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# Monoclonality of Smooth Muscle Cells in Human Atherosclerosis

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**Atherosclerotic plaques contain a large monoclonal population of cells. Monoclonality could arise by somatic mutation, selection of a pre-existing lineage, or expansion of a pre-existing (developmental) clone. To determine the monoclonal cell type in plaque and learn when monoclonality arises, we studied X chromosome inactivation patterns using methylation of the X-linked human androgen receptor gene. Assays based on polymerase chain reaction were performed on samples of known cellular composition, microdissected from histological sections of human arteries. In atherosclerotic vessels, the majority of medial samples (7/11 coronary and 2/3 aortic) showed balanced (paternal and maternal) patterns of X inactivation, indicating polyclonality. In contrast, most samples of plaque smooth muscle cells showed a single pattern of X inactivation (3/4 aortic plaques and 9/11 coronary plaques; $P < 0.01$ *versus* media), indicating that plaque smooth muscle cells are monoclonal. Samples of plaque containing inflammatory or endothelial cells showed balanced X inactivation, also demonstrating polyclonality. Multiple plaques from a given patient showed no bias toward one allele, indicating there was no X-linked selection of cells during plaque growth. To determine whether plaques might arise from pre-existing clones (large X inactivation patches), we then studied 10 normal coronaries with diffuse intimal thickening. Six of the ten coronaries showed skewed X inactivation patterns in normal media and intima, suggesting the patch size in normal arteries is surprisingly large. Thus, smooth muscle cells constitute the monoclonal population in atherosclerotic plaques. The finding that normal arteries may have large X inactivation patches raises the possibility that plaque monoclonality may arise by expanding a pre-existing clone of cells rather than generating a new clone by mutation or selection. *(Am J Pathol 1997, 151:697–706)***

Atherosclerotic plaques are generally considered to arise by proliferation of cells within the arterial intima in response to injury.[1] Although this notion is widely held, there is very little direct evidence in humans. For example, although fat-fed swine or rabbits show transient smooth muscle cell replication,[2–4] studies of human lesions show that replication rates are low (<1%) in the vast majority of plaques.[5–7] The best evidence that atherosclerosis involves cell proliferation comes from the observation that most plaques contain a large monoclonal population of cells.[8–11] Although the time course of monoclonal expansion is unknown, monoclonality can result only from many generations of cell replication.

Evidence for monoclonality in atherosclerosis comes from studies of X chromosome inactivation patterns. According to Lyon's hypothesis, X chromosome inactivation occurs randomly during early embryogenesis, and all progeny of a given cell inactivate the same X chromosome (either paternally or maternally derived). Lesions resulting from the proliferation of multiple cells, such as healing skin wounds, exhibit a mixed pattern of X chromosome inactivation.[10] In contrast, lesions resulting from the proliferation of a single cell, such as human tumors, exhibit a monotypic pattern of X inactivation (ie, maternal or paternal but not both).[12] Studies using the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) to determine X chromosome inactivation patterns showed that, whereas aortic media contained a mixed pattern of G6PD expression, most atherosclerotic plaques contained a single isoform of G6PD.[8–11] These data imply that atherosclerotic plaques arise from single progenitor cells.

Although plaque monoclonality has been confirmed several times since its discovery over 20 years ago, there has been little progress in understanding its pathogenesis. We do not yet know when monoclonal expansion occurs. Similarly, we do not know what cell type makes up the clone, although immunological studies have ruled out the plaque lymphocyte.[13] Progress has been slow

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This manuscript is dedicated to the memory of Dr. Earl Benditt, who died on May 27, 1996. Dr. Benditt was a trusted mentor and friend who contributed much to the scientific community. He will be greatly missed.

**Table 1.** Patient Characteristics

| Case | Age | Autopsy or transplant | Underlying disease | Sample location and number | Medial X-inactivation (% allele 1) | Intimal X-inactivation (% allele 1) |
|---|---|---|---|---|---|---|
| 1 | 45 | Autopsy | Myotonic dystrophy with DVT/PE | Aortic plaque | N/A | Allele 2 (14%) |
| 2 | 84 | Autopsy | Alzheimer's disease with bronchopneumonia | Aortic plaque | Balanced (49%) | Allele 1 (86%) |
| 3 | 56 | Transplant | Dilated cardiomyopathy | Aortic plaque 1 | Balanced (38%) | Allele 2 (22%) |
| | | | | Aortic plaque 2 | Allele 2 (22%) | Balanced (49%) |
| | | | | RCA plaque 1 | Balanced (53%) | Allele 2 (2%) |
| | | | | RCA plaque 2 | Balanced (68%) | Allele 1 (81%) |
| | | | | LCX plaque | Allele 2 (28%) | Allele 2 (7%) |
| 4 | 41 | Transplant | Dilated cardiomyopathy | LAD plaque | Allele 2 (29%) | Allele 2 (21%) |
| | | | | LAD DIT | Balanced (58%) | Balanced (65%) |
| | | | | RCA DIT | Allele 1 (74%) and Allele 2 (30%)* | Allele 2 (27%) |
| 5 | 57 | Transplant | Ischemic heart disease | LCX plaque 1 | Balanced (36%) | Allele 2 (2%) |
| | | | | LCX plaque 2 | Allele 2 (19%) | Allele 2 (24%) |
| | | | | LAD DIT | Allele 2 (11%) | Balanced (42%) |
| | | | | LCX DIT | Allele 2 (18%) | Allele 2 (28%) |
| 6 | 39 | Transplant | Dilated cardiomyopathy | LAD plaque | Allele 1 (98%) | Allele 1 (90%) and Allele 2 (15%)* |
| | | | | LCX plaque | Balanced (57%) | Balanced (61%) |
| | | | | RCA DIT 1 | Balanced (41%) | Balanced (52%) |
| | | | | RCA DIT 2 | Balanced (56%) | Allele 1 (77%) |
| | | | | RCA DIT 3 | Allele 1 (89%) | Allele 1 (96%) |
| 7 | 53 | Transplant | Dilated cardiomyopathy | LAD plaque | Balanced (65%) | Allele 2 (18%) |
| | | | | RCA plaque 1 | Balanced (51%) | Allele 1 (71%) |
| | | | | RCA plaque 2 | Balanced (48%) | Balanced (50%) |

LAD, left anterior descending coronary; RCA, right coronary artery; LCX, left circumflex coronary; DIT, diffuse intimal thickening; DVT, deep venous thrombosis; PE, pulmonary embolism; N/A, data not available due to failure of PCR amplification.
*These cases showed separate regions skewed to allele 1 and allele 2.

due, in part, to limitations in the G6PD assay. G6PD analysis is performed on protein extracts of grossly dissected tissue samples. As atherosclerotic plaques contain multiple cell types, including smooth muscle cells, endothelial cells, macrophages, and T cells, it is not possible to determine which cell type was detected as the monoclonal population. Furthermore, gross dissection does not provide the resolution required to analyze very small or developing lesions. Poor resolution therefore hinders determining when monoclonal expansion takes place. Finally, the population that can be studied is very limited. Heterozygosity at the G6PD locus occurs only in one-third of African-American females, limiting availability of tissue and restricting analysis to a small subset of the general population.

The current study circumvented many of the G6PD limitations by using a polymerase chain reaction (PCR)-based assay for X chromosome inactivation. This method can be applied in a semi-histological fashion to regions of plaque microdissected from histological sections. The assay determines methylation patterns of the human androgen receptor gene, a highly polymorphic locus on the X chromosome for which 90% of women are heterozygous.[14] Our data confirm the presence of monoclonal cells in aortic plaques, extend the finding to the coronary artery, identify smooth muscle cells as constituting the monoclonal population, and provide the unexpected finding that normal arteries may have large patches where X inactivation is skewed in the same direction.

## Materials and Methods

### Tissue Acquisition and Immunohistochemistry

Data for patients enrolled in the study are given in Table 1. Samples of aorta were obtained from female patients at autopsy or in one case after cardiac transplantation. The endothelium was wiped away with gauze, and samples containing grossly evident atherosclerotic plaques were fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid). Samples of coronary arteries were obtained from the explanted hearts of transplant recipients and fixed in methyl Carnoy's solution. After routine processing and paraffin embedding, histological sections were examined, and samples containing fibrous plaques or diffuse intimal thickening were chosen for further analysis. After determining homo- or heterozygosity at the androgen receptor locus (see below), 4-$\mu$m sections were immunostained with antibodies to mark smooth muscle cells (IA4, anti-smooth muscle $\alpha$-actin; Dako, Carpinteria, CA[15]), macrophages (KP1, anti-CD68; Dako[16]), T cells (anti-CD3; Dako), and endothelial cells (*Ulex europaeus* lectin; Vector Laboratories, Burlingame, CA[17,18]), as recently described.[19] A map of the artery was drawn recording the distribution of cell types.

### Microdissection

After obtaining sections for immunostaining, a piece of transparent cellophane shipping tape was placed over

the surface of the block and an adjacent 50-μm section was cut. The microtome was carefully cleaned between blocks to avoid carryover of tissue contaminants, and a new blade was used with each patient sample. The 50-μm section was attached to a glass slide using the tape and examined under a dissecting microscope. When transilluminated, it was possible to distinguish clearly the lumen, intimal, medial, and adventitial layers as well as landmarks such as adventitial nerves and vasa vasorum, collagen- or elastin-rich regions, and calcification. With the immunostained sections as a guide, samples were dissected by hand from the 50-μm section using a number 11 scalpel. The scalpel blade was always changed between slides and sometimes between samples when contamination was evident.

In diseased arteries, samples were obtained from smooth-muscle-rich regions of the atherosclerotic plaque, including the fibrous cap, base, and shoulder, as well as from the media. The size of the plaque samples varied, depending on the anatomy of the lesion and the amount of inflammation present. Typical plaque samples were ~1.0 × 0.5 mm from the aorta and ~0.6 × 0.3 mm from coronary arteries. In most cases, the media immediately underlying the plaque was sampled; occasionally, severe medial atrophy necessitated sampling media from the opposite side of the artery. The medial samples were matched in length to the plaque samples but were nearly full thickness. In arteries with diffuse intimal thickening, samples were obtained as full-thickness intima or media but were smaller laterally (~0.4 × 0.2 mm) to provide better spatial resolution. The endothelium was excluded from the luminal surface of coronary samples, and all dissections excluded the adjacent layer; eg, small amounts of media were left behind at both the intimal and adventitial borders. In the initial phases of this study, extensive microdissections were performed to include inflamed and vascularized regions of the plaque. Later phases focused on smooth muscle cells. The location of the microdissected samples was recorded on hand-drawn arterial maps.

## *Analysis of X Chromosome Inactivation*

The androgen receptor contains a trinucleotide (CAG) sequence in its first exon, repeated from 14 to 31 times.[14] Approximately 90% of all females are heterozygous at the androgen receptor locus, thus permitting distinction between the paternally and maternally derived X chromosomes. The CAG length polymorphism is closely linked to several methylation-sensitive restriction sites, allowing discrimination between active (unmethylated) and inactive (methylated) loci. X chromosome inactivation patterns are determined by first digesting genomic DNA samples to cut the active gene (rendering it unsuitable for PCR amplification), followed by PCR amplification of the inactive gene. When both X-linked alleles are approximately equally represented, the tissue is classified as polyclonal, but when the population is significantly skewed toward a single allele, the tissue is considered monoclonal.

In practice, the microdissected samples of paraffin-embedded tissue were placed in a DNA extraction buffer containing 50 mmol/l Tris, pH 8.0, 0.5% sodium dodecyl sulfate, 1 mmol/l EDTA, and 100 μg/ml proteinase K.[20] Preliminary studies showed that paraffin extraction with xylene was not necessary. The samples were incubated at 55°C for 48 hours, with fresh proteinase K added after the first 24 hours. The solution was extracted once with an equal volume of phenol, once with phenol/chloroform/isoamyl alcohol (25:24:1), followed by chloroform/isoamyl alcohol (24:1). To remove potential metal ions, the solution was mixed with 0.24 vol of 25% ion exchange resin (Instagene, BioRad, Richmond, CA). The DNA was precipitated with ammonium acetate/ethanol, using 100 μg of glycogen (Boehringer Mannheim, Indianapolis, IN) as carrier. The pellet was washed with 70% ethanol and resuspended overnight at 4°C in ~25 μl of water.

For restriction digestion, 2-μl samples of DNA were digested overnight at 37°C with 10 U of the methylation-sensitive endonuclease *Hpa*II (40 U/μl; Boehringer Mannheim) in a reaction volume of 2.5 μl. Separate aliquots of undigested DNA were prepared and incubated in the same solution without enzyme as controls for each sample. After heat inactivation of the endonuclease, the entire reaction volume was used as template for PCR analysis. PCR was performed with a $\gamma^{32}$P-end-labeled sense primer with the sequence 5′ TCCAGAATCTGTTCCAGAGCGTGC 3′ and an unlabeled antisense primer with the sequence 5′ TGGGGAGAACCATCCTCACC 3′. Each 25-μl PCR reaction contained 0.625 U of *Taq* polymerase (Promega, Madison, WI), 1 μmol/L PCR primers, 200 μmol/L dNTPs, 1 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris, pH 9.0, 0.1% Triton X-100, and 3% dimethylsulfoxide. After a 5-minute denaturation at 94°C, amplification was accomplished by 40 cycles consisting of denaturing at 94°C, annealing at 67°C, and extension at 72°C, each for 30 seconds. Aliquots of 5 μl from each PCR product were heat denatured and electrophoresed on a 7% polyacrylamide sequencing gel containing 42% urea for 4.5 hours at 32 W. The gels were dried and autoradiography performed at −70°C.

## *Quantitative and Statistical Analysis*

Autoradiograms were scanned on a Hewlett Packard model 3C/T flatbed scanner, and allele intensities were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Data are expressed as the percentage of the number 1 (upper) allele. To obtain objective criteria for classifying a sample as monoclonal, a series of 17 uterine leiomyomas were examined. Leiomyomas are benign, smooth muscle tumors, which have long been known to be monoclonal.[12] We defined samples as monoclonal when they fell within 2 SD of the values for leiomyomas (0 to 30% allele 1 for type 2 tumors and 70 to 100% for type 1 tumors; data not shown). A $\chi^2$ analysis was performed to determine whether skewing rates differed significantly between samples of media and atherosclerotic plaque. A $P$ value $<0.05$ was considered statistically significant.

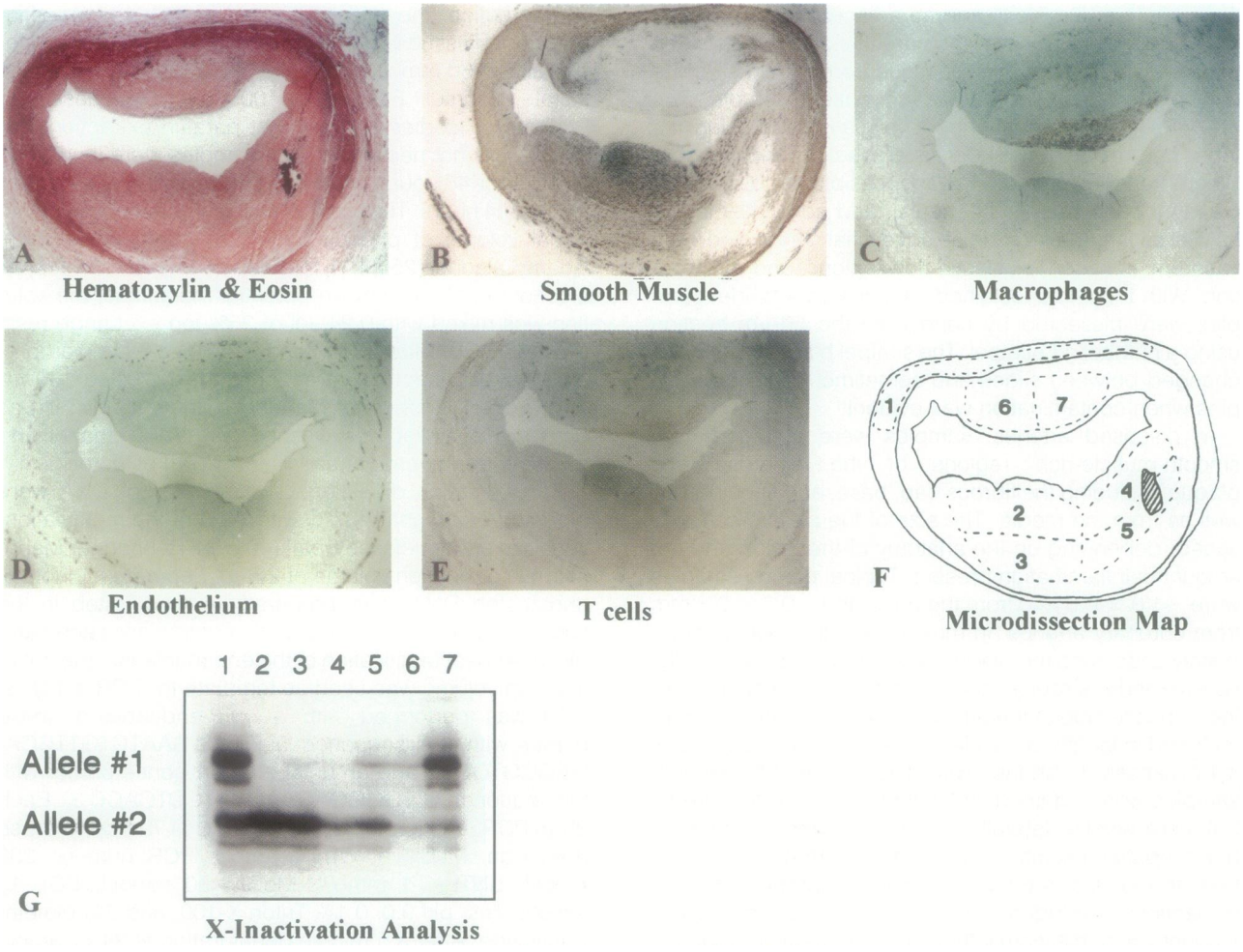


**Figure 1.** X inactivation in a coronary atheroma. **A:** H&E-stained section of a right coronary artery from a patient with dilated cardiomyopathy (patient 3 in Table 1). There is an eccentric, fibrocellular plaque in the lower half of the artery, associated with atrophy of the underlying media. The base of the plaque contains inflammatory cells and vasa vasorum. There is focal calcification (purple nodule). The upper aspect of the vessel is inflamed and contains lipid-laden foam cells, seen better at higher magnification. Adjacent sections were immunostained in **B** to **E**. All original magnifications, ×63. **B:** Smooth muscle cells identified by staining with anti-smooth muscle α-actin. The media and fibrous plaque are rich in smooth muscle cells. Note the α-actin-negative regions associated with inflammation. **C:** Macrophages identified by staining with anti-CD68 antibodies. The foam-cell-rich region in the upper aspect of the intima is rich in macrophages. Macrophages are also present at the base of the lower, fibrous plaque near the vasa vasorum and in the α-actin-negative region near the calcification (seen better at higher magnification). **D:** Endothelial cells identified by staining with the lectin *Ulex europaeus*. Endothelial cells are present at the luminal surface and also within the vasa vasorum of the plaque and adventitia. **E:** T cells identified by staining with anti-CD3 antibodies. T cells are present in the foam-cell-rich region of the upper lesion, in the base of the fibrous plaque in the lower aspect (seen better at higher magnification), and in the adventitia. **F:** Microdissection map showing the location of samples taken for analysis of X chromosome inactivation patterns. **G:** X chromosome inactivation analysis determined by methylation of the human androgen receptor gene. Only methylated (inactive) genes survive restriction digestion and can be amplified by PCR. The presence of two alleles indicates a polyclonal population, whereas the presence of a single allele or marked skewing toward one allele indicates a monoclonal population. Only *Hpa*II-digested samples are shown for clarity. The coronary media (**lane 1**) is clearly polyclonal, showing 53% allele 1 by densitometry. The noninflamed region of plaque smooth muscle (**lane 2**) shows a monoclonal population (2% allele 1). When samples include vasa vasorum, macrophages, or T cells (**lanes 3** to **7**), both alleles are observed, although they are often skewed (5, 3, 28, 50, and 68% allele 1, respectively).

## *Results*

Clinical data for the patients included in this study are given in Table 1, along with the results of X inactivation analysis for each arterial sample. A total of 4 aortic plaques from three patients, 11 coronary plaques from five patients, and 10 coronary segments with diffuse intimal thickening from four patients were examined. In atherosclerotic coronary arteries, 7 of 11 samples of media showed a mixed X inactivation pattern; ie, both alleles were equally represented after PCR amplification. This indicates that these medial samples contained a polyclonal population; four medial samples were significantly skewed to one allele (see below). In contrast, 9 of 11 coronary plaques contained samples significantly skewed to one allele, indicating a monoclonal population of cells was present ($P < 0.01$ media *versus* plaque). As shown in Figure 1, the monoclonal cells were clearly smooth muscle cells. When regions containing plaque vasa vasorum, T cells, or macrophages were analyzed, both alleles were represented, although the samples frequently still showed skewing.

Three of four aortic plaques showed marked skewing of X inactivation, indicating that a monoclonal population of cells was present (Figure 2A). Skewing of

plaque samples was observed only when samples came from relatively pure populations of smooth muscle cells. Samples with vascularization or inflammation always showed both alleles, indicating they contained a polyclonal population. Two of three samples of aortic media showed a mixed X inactivation pattern, one sample was skewed to a single allele, and amplification failed in one additional sample.

When multiple plaques from a single patient were examined, the different plaques showed independent patterns of X inactivation. In one case, two adjacent plaques within a patient's right coronary artery had opposite X inactivation patterns (Figure 2B). Independent X inactivation patterns rule out the possibility that some X-linked selection pressure caused skewing of the cell population within the plaque. This finding also eliminates the possibility that all plaques within a patient resulted from a common progenitor during embryological development, for example, via some metastasis-like mechanism. Similar data have been reported by previous investigators using G6PD analysis.[8,9,11]

An unexpected finding was the presence of skewing within the media in 4 of 11 coronary arteries with atherosclerosis. In all cases with medial skewing, the overlying plaque was skewed in the same direction. In one case, however, the coronary plaque was composed of two nodules separated by a cleft (Figure 3). One nodule was skewed to allele 1, similar to the media. The other nodule was skewed to allele 2. This indicates that these two nodules had distinct cells of origin and suggests that this plaque arose by clonal expansion of two cells within a short distance of each other. We have observed similar findings in the aorta, where an apparently single plaque had two separate clonal populations contained within, although in this case there was no skewing within the media (data not shown).

The finding of medial skewing prompted us to examine normal coronary arteries with diffuse intimal thickening to determine whether plaques might have arisen from tissue with large, pre-existing X inactivation patches. Of 10 coronary segments examined, 5 had at least one region of diffuse intimal thickening with skewed X inactivation patterns, and 6 had at least one region of media with such skewing (5/6 were in the same arteries; Figure 4). Considering all biopsies irrespective of location, 5/13 biopsies of diffuse intimal thickening and 9/14 biopsies of media were significantly skewed. These data suggest that there are relatively large X inactivation patches in diffuse intimal thickening, the presumed precursor tissue to atherosclerotic plaques. The higher frequency of medial skewing in arteries with diffuse intimal thickening compared with those with atherosclerosis is likely due to smaller biopsy size in the former group, not related to any biological variation.

## *Discussion*

The principal findings of this study are 1) the majority of atherosclerotic plaques have a monoclonal population of cells, 2) the monoclonal population is composed of smooth muscle cells, 3) monoclonality is present both in aortic and in coronary plaques, 4) although most samples of medial smooth muscle are polyclonal, a significant minority also show monoclonal characteristics, and 5) smooth muscle cells from diffuse intimal thickening also can show monoclonal characteristics.

It is important to note that we defined monoclonality based on the X inactivation ratios of uterine leiomyomas, known monoclonal smooth muscle tumors.[12] We wished to determine whether samples of vascular smooth muscle showed monoclonal X inactivation patterns. The use of leiomyomas as a gold standard allowed us to survey vascular samples from multiple patients and determine their clonal characteristics. An alternative strategy would have been to obtain multiple samples from a single patient and then compare the distribution of X inactivation ratios for media, diffuse intimal thickening, and atherosclerotic plaques. Although this latter approach would remove the need for comparison with leiomyomas, such extensive sampling would have diminished significantly the number of patients included in this study. Furthermore, comparison with leiomyomas made possible the unexpected finding that some samples of media and diffuse intimal thickening also exhibited monoclonal characteristics.

A monoclonal proliferation cannot be explained by a straightforward reaction of the vessel wall to injury. In healing skin wounds, for example, the fibroblasts in dermal scars arise from multiple progenitors,[21] giving a mixed pattern of X inactivation. The monoclonality of plaque smooth muscle cells indicates that either the vessel wall is fundamentally different from the skin or that the nature of cell injury in atherosclerosis is somehow unique. In general terms, there are at least three possible mechanisms leading to monoclonality of plaque smooth muscle cells (Figure 5): 1) expansion of a large, pre-existing patch of smooth muscle cells; 2) selection of a pre-existing lineage of smooth muscle cells; and 3) creation of a new lineage of smooth muscle cells.

In the first case, plaque smooth muscle cells might arise from proliferation within a large, pre-existing patch (an anatomic cluster of cells with a common pattern of X inactivation; Figure 5A) of smooth muscle cells. Although, strictly speaking, the plaque cells would be clonally derived from a common founder, the initial clone would have been established during normal development and growth of the vessel wall. Any stimulus for proliferation contained within that patch, such as a subendothelial inflammatory infiltrate, would cause patch expansion and apparent monoclonality. In other words, monoclonality would be a necessary consequence of any localized stimulus for proliferation.

To address the question of patch expansion, it will be necessary to determine the X inactivation patch architecture of the normal artery. Previous studies[8,9,11] have reported most samples of media or diffuse intimal thickening to have a mixed X inactivation pattern, suggesting patch size is small in both tissues. An important caveat to keep in mind, however, is that the likelihood of detecting skewing within a patch depends on the size of the biopsy taken. In most previous studies, full-thickness intimal or

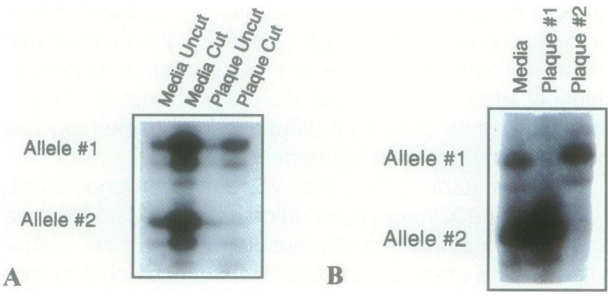


**Figure 2. A:** X inactivation in an aortic atheroma (patient 2 from Table 1). Samples of DNA without restriction digestion (uncut) yield two alleles, indicating the patient is heterozygous. The media shows two alleles after digestion (media cut; 49% allele 1), indicating a polyclonal population. In contrast, DNA from the plaque was markedly skewed to allele 1 after restriction digestion (plaque cut; 85% allele 1), indicating it contained a monoclonal population. **B:** Independent patterns of X inactivation in adjacent plaques. Two plaques from one patient's right coronary artery were examined (patient 3 from Table 1). Only *Hpa*II-digested samples are shown for clarity. The media contained a mixed X inactivation pattern (53% allele 1). Plaque 1 was markedly skewed to allele 2 (2% allele 1), whereas plaque 2 was markedly skewed to allele 1 (81% allele 1).

medial samples were obtained, and the lateral dimensions were as great as 6 mm. In the current study, with typical sample sizes of ~0.5 × 0.3 mm, we observed surprisingly frequent skewing in coronary media (4/11 arteries with atherosclerosis and 6/10 arteries with diffuse intimal thickening). This indicates that our sample size was approaching the size of the patch in normal media and intima and provides direct evidence for the patch expansion hypothesis. The presence of large patches in diffuse intimal thickening is of particular interest because the intima is thought to be the tissue from which plaques arise.[22]

The most direct analysis of vascular patterning came from Mikawa and Fischman,[23] who used retroviral tagging to trace progeny of single smooth muscle cells in chick coronaries. Their studies showed that, when tagged at the time of migration into the heart, a single smooth muscle progenitor contributed cells to several millimeters of the vessel wall's length. Each segment, however, contained a mix of tagged and untagged cells, arranged in transverse stripes each approximately one cell in width. The presence of stripes indicated that more than one progenitor contributed cells to each segment. Cross sections showed the tagged cells occupying a single layer of the arterial media; ie, they were not transmural. Their tagging approach is probably comparable to analysis of X inactivation patches, as each marks progenitor cells before the coronaries form. Although extrapolation to the human must be done with caution, the presence of single-cell stripes along the coronary segment suggests that the patch size in developing arteries is quite small. It is not known how the architecture of these patches would change as the arteries grow. We might speculate, however, that the formation of elastic lamellae isolates cells anatomically, particularly in the intima, providing a simple structural basis for clonal expansion.

If plaques do not arise from large, pre-existing patches, then the plaque smooth muscle cell clones must somehow be unique compared with the cells that did not form the plaque. This difference, in turn, must either have preceded the injury or arisen concomitantly. A second mechanism, therefore, is that vascular injury selects a pre-existing, unique lineage of smooth muscle cells to form the plaque (Figure 5B). Selection could be on the basis of cell proliferation, migration, or survival. For the selection to result in clonal expansion, the uniquely suited progenitor cells would have to be infrequently spaced in the normal artery. There is increasing evidence that smooth muscle cells within the artery wall are actually a heterogeneous mix of discrete lineages. Cells cloned from rat pup aortas show marked variations in their growth rates, serum dependence, growth factor and matrix production, and morphology.[24] Similarly, Frid et al[25] have shown heterogeneity of smooth muscle cells within the bovine pulmonary arteries *in vivo*. Large clusters of cells existed in the vessel wall that did not express the smooth muscle marker metavinculin. Interestingly, when medial smooth muscle cells were induced to proliferate *in vivo* in response to hypoxia, proliferation occurred only in metavinculin-negative cells.[26] This finding argues that the metavinculin-negative cells were functionally distinct from the surrounding cells.

If the human artery has distinct types of smooth muscle cells, it is possible that an atherogenic stimulus would recruit only one subset. It is interesting to note that Pearson et al[27] found that arterial thrombi became monoclonal as they were organized, presumably by ingrowth of smooth muscle cells. Given that the thrombi had broad attachments to the vessel wall (centimeters), a plausible explanation for their monoclonality is selection of a subset of smooth muscle cells during the process of organization. This finding raises the possibility that multiple forms of vascular injury could result in a monoclonal proliferation. It will be important to determine whether other, nonatherosclerotic forms of vascular injury also result in a monoclonal proliferation.

The third possible mechanism for monoclonality is creation of a new lineage of smooth muscle cells (Figure 5C), either through genetic mutation or through epigenetic changes (such as heritable changes in DNA methylation or creation of a stable phosphorylation loop). The original explanation offered for monoclonality was that plaque cells were mutated, eg, by a virus or chemical mutagen.[8] In this view, the plaque is analogous to a benign neoplasm. There is some evidence to support this notion. For example, infection with herpes virus can produce lesions in birds closely resembling human atherosclerosis, and herpes viral DNA has been detected in human plaque by Southern hybridization.[28] Treating fat-fed cockerels with a variety of tumor promoters, including polynuclear aromatic hydrocarbons, enhances lesion formation in the aorta.[29] There is a report that DNA from human plaques can transform fibroblasts *in vitro*[30]; however, this result could not be replicated by another group,[31] and the sequence responsible for the putative transformation was not isolated. Cytogenetic studies have reported a variety of karyotype abnormalities in smooth muscle cells from plaque, some of which were frequent

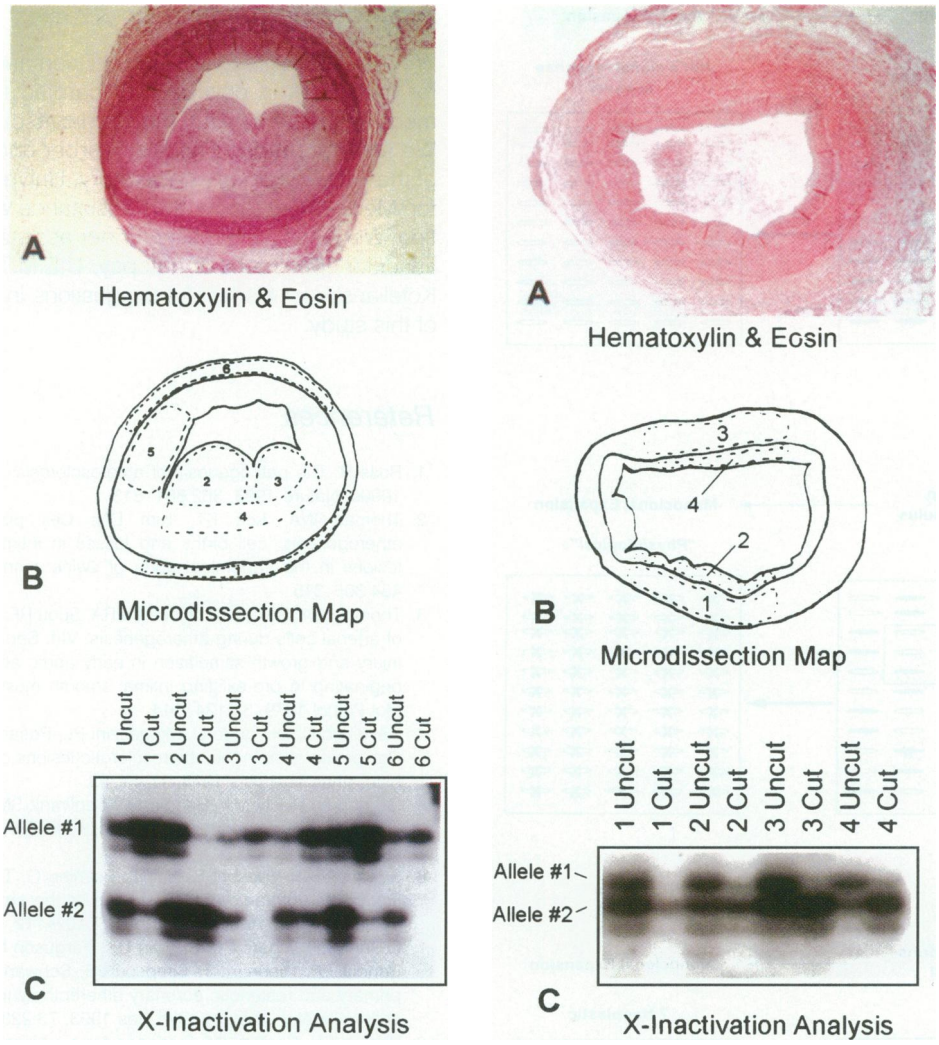


**Figure 3.** (**Left**) Skewing of X inactivation ratios in media, diffuse intimal thickening, and fibrous plaque. **A:** Section of left anterior descending coronary artery from a female patient with dilated cardiomyopathy (patient 6 from Table 1). The lower aspect of the artery has a fibrous plaque containing two nodules separated by a cleft. Immunostaining demonstrated the nodules to be free of inflammatory cells and endothelium, although these cell types were present at the base of the plaque. The upper aspect of the artery contains diffuse intimal thickening. Original magnification, ×63. **B:** Microdissection map showing samples taken for X inactivation analysis. **C:** X chromosome inactivation analysis. Samples of the normal media (1 cut and 6 cut) were skewed to allele 1 (68 and 98% allele 1, respectively), suggesting the entire circumference to be one patch. Similarly, samples of diffuse intimal thickening (5 cut) and one nodule of the plaque (3 cut) were also skewed toward allele 1 (77 and 90% allele 1, respectively). Interestingly, the other nodule of the plaque (2 cut) was markedly skewed toward allele 2 (15% allele 1), indicating that this nodule had a different cell of origin from the other regions sampled. Undigested samples (uncut) all showed balanced representation of both alleles.

**Figure 4.** (**Right**) X inactivation patterns in a left circumflex coronary artery with diffuse intimal thickening (patient 5 from Table 1). **A:** H&E staining showing diffuse intimal thickening with no lipid deposition or inflammation. Immunostaining showed the intima to be rich in smooth muscle cells, with virtually no inflammatory cells or vascularization (not shown). Magnification, ×95. **B:** Microdissection map showing samples taken for X inactivation analysis. **C:** X chromosome inactivation analysis. Samples of media (1 cut and 3 cut; 18 and 30% allele 1, respectively) and diffuse intimal thickening (2 cut and 4 cut; 34 and 28% allele 1, respectively) all showed skewing toward allele 2, suggesting the X inactivation patch size was relatively large in this normal artery. Note that in this patient the two alleles are much closer in size than in previous examples. Undigested samples (uncut) all showed balanced representation of both alleles.

enough to meet cytogenetic criteria for clonality.[32] Unfortunately, no single chromosomal abnormality occurred with sufficient frequency to suggest either a pathogenic mechanism or a potential disease marker. Thus, at present there is no clear picture of genetic changes that may predispose to plaque formation. To our knowledge, however, no one has investigated possible roles of mutations in tumor suppressor genes or oncogenes. Two recent papers from Spandidos's laboratory,[33,34] however, report evidence of genomic instability in microsatellite DNA. Microsatellite instability is commonly present in tumor cells.[35]

In summary, these data indicate that smooth muscle cells constitute the monoclonal population of atherosclerotic plaques. Although monoclonality still may arise by lineage selection or mutation, the finding that smooth muscle cells from normal arteries can show monoclonal characteristics provides evidence that plaque monoclonality may result from expansion of a pre-existing (developmental) clone of cells.

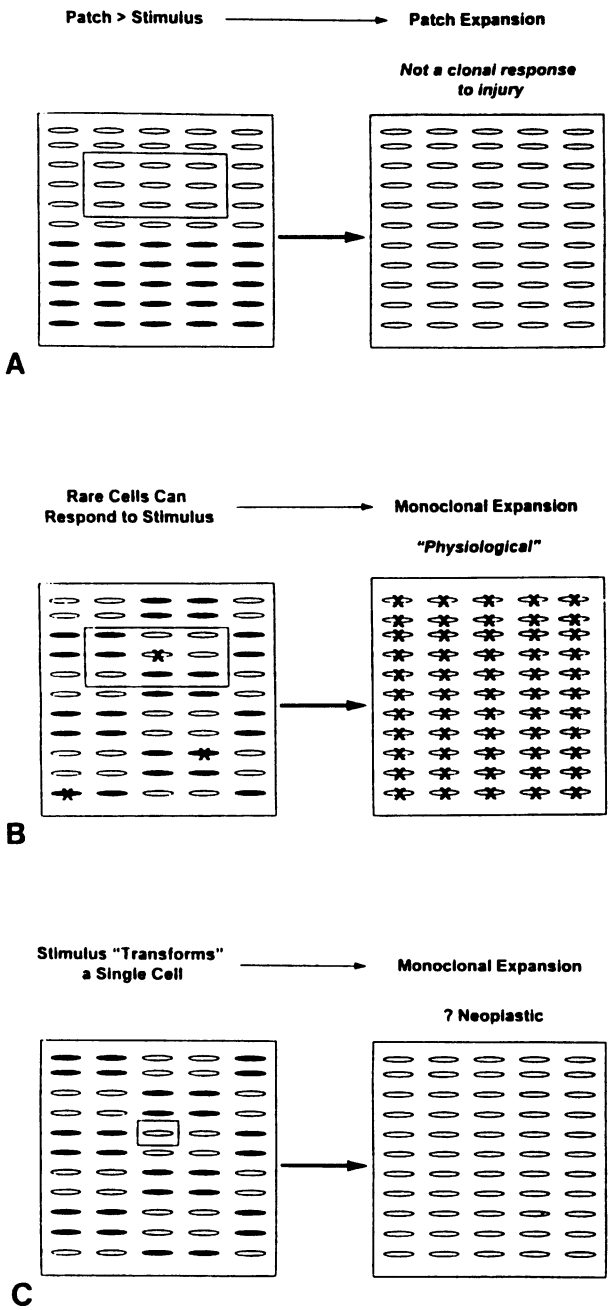


**Figure 5.** Possible mechanisms for plaque monoclonality. Patch sizes (groups of cells with like patterns of X chromosome inactivation) are indicated by clusters of black and white cells. The size of the stimulus for proliferation is shown by the box. The lesion resulting from proliferation is shown at the right. Reproduced from Schwartz et al[36] with permission. **A:** Developmental mechanism. If normal growth and development results in a large patch size, a relatively small stimulus for proliferation could be contained entirely within a single patch. If so, the resulting lesion would be composed entirely of cells exhibiting the same pattern of X chromosome inactivation. Although the cells within the lesion would be monoclonal, in that they derived from a common developmental founder, the proliferation would not be a monoclonal response to injury. The phenomenon could be termed patch expansion. **B:** Lineage selection. If the vessel wall is composed of heterogeneous smooth muscle cells, with only rare cells able to respond to the injury (as indicated by the X), a relatively large stimulus for proliferation might induce only one cell to proliferate and form the atherosclerotic plaque. Selection could occur on the basis of proliferation, migration, or survival. **C:** Neoplastic mechanism. If the stimulus for proliferation results in a heritable change within a single cell, eg, by chemical or viral mutagenesis, the resulting lesion could be derived from a single cell. This is the mechanism by which all human tumors are thought to arise.

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