepts of the end stages of atherosclerosis suggest that plaque integrity, rather than plaque mass, is the critical feature in atherosclerotic progression. If this is true, cell proliferation may be beneficial by creating the matrix that prevents plaque rupture. Finally, there is a major conceptual problem with the Virchow hypothesis. We have known for 20 years that atherosclerotic lesions are clonal (9). That is, we know that lesions in females heterozygotic for an X-linked marker, G6PD, usually express only one or the other allele. The discoverer of this phenomenon, Earl Benditt, interpreted this as evidence that the plaque was neoplastic in origin. Whether or not he was correct as to the mechanisms leading to clonality, it is difficult to reconcile plaque clonality with the sort of diffuse responses to injury proposed by Virchow.

Evidence of clonality does not necessarily prove that the marker used to detect clonality, including a somatic mutation, has etiologic significance. Using an X-linked CAG polymorphism in the human androgen receptor, we now know that large patches of single allotype exist even in normal arterial walls (10). This implies that clonal expansion (defined as focal replication without mixing with neighboring clones) must, at least to some extent, be a normal feature of the human arterial wall. Plaques may represent much larger monoclonal patches. All we can say is that the marker appeared early in the process of forming the clone. This may happen, and in the case of X-inactivation presumably does happen, prior to any pathologic process involved in forming the lesion.

Regardless of the genetic basis for clonal formation, clonal expansion tells us something very interesting: The smooth muscle cells of the plaque represent the result of a proliferative process. Knowing when clonal expansion occurs, then, may be useful as an "archaeological" tool to tell us about an early event in the formation of these critical lesions.

There are several key issues. (*a*) How does smooth muscle proliferation contribute to the origin or progression of lesions? (*b*) If the wall is clonal, does the atherosclerotic clone arise from clones that differ from other smooth muscle cells in a pathogenetic fashion? (*c*) Is clonal expansion of the plaque associated with genetic changes in the plaque clone?

EVIDENCE OF PROLIFERATION IN ATHEROSCLEROSIS

In retrospect, Benditt's interpretation of lesion clonality as evidence of a neoplastic origin may have been the inevitable result of the simple fact that the atherosclerotic plaque is the only focal overgrowth of smooth muscle cells seen in vessel walls. Surprisingly, the identity of the plaque cells was not established until modern times. Virchow noted proliferation of vessel wall cells,

although he was unclear about the difference between smooth muscle cells and fibroblasts (11). The identification of smooth muscle cells as the mesenchymal cells responsible for forming atherosclerotic lesions was accomplished in the 1960s after Geer (12), Haust et al (13), and Parker & Odland (14) examined plaques by electron microscopy. These workers, followed by Ross (1), Campbell et al (15), and Fischer-Dzoga et al (16), made the reasonable assumption that the accumulation of smooth muscle was a response to injury in a smooth muscle–rich tissue, just as gliosis is characteristic of responses to injury in the brain. Clonality made this equation hard to accept. Knowing that the plaque was the only form of focal smooth muscle overgrowth in artery walls, Benditt made the analogy to uterine leiomyoma, a neoplasm already known to be monoclonal based on protein gels used to measure the two allotypes of an X-linked gene, G6PD (17). Interestingly, like atherosclerotic plaques in all humans, uterine leiomyomas can be found in the uteri of most women. Benditt's application of the G6PD method, while limited in spatial resolution by the need to isolate enough protein to run the gel, suggested that patch sizes in normal wall were small (18) while the size of regions showing one or the other allele of G6PD in the plaques was large, often as large as the plaque itself. The result was confirmed by other groups (19–21).

It is important to distinguish Benditt's observation from his hypothesis. He observed that the patch size—the adjacent area occupied by a single G6PD allotype—in atherosclerotic plaques was large. He showed that the patches in plaques were too large to have arisen by chance, leaving the obvious conclusion that the large patches must represent clones. So clonality is probably a fact. In contrast, Benditt's attempt to explain clonality took the form of a hypothesis proposing that these clones arise by rare, somatic genetic events. That is, either a virus or a mutation, as we usually assume, is the etiology of clones seen in neoplasms. This monoclonal hypothesis is probably better called a neoplastic hypothesis. In either case, the concept that lesions arise by rare genetic events seemingly violates conventional wisdom about origins of the plaque as a reaction to injury caused by local accumulation of lipid.

In an effort to reconcile Benditt's observation with the conventional wisdom, other investigators suggested that monoclonality might arise by selection of a subset of cells or by some mechanism involving death of the plaque cells with overgrowth of the few remaining cells in any manner similar to dilute plate cloning in vitro (1, 22, 23). These alternatives remain viable hypotheses, though little has been done to test them. Moreover, it is important to realize that the concept of monoclonal expansion is central to modern thought about the origins of most neoplasms. Thus, in attempting to be consistent with conventional wisdom in atherosclerosis, Benditt's detractors come up against conventional wisdom in tumor biology.

Benditt's detractors were also concerned about the possibility that G6PD, a protein, might inaccurately represent changes at a genomic level. For example, one might imagine that some sort of unexpected mechanism was operating at the level of transcription and translation. Recently, however, Casalone et al used karyotypic analysis and confirmed, in 13 of 18 primary cultures of plaque smooth muscle, that chromosomal rearrangements were present (24). The limitation of karyotypic analysis is that the number of cells studied in any one culture is small. Moreover, even in primary culture, a monoclonal expansion of cells showing some chromosomal rearrangement could be due to selective outgrowth of a small population of cells that had arisen in the plaque. Nonetheless, the homogeneity within each specimen, though not among the different specimens, supports Benditt's observation and suggests that the tissue used to derive these cultures was clonal. The genetic rearrangements seen by Casalone et al may themselves be interesting, as is discussed below.

More extensive evidence for plaque monoclonality has come from our recent studies. We have been able to use the CAG polymorphism of the X-linked androgen receptor to study X-inactivation via DNA methylation patterns. Because our new method is DNA based, we were able to rule out possible artifacts due to protein expression. Microdissection of multiple portions of plaques confirmed, for the first time, that lesions in the coronary arteries are monoclonal, and identified the smooth muscle cell of the fibrous cap as the monoclonal cell (Figure 1*a*–*c*, see color section, pp. C-1, C-2) (20). However, we also found rather large clonal patches even in normal diffuse intima thickening of people over age 40 (Figure 2*a*-*c*, pp. C-3, C-4). Nonetheless, these patches are much smaller than the clones that comprise the fibrous cap of atherosclerotic lesions. Based on these experiments, our conclusion is that the smooth muscle cells of the atherosclerotic plaque in humans are true clones. The critical question is whether the plaque grows from a single cell in the adult artery or by hyperplasia within a preexisting clone. If the latter, then we need to ask if that clone has special properties or if the proliferative process occurs randomly within preexisting patches.

MECHANISMS OF CLONALITY: EMBRYOLOGIC ORIGINS OF VASCULAR SMOOTH MUSCLE

The most important layer for our discussion is the intima, the layer where atherosclerotic lesions arise. Studies suggest that atherosclerotic plaques may begin in preexisting intimal cell masses (19, 25–27). If these masses are already clonal, then the atherosclerotic plaque could be clonal because it arises in preexisting intimal clones. Thus, clonality could be the result of developmental processes. Before considering mechanisms of clonality, we consider the formation of the smooth muscle cells coats of the vessel wall.

The origin of the mesenchymal cells giving rise to smooth muscle is poorly understood. While endothelial cells of the vascular tree and endodermal cells arise by gastrulation and form primitive tubular structures, the remaining organ structures arise by branching morphogenesis or angiogenesis of the primary endothelial or endodermal tubes. In contrast, smooth muscle cells seem to arise locally from the surrounding mesenchyme (27–31). This origin may have profound implications for localized differences, including the localization of atherosclerotic lesions. For example, all mesenchyme of the head and neck, including smooth muscle of the vessel wall, is derived from ectoderm (so-called mesectodermal cells) rather than mesoderm, as in the lower body (32). These mesectodermal smooth muscle cells have unique properties, including higher synthesis of elastin (33–36). This difference in synthesis implies that there are genetic differences between mesodermal and mesectodermal smooth muscle. Such differences could explain the localization of atherosclerotic lesions to specific sites or specific layers of vessels. Moreover, Cunha et al showed that the matrix produced by the cells underlying other epithelia determine the phenotype of the epithelial cells. It is intriguing to wonder if the extent of local differentiation of smooth muscle cells determines the phenotype of the overlying endothelium in a similar manner (37).

The first hints of which factors are involved in the recruitment of smooth muscle cells to the vessel wall come from recent studies of knockout mice. In the platelet-derived growth factor (PDGF) system, deletion of the PDGFβR leads to a neonatal lethal phenotype (38–40). The reasons for this are not entirely clear, but it seems to be the result of a localized hemorrhage in the smallest arteries that lack mural smooth muscle or pericytes, the form of smooth muscle cells forming a single cell layer around the smallest blood vessels (31, 41). This lack of pericytes might suggest that pericytes arise by migration from the smooth muscle cells of the larger vessels along with endothelial cells, forming smaller vessels during angiogenesis. However, a recent study with tetraparental, chimeric mice made from PDGFβR knockout and wild-type cells suggests that PDGFβR has an even wider role. In animals chimeric for this receptor, all vascular smooth muscle, visceral smooth, cardiac, and skeletal muscle show a marked enrichment in the wild-type smooth muscle cells with the PDGFβR (D Bowen-Pope, JR Crosby, RA Seifert, P Soriano, personal communication). Thus it is likely that this receptor plays a critical role in some early step of the recruitment of smooth muscle progenitors to surround the endothelial or epithelial tubes. This could be induction of migration of mesenchymal cells leading to their aggregation around the epithelium, or it could be an effect on proliferation.

If preexisting intimal masses are the progenitor of the plaques, we still have two problems: How do the masses arise, and what are their special properties

that encourage atherogenesis? Essentially nothing is known about the first question. The clonal origins of intimal cells could reflect trapping of rare cells during early embryogenesis, migration of rare cells across the internal elastic lamina at later times, or, as originally proposed by Benditt, a mutation that for some reason only occurs in intimal cells. Williams & Tabas addressed the latter question: the unique properties of the intimal mass that contribute to atherogenesis. They suggested that the critical property is synthesis of proteoglycans that trap lipoproteins (42). Interestingly, we found that a subset of smooth muscle cells overexpresses versican, a proteoglycan that is concentrated in plaque and binds lipoproteins (43). Finally, it is possible that plaques localize in the intima because of a localized immunologic response (44). Bobryshev & Lord showed that a subset of intimal cells contain markers characteristic of the dendritic cells seen elsewhere in the body (45). Such cells, like endothelial cells or macrophages, can process antigen and might initiate immune responses in the plaque (46).

While these hypotheses for the origin of the plaque/clone in the intima differ in many ways, they share one important feature: They all depend on the assertion that intimal smooth muscle cells have features distinct from medial smooth muscle cells. It could be that the origin of clones and the focality of lesions reflects the diversity of the intimal smooth muscle cells themselves. A recent paper by deRuiter et al suggests a unique hypothesis about how such diversity might arise. They present evidence that endothelial cells can delaminate and move into the subjacent vessel wall (47). While the event they observed was quite early, Krug et al (48) and Sugi & Markwald (49) described something similar for the origin of cells in the endocardial cushions. Perhaps the endothelial cells give rise to subpopulations of intimal cells (47), and these comprise a special subpopulation that provides the cells with the unique properties necessary for localization of atherosclerotic lesions. The origin of such unique cells, moreover, may not be restricted to the local endothelium. A number of studies in the transplantation and marrow literature suggest that peripheral blood contains CD34-positive cells with the potential to form several cell types, including smooth muscle cells, as well as endothelial cells (50–53). Moreover, a recent study of meter-long, impermeable vascular grafts showed what appeared to be isolated colonies of endothelial cells and smooth muscle cells forming localized intimal masses (54). Thus, reconsidering conventional wisdom regarding the origin of the intima from the media may have critical implications for the early events leading to ontogeny and localization of atherosclerotic plaques.

We know little about the properties of intimal cells or about their origins, despite the extensive evidence that these unique cells provide the site of origin of atherosclerotic plaques. There is a literature on the formation of the intima of the ductus arteriosus, and it seems clear that this process, which occurs spontaneously and before birth, begins with the formation of large amounts of

hyaluronic acid in the intima, followed by migration of medial smooth muscle cells into the resulting proteoglycan-rich lake (55–58). While it seems likely that migration plays a similar role in formation of the intima at sites prone to form atherosclerotic lesions, we currently lack experimental or genetic data in vivo and even have little descriptive data to allow us to speculate on how this important tissue is formed.

MECHANISMS OF CLONALITY: WHEN DOES CLONALITY ARISE?

If the plaque smooth muscle is clonal, does this clonality contribute to the etiology of the lesions? A critical question is whether clonality precedes or follows development of recognizable atherosclerotic lesions. Pearson et al (59) sampled 6-mm segments of nonatherosclerotic aortic intima and, using Benditt's G6PD method, showed them to be polyclonal. This suggests that monoclonality arises during the process of atherogenesis. However, Mikawa & Fischman used viral tagging to look at the origins of the coronary arteries in birds. Their data show an intriguing spiral pattern of labeled cells, suggesting that clonal expansion might occur during the development of the media (60). The resolution of their images and the frequency of tagged cells, however, make this difficult to prove. Perhaps more relevant to the issue of human lesions is a DNA-based method we developed that employs polymerase chain reaction (PCR) amplification of microdissected portions of sections through plaques or vessel walls (20). Our recent data, taking advantage of the much higher spatial resolution possible with microdissection and PCR amplification of the CAG polymorphism, show patch sizes as large as 4 mm in diffuse intima thickening of arteries from patients more than 40 years old (Figure 2*a*–*c*, pp. C-3, C-4). Thus clonal expansion is a normal property of the intima and is even seen, though more rarely, in the media.

At this point, we do not know how early in vascular development clones or areas of the intima with large patch sizes appear. However, limited data from morphometric studies in humans show a rapid rate of growth of the intima over the first six months of life in the left anterior descending coronary artery (LAD), a site likely in later life in humans to develop atherosclerotic lesions (25, 42, 61–63). Our preliminary data (Figure 3, p. C-5) suggest an even more rapid process occurring near birth, and it is already known that another intima, the intima of the ductus arteriosus, develops over a few weeks, spontaneously, and before birth (55). If these early intimal masses are clonal, clonality could be a very early event if the masses form by replication with little migration from the media.

If the intima has large clones, these may be the site of origin of atherosclerotic lesions. Thomas and coworkers claimed, on the basis of elegant cell ki-

netic studies, that atherosclerotic lesions in fat-fed swine developed in preexisting intimal cell masses (64). These data also showed that sizable lesions arose with only a few cell divisions, consistent with origin from a preexisting clone. Interestingly, when this same group fat fed hybrid hares heterozygous for an X-linked marker, one animal showed monoclonal foci in its lesions (22). Finally, Casalone's studies (as cited in 24) showed monoclonal chromosomal rearrangements in smooth muscle cells cultured from plaques. While they suggested that chromosome instability may be a secondary change in a tissue made hyperplastic by response to mechanical and biological injury, the data are hard to interpret unless the clonal event is very early, since any other sequence would not be expected to produce homogenous chromosomal rearrangements within cells from each plaque.

USE OF CLONALITY TO DETERMINE WHEN INTIMAL CELLS PROLIFERATE

Regardless of the mechanism leading to the clonality of atherosclerotic lesions, clonality implies that smooth muscle cells comprising the plaque must have undergone many rounds of cell replication. This is true even if one posits that the clonality arises as amplification of cells surviving rounds of cell death (64). The time course of proliferation or death, however, is unclear. Studies using both ex vivo labeling with thymidine and measurements of the frequency of replicating smooth muscle cells by PCNA staining or in situ hybridization for [H^3]histone have all shown the same thing: The incidence of replication in both early and advanced atherosclerotic lesions is low (3–8). If replication is not prominent at later stages of lesion development, then clonal expansion may be a very early event.

The observations of low levels of replication in advanced plaques combined with evidence that the wall has the ability to develop large clonal patches even without atherosclerosis suggest that monoclonality could be the result of a proliferative burst in the intima over the first few months of life. Preexisting clonal patches could occur by proliferation of a very few cells trapped in the intima. If this is the way the intima generally forms, we would expect clusters of clonal intimal cells, patches, to be large in the earliest accumulations of intimal cells at sites where we know atherosclerosis will develop in later life. The most obvious such site is the intima of the LAD (26, 65). In a collaborative study of neonatal human LAD coronary arteries with Bruce McManus (Figure 3, p. C-5), about 40% of these vessels showed focal, highly smooth muscle–rich intimal masses within a few days of birth. We have seen only rare intimal masses prior to birth, suggesting that the intima of the LAD, like the intima of the ductus, may form rapidly and spontaneously. In a mass like that in Figure 3 (p. C-5),

only a few doublings would be required to account for the mass of the final plaque. The question is, are masses like that already clonal?

A variation of this developmental theme occurs if we imagine that the intima is polyclonal but contains rare cells able to proliferate in response to injury or to survive episodes of cell death occurring during plaque development (22). Pearson et al, for example, found that samples of fatty streaks were occasionally skewed toward one X allele, suggesting clonal expansion might be occurring in these early lesions (59). This observation is surprising given the prevalence of leukocytes in fatty streaks and suggests a rapid clonal expansion of the smooth muscle cells. If clonal response to injury is a typical response of the vessel wall to injury, the formation of monoclonal lesions might be expected even following injury. Interestingly, Pearson et al also described development of clonality in an organizing thrombus, perhaps due to the invasion of the thrombus by rare cells able to proliferate or resist cell death in that environment (66).

The idea of clonal expansion as a response to injury during plaque evolution is not necessarily inconsistent with the data showing low replication rates in plaques (3–6, 8, 64). Replication might occur episodically, or indolent replication occurring over many years might account for substantial lesion growth. Moreover, Thomas, Pearson, and their collaborators have suggested that clones could arise by selection in the presence of extensive cell death in a plaque (19, 22, 67). Their suggestions were based on kinetic studies or analysis of development of clonal drift in lesions. Just such an incidence of cell death is suggested by recent reports of apoptotic cells in atherosclerotic plaques (68–72). While the emphasis in these studies has been on a role for apoptosis in plaque rupture (68), it is also conceivable that cell death plays key roles in earlier stages of lesion progression via selection or via clonal isolation as a result of repeated episodes of cell death.

ARE PLAQUE SMOOTH MUSCLE CELLS DIFFERENT FROM MEDIAL SMOOTH MUSCLE?

If plaques were to arise by some sort of mutation, then the plaque smooth muscle cell, like other neoplastic cells, might be expected to have properties that distinguish it from normal, medial smooth muscle cells. In fact, about 80 genes have been reported as showing differential expression between the plaque smooth muscle cell and the medial smooth muscle cell in vivo (27). Most of these have been found as a result of informed guesses about genes likely to be important in processes posited to be important within the plaque. Because the plaque is a highly inflamed environment, however, such plaque smooth muscle–specific gene expression may simply reflect the presence of cytokines in the milieu. This could be especially relevant, for example, to the expression of tissue fac-

tor in the lesion (73). Tissue factor is believed to be critical to the plaque's procoagulant properties; however, it seems likely that the overexpression is the result of action of cytokines released by plaque macrophage (74).

We have looked in the plaque for several specific genes of possible relevance to forming a unique intimal cell type. These include two Hox genes, B7 and C9, identified by library screening as distinguishing human fetal from adult cells (75); β3 integrins known to be required for migration of human smooth muscle cells on osteopontin, a gene we had previously cloned as showing differential expression in a subset of rat smooth muscle cells and in atherosclerotic plaques (76–80); transforming growth factor (TGF)-β and a TGF-β–inducible gene, "βIG" (81); p53; H19, a developmentally expressed but nontranslated mRNA (82); cytomegalovirus; nonmuscle myosin; and versican in plaques or restenotic tissue (83–86). The results of these efforts have been mixed. The HOX genes, β3 and β2 integrins, nonmuscle myosin, and p53 did not show differential expression in plaque smooth muscle. H19, versican, TGF-β, and βIG were overexpressed; however, there are a lot of genes overexpressed in the plaque, and the role of cytokines in control of transcription of these genes is not known.

Another approach has been to compare expression in smooth muscle cells from two-week-old rats (pup cells) with gene expression in cells from the adult rat. Table 1 shows the result of this effort. Although several of these genes show differential regulation in the smooth muscle cells forming a neointima, only three of the genes identified in this way have turned out to be overexpressed in the human plaque (80, 87).

These studies do not rule out the existence of genes that mark a unique plaque or intimal smooth muscle cell. Indeed, it is somewhat surprising that so little has been done to look at the possibility that such cell type–specific markers exist.

Table 1 Genes differentially expressed by pup vs. adult cells (1992–1996)

PDGF-B	Growth factor	(146)	Osteopontin[a]	Matrix molecule	(147)
p450IA1	Mixed function oxygenase	(148)	Elastin[a]	Matrix molecule	(149)
ZO-2	Tight junction protein	(150)	Type VIII collagen	Matrix molecule	(151)
HoxB7	Homeobox	(152)	Versican[a] (adult)	Matrix molecule	(153)
HoxC9	Homeobox	(152)	Tenascin splice forms	Matrix molecule	(154)
PDGF α rec. (adult)	Growth factor rec.	(153)	FGF R3 (pup)	Matrix molecule	(155)

[a]Overexpression in the human plaques.

MUTAGENIC AND VIRAL MECHANISMS FOR SMOOTH MUSCLE CLONAL EXPANSION

Our data on the presence of large patches as a normal property of the vessel wall does not rule out the possibility that genetic changes, mutations, or viral infections produce intimal cells with a selective advantage. The advanced plaque may be mutagenic as a result of lipid peroxidation and the production of superoxide (88, 89). Apoptosis, a frequent finding in plaques, is characterized by DNA breaks. It is possible these breaks might occur in a form fruste, resulting in strand breaks that could account for the chromosomal rearrangements described by Casalone (see 24; 68, 69, 71, 90–92). Viral presence in the plaque or serologic evidence of infection has also been shown in several studies of humans (84, 93–96). The significance of finding viri in plaque or serologic evidence is often difficult to determine, since the virus may be present as a secondary event or may even be a normal resident of the vessel wall, as appears to be the case for cytomegalovirus despite a recent report suggesting a unique association with restenosis (84, 97). The most impressive evidence for a viral etiology of atherosclerosis is in aves. Studies of Marek's disease in pathogen-free chickens showed that infection with an oncogenic lymphotropic herpesvirus resulted in the development of atherosclerotic lesions (98, 99).

More impressive evidence for a mutagenic origin of smooth muscle clonality comes not from the vessel wall but from the uterus. Uterine leiomyomas are of obvious interest, since monoclonality of the leiomyoma was the reason Benditt looked at plaques in the first place. Moreover, while the uterine leiomyoma is usually considered a neoplasm, the lesions are, like atherosclerotic plaques in all people, present to some extent and frequency in all women. It is reasonable to think of this "tumor" as something normal that gets exaggerated in a certain subset of clinically relevant individuals. Nonetheless, even if these are normal events, there is an abundant literature showing chromosomal rearrangements in uterine leiomyoma. Chromosomal abnormalities reported in uterine smooth muscle cells (SMC) from leiomyomas include deletion on the long arm of chromosome 3, rearrangements at 6p, translocation between chromosomes 6 and 10, deletion at 7q, translocation at 7q22, rearrangements at 10q22, translocations within 12q13-15 to other loci (especially on chromosome 14), trisomy 12, and abnormalities in 13q (24, 100–112). In uterine leiomyomas, these rearrangements are present even in vivo*,* suggesting that they could be causal to the development of the clone that becomes the leiomyoma (105, 113).

The rearrangements most usually discussed are on chromosome 12, region q13-q15 (111). This locus contains a particular gene, HMGI-C, which is frequently rearranged in soft tissue neoplasms (114, 115). The primary transcript of HMGI-C is associated with adipogenesis; however, the gene has a long in-

tron that is fragile and frequently becomes translocated. Apparently the 5' part of the gene transcribes a DNA binding sequence with broad ability to enhance transcription, not as a conventional enhancer, but altering the conformation of DNA (112, 115, 116). Expression of HMGI-C itself is primarily embryonic and, in proliferative cells in vitro, levels in adult tissue are low; to our knowledge, overexpression of the primary transcript has not been identified in a neoplasm in vivo. A second region of interest in leiomyomas is 6pq21-23, which also codes an HMG, HMGI-Y (115, 117, 118). Thus the phenotype induced by a translocation in one of these HMG genes depends on the recipient site; often in tumors induced by these translocations the targets have been transcription factors. Intriguingly, in one case a rearrangement of HMGI-C gene was associated with a myxoid, smooth muscle–derived neoplasm similar in morphology to the tissue seen in restenotic atherosclerotic lesions (114).

Of course mutations could be a secondary event rather than a cause of clonal expansion. Mutations in plaque cells could be an epiphenomenologic result of the environment of the pathologic cell mass. This issue is relevant to studies in human plaque that attempted to identify an elevation of mutational frequency. As already mentioned, Casalone et al reported clonal chromosome abnormalities in primary cell lines from human atherosclerotic lesions (24). They suggested that chromosome instability was secondary to mutagenic properties of the plaque. Similarly, Spandidos et al recently reported an unusually high incidence of somatic mutations (microsatellite instability) in DNA extracted from the plaque (119–121). Finally, a series of papers from Parkes et al have shown that plaque-derived DNA and even DNA derived from cultured plaque smooth muscle cells have enhanced transforming activity for cultured 3T3 cells (117). These intriguing papers are all limited by the preliminary quality of the data, lack of evidence for reproducibility of the clonal patterns from different regions of the plaque, lack of appropriate tissue controls for the complex plaque, and failure of the investigators to determine whether the polymorphisms are in smooth muscle cells or other cells in the plaque. Nonetheless, combined with Casalone's data, evidence of microsatellite heterogeneity, if clonal, would imply that some mutation had occurred early in the ontogeny of the plaque, and the data of Parkes et al would suggest that such a mutation could be able to promote clonal expansion.

Studies implicating mutations in smooth muscle cells would be more convincing if one could demonstrate that some specific genetic change occurs consistently in plaque smooth muscle cells and produces changes likely to explain plaque progression or localization. The obvious approach to identifying such genetic changes is to explore properties of cells in culture: those isolated from local influences that might stimulate expression of specific genes. Paradoxically, plaque smooth muscle cells in vitro have properties opposite to what

might be expected to lead to clonal expansion in vivo. In our work and that of others, these cells grow poorly, have a shortened replicative life span, and show an elevated apoptotic rate relative to smooth muscle cells cultured from the tunica media (4, 9, 70, 122, 123). Of course, this paradox may simply mean that the cultured cells have properties that reflect in vitro selection from the population comprising end-stage lesions, rather than properties of the cells that gave rise to the early plaque.

THE CELL TYPE COMPRISING THE CLONE: MONOCLONALITY OF THE PLAQUE SMOOTH MUSCLE CELL

Until recently we merely assumed that the clone comprising the plaque is comprised of smooth muscle cells. This was not formally proven until this year. Benditt's original method, using protein gels to measure the two allotypes of G6PD, suggested that patch sizes are small (18). However, he could not confine the analysis to areas comprised of single cell types. In 1991, Stemme et al amplified the T-cell receptor sequences in the plaque and showed that the lymphocytes were polyclonal (44). Their data rule out the intriguing possibility that plaques arise as monoclonal autoimmune responses and left the intimal smooth muscle cell as the likely source of clonality.

We decided to develop new methods for analysis of tissue that would avoid the obvious problem that human plaque comprises many cell types (20). An X-chromosome inactivation assay based on the human androgen receptor was adapted (124, 125). The androgen receptor is an X-linked gene with a polymorphic trinucleotide repeat (CAG) in its first exon. Of females, 90% are heterozygotic at this locus. In the immediate vicinity of the CAG repeat are several methylation-sensitive restriction sites. Digestion with a methylation-sensitive endonuclease will destroy the active androgen receptor gene, leaving the methylated, inactive gene intact. The intact gene can then be amplified by polymerase chain reaction (PCR), using primers that flank both the polymorphism and the restriction sites. Thus, analyses can be performed at both a genomic and an mRNA level. If both alleles amplify, it means the original tissue contained a mixture of cells with each X-inactivation pattern and is therefore polyclonal. If only one allele amplifies, the tissue is termed monoclonal. We initially validated the method using uterine leiomyomas and then demonstrated identical results from fresh-frozen samples versus histological sections.

Figure 1*a–c*, (pp. C-1, C-2) shows the X-inactivation pattern in an eccentric fibrocellular plaque. Specific regions of plaque and underlying media were microdissected from paraffin sections. Genomic DNA was extracted and digested with a methylation-sensitive endonuclease. The intact androgen recep-

tor gene was amplified by PCR, and the radiolabeled product was resolved on a sequencing gel. Because we can analyze minute portions of tissue, we were able to localize the monoclonal region of the plaque and identify the cell types present in that region. Digested samples of the media yielded two alleles, indicating that the medial sample was polyclonal. In contrast, samples of the plaque containing predominantly smooth muscle yielded a single allele, indicating that the smooth muscle cells in this lesion were monoclonal. When we sampled the base of the plaque, which contained inflammatory cells and microvessels, the second allele appeared. Similarly, when we sampled the inflamed region on the opposite side of the vessel, both alleles were amplified. This indicated that the intraplaque microvascular endothelial cells and inflammatory cells were polyclonal. To date, we have shown that monoclonality occurs in plaques of the aorta, coronaries, and carotid arteries. Plaques with a mixed X-inactivation pattern appear to be contaminated by inflammatory cells. As expected, X-inactivation patterns are random within a given patient; that is, the two possible monoclonal patterns are present within the same person. This rules out X-linked selection of parental cells for the growth of atherosclerotic plaques. Randomness of X-inactivation also rules out a common progenitor for all plaques, for example via some metastasis-like mechanism.

HOW DOES VESSEL WALL CLONALITY ARISE AND DOES IT MATTER?

As the emphasis on proliferation as a key event in atherosclerosis has decreased, it has become common to read that smooth muscle migration is the critical step of the formation of atherosclerotic lesions. Most of this concept is based on animal models of balloon injury to normal vessels. The normal vessels of our species, however, spontaneously, without any obvious injury, form an intima at sites likely to go on in a person's later life to form the atherosclerotic lesion (26, 27, 42). As we suggested, this intima may already be clonal, i.e. having a large patch size. If so, then clonality may be an epiphenomenon, true but not clinically relevant. The question of how clonality arises, therefore, is likely to be critical to determining whether the properties of these clones are significant to our understanding of the disease process.

We have considered five possible ways clonality might arise.

Migration/Trapping Hypothesis

Migration across the IEL and trapping of rare cells during development could produce intimal clones that eventually become plaques. This is consistent with the appearance of intimal masses, as in Figure 3 (p. C-5), and with several studies by pathologists that relate the distribution of intimal masses in neonatal or in-

fant arteries to the distribution of atherosclerotic lesions in adults (25–27, 62, 65, 126–131). This is an example of a developmental origin that may well be critical to the pathogenesis of the lesion without itself being a therapeutic target.

Stem Cell Hypothesis

The vessel wall might contain rare stem cells with the ability to replicate. This is similar to the mutation hypothesis (below), except that it supposes that the subset arises developmentally without a need for mutation or viral effect. Of course stem cells might give rise to intimal masses as well, especially given the recent evidence (as reviewed above) that intimal cells could arise from circulation.

There is very little evidence that such stem cells exist. However, it is important to note, as we discussed above, the complex origins of vascular smooth muscle cells. This leads us to believe that the vessel wall comprises diverse smooth muscle subtypes, and it is possible that one or more of these have special properties that could give rise to a clonal expansion (32). Perhaps the most impressive evidence for such diversity is in the pulmonary artery, where even the medial smooth muscle cells belong to two distinct phenotypes that may contribute in distinct ways to proliferative responses to injury (132).

Of course, if plaques arise from a unique subset of smooth muscle cells, the properties of those cells could comprise important therapeutic targets, as suggested by Williams & Tabas (42).

Apoptotic Selection Hypothesis

Clones could arise as a result of cell death, with expansion of rare cells having a selective advantage in the ability to survive death stimuli.

Our own 20-year-old data in the developing intima of the rat showed high rates of spontaneous cell death (133). Recent as well as older studies document extensive cell death in plaques (68–71, 133–135), and Janakidevi et al reported that feeding rabbits cytotoxic oxidized sterols accelerated the drift of these experimental lesions toward clonality (22).

Paradoxically, we found that plaque smooth muscle cells have an elevated apoptotic rate in vitro (70). The relevance of this phenomenon to clonal expansion in vivo is hard to explain, especially since the in vitro behavior is p53-sensitive and associated with replication (136). Perhaps the cells that comprise the plaque are postmitotic, and those cells we obtain in vitro represent a subset that has not been selected because it has not been made to replicate. In support of this line of reasoning, numerous studies of cultured plaque smooth muscle cells have shown that these cells have a shortened replicative life span and were able to lengthen this by transforming the plaque cells with the antiapoptotic oncogene, bcl2 (70, 122, 123, 137, 138).

Apoptosis in the plaque is also, potentially, an important issue in the ultimate breakdown of the fibrous cap leading to plaque rupture (139).

Organizing Thrombus Hypothesis

Clones could arise in organizing thrombus if only a few of the immigrating cells survive and proliferate. Clonality arising in an organized thrombus has been reported (67). This could, however, be consistent with invasion of a thrombus by cells from a preexisting intimal clone.

There is no obvious therapeutic significance to this mechanism.

Mutation Hypothesis

The atherogenic environment might produce clones as a result of a rare mutagenic or viral event permitting expansion of cells. These cells might have a selective advantage either in ability to replicate or in ability to survive death stimuli. This is the usual interpretation of clonality in a cell mass.

This is certainly the most intriguing hypothesis for many reasons. Perhaps the most important is the possibility that such mutations, like those frequently associated with cancer, may occur by loss of heterozygosity and therefore lead us to identify new genetic traits that contribute to lesions predilection.

Table 2 summarizes the implications of the five hypotheses.

Table 2 Predictions of clonality based on alternative mechanisms of clonality

	Migration trapping	Stem cell	Apoptotic selection	Organizing thrombus	Mutation
When clonality arises	Before plaque	Before plaque	After plaque	After plaque	Concomitant with or after plaque starts
When intimal SMC replicate in intima or plaque	Early in development	Unclear; probably chronically	Chronically during plaque formation	In organizing thrombus	After plaque starts
Special properties of cultured SMC	Not necessarily	Yes, cells in vitro may show enhanced growth or resistance to death	Yes, unless selection is severe and random	Not necessarily	Yes
Present in vascular responses to trauma	Maybe, if only intima contributes	Yes	No	Depends on kind of trauma	No
Therapeutic significance?	Not obvious	Possible	Likely	Not obvious	Likely

SUMMARY

The importance of smooth muscle proliferation to the ultimate clinical outcome of atherosclerotic plaque may depend on which of these five hypotheses explains clonality. For example, several of the hypotheses might be called developmental; that is, they suggest that monoclonality is an inevitable result of the processes forming the vascular intima or, perhaps, expansion of a stem cell population. These developmental hypotheses may only be significant insofar as they explain the localization of lesions. It seems likely that the process shown in Figure 3 (p. C-5) is an early event in the process of atherogenesis, albeit an event that may not be targetable clinically if it occurs in all of us. Nonetheless, it is intriguing to read papers on the distribution of intimal masses in children as a predictor of lesion localization and severity in the adult (19, 26, 42, 65, 126, 127).

In contrast, the mutation, apoptosis, and stem cell hypotheses may lead us to speculate on special properties of the plaque smooth muscle cell that could contribute to lesion outcome. For example, there is reason to believe that smooth muscle proliferation is itself an important clinical target at a critical stage of the disease: the formation of lesions that lead to premature, sudden cardiac death where the predominant histological feature is intimal hyperplasia, not lipid accumulation (140–142). Moreover, it is intriguing to note the requisite role of the intima in closure of the ductus arteriosus and to wonder whether intimal cells in other sites may contribute to the pathologic narrowing that occurs as plaques progress, apparently independently of the mass of the plaque itself (55, 143).

We need to finish with a caveat. Animals have, to date, failed to demonstrate monoclonality (19, 27, 59, 67, 144, 145). Does this mean that the processes studied in such models are irrelevant to the process forming an intima in humans?

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Figure 1 X-inactivation patterns in a coronary atheroma. (1a) Hematoxylin (H)- and eosin (E)-stained section of the coronary artery of a patient with dilated cardiomyopathy. There is a fibrocellular atherosclerotic plaque on the lower half of the artery wall. The upper portion contains a lipid-rich region with numerous foam cells. Note the medial atrophy underlying the plaque in the lower half of the artery.

(1b) Microdissection map showing regions of the artery wall taken for X-chromosome inactivation analysis from a serial 50-thick section. Immunostains (not shown) demonstrated that segments 1 (media) and 2 (fibrous cap) contained virtually no endothelial or inflammatory cells. Inflammatory cells were present in the base and in calcified regions of the plaque (segments 3-5), as well as in the foam cell-rich region of the upper plaque (segments 6 and 7). (continued next page)