Atherosclerotic Plaque Smooth Muscle Cells Have a Distinct Phenotype


Objective—The present study addresses the question, “Are plaque smooth muscles cells (SMCs) genetically distinct from medial SMCs as reflected by the ability to maintain a distinctive expression phenotype in vitro?”

Methods and Results—Multiple cell strains were developed from carotid endarterectomy specimens, and quadruplicate array hybridizations were completed for each sample. A new normalization protocol was developed and used to analyze the data. Permutation analysis suggests that most of the significant differences in expression could not have occurred by chance. A broad pattern of significant expression differences, consisting of almost 5% of the genes probed, was detected. Quantitative polymerase chain reaction (QPCR) confirmation was found in 70% of a subset of genes selected for validation.

Conclusions—The SMC cultures were nearly indistinguishable by morphological features, population doubling time, and sensitivity to cell death induced by Fas cross-linking. Surprisingly, array expression analysis identified differences so extensive that we conclude that plaque and medial SMCs are distinctly different SMC cell types. (Arterioscler Thromb Vasc Biol. 2004;24:1283-1289.)

Key Words: cell biology ■ genomics ■ gene expression ■ gene regulation

Three decades ago Benditt and Benditt presented evidence that atherosclerotic lesions were monoclonal.1 Subsequent work using a polymorphism in the human androgen receptor gene demonstrated that the cell responsible for clonality was the smooth muscle cell of the fibrous cap.2 The critical question now is: How does monoclonality arise?

Benditt proposed that monoclonality of the plaque cap was the result of a mutation or viral event as in neoplasia.3 This hypothesis is not consistent with the current consensus that the atherosclerotic plaque is an inflammatory lesion where smooth muscle cells (SMCs) arise as a fibrotic reaction encapsulating the inflammatory necrotic core.4 Malignant neoplasms, however, are not the only instance of clonality. For example, another clonal smooth muscle mass, uterine leiomyoma, appears in most females as a focal overgrowth of SMCs.5 In this case a “normal,” or at least very common, monoclonal overgrowth becomes clinically significant with age. Other forms of monoclonality also occur during development. For example, in the hair follicles and crypts of the intestine, clones present themselves as well defined mini-organs. In vessels themselves, large patches of clonal cells distribute in a spiral formation throughout the avian coronary artery.6

Thus, we should consider the possibility that plaque clonality represents a normal process during proliferation and development. This concept is consistent with the description of lesions by an American Heart Association (AHA) consensus committee led by Stary.8 The committee noted that most lesions occur at sites where preexisting intimal cell masses localize. Lipid deposition appears within this intimal mass.

Interpretation of plaque monoclonality as part of normal development contrasts with current concepts of neoplasia as the result of the gain or loss of function of an oncogene or tumor suppressor gene.9–11 These oncogenic events, resulting from a mutation in DNA sequence, or insertion of a viral sequence, produce expression phenotypes uniquely associated with a given type of tumor.12,13 If plaques have a mutational or viral origin, plaque SMCs should display different properties from other SMCs. Such properties could account for the localized accumulation of lipid as suggested by Williams and Tabas.14 Genetically distinct plaque SMCs could also arise if, as we have suggested, the lesions originate in a subset of SMCs with a distinct history of developmental differentiation.6,7

In support of a mutational hypothesis, a diverse body of literature has identified mutations, translocations, modifications of DNA, and phenotypic properties of SMCs derived from plaque.15–26 This literature, however, has not established
a description of the unique properties of the plaque cells. It is possible that large-scale expression analysis would recognize different mechanistic signatures that would reflect the mechanisms giving rise to these cells. To approach this goal, we have recently completed a study of the fibrous cap in vivo using pooled tissue samples from human carotid plaque, intima, and media. In the in vivo study, we found a small but statistically significant set of genes with differential expression in the cap versus other smooth muscle layers of the artery (L. Adams, unpublished data, 2004). To remove the possibility that this distinct pattern resulted from the cells’in vivo milieu, we extended the comparison to cell strains of plaque and medial SMCs isolated and grown under identical conditions.

Methods

All protocols are described in the online Methods (available at http://atvb.ahajournals.org).

Data Normalization

There are many sources of systematic variation in cDNA microarray experiments, and the term normalization refers to the process of removing such variation. Our method is as follows. The median of log_{10}-transformed expression values for each gene is calculated, giving a rough estimate of expression level for that gene. Then, for each exposure of each filter we compute a smooth “local” regression line between the log intensities for that exposure and the overall level estimated above, using the “loess” function (from the “modreg” library of R) with default parameters. These regression curves reflect broad trends in the data, but are insensitive to local deviations. The “normalized” log-level for each gene in the exposure is the value as predicted by the loess line from the measured value, that is, the normalized expression of gene i in exposure j is \( \hat{y}_{ij} = f(x_{ij}) \), where \( f(x) \) is the loess regression of \( y \) on \( x \), \( y = \text{median}(x_0, x_1, \ldots, x_j) \), \( x_j = \log_{10}(x'_{ij}) \), and \( x'_{ij} \) is the measured intensity of gene i in exposure j. Finally, normalized values for multiple exposures of a single hybridization on 1 filter are combined by taking the per gene medians of the multiple values computed above.

Statistical and Cluster Analysis

The normalized data were analyzed by a number of methods including t-test (Excel built-in function, assuming unequal variances, i.e., heteroscedastic Welch test), significance analysis of microarrays (SAM)\(^{28} \) (visit http://www-stat.stanford.edu/~tibs/SAM/index.html), and Treeview.\(^{29} \) Other statistical analyses were performed using the R statistics package, version 1.6.1 (visit http://www.r-project.org).

Quantitative PCR

The principles of this method have been described.\(^{30} \) A complete description of our protocol, templates, and oligonucleotides are provided in online Methods.

Results

Characterization of Human Carotid SMCs

We used an enzymatic dispersion protocol to isolate SMCs,\(^{31} \) and SmGM2 media (which contains recombinant epidermal growth factor [EGF], fibroblast growth factor [FGF], and insulin) to establish cultures that survived multiple passages. During the study, 11 of 13 cell strains were analyzed for their behavior in culture. Cell-doubling times were determined from growth curves over 6 to 7 weeks of continuous culture. Plaque cells replicated slightly faster than medial cells, with a population doubling time of 5 versus 7.5 days (Figure 1A). Effects of changing cells to serum-free media were assessed. Two of the 5 plaque cell strains went through an initial period of noticeable cell loss but these 2 strains, as well as all of the remaining cells, displayed no obvious difference over the 3 weeks during which the cells were maintained in the growth arrested state. The morphological phenotype at low density in serum-free media was similar (Figure 1B and 1C), as was staining with antibody to \( \alpha \)-smooth muscle actin (not shown). We looked at sensitivity to Fas receptor cross-linking in the presence of cycloheximide and the antibody CH-11.\(^{32} \) All of the cell strains were induced to die by Fas receptor cross-linking. Dose dependence and timing of cell death were very similar in most strains (Figure 1, available online at http://atvb.ahajournals.org). Table I (available online at http://atvb.ahajournals.org) summarizes the array expression data for a number of smooth muscle marker genes. There was a significant elevation in \( \alpha \)-smooth muscle actin and non-muscle myosin in the medial SMCs, whereas other SMC marker genes, including smoothelin, caldesmon, SM22-\( \alpha \), and calponin 3 were similar.

Expression Analysis

We used Research Genetics GF211 filter arrays containing 4046 known genes. Because array media, including filter and glass platforms,\(^{33} \) exhibit variations within hybridization sets, techniques were developed to compare and combine data from multiple exposures and hybridizations, while simultaneously addressing the varying exposure times and nonlinear effects. As explained in Methods, expression measurements were matched to each other by a novel technique displaying some similarities to described disparate approaches.\(^{33,34} \) Assuming that most genes are not differentially expressed across the set of experiments and that intensities measured in each exposure are an unspecified but smoothly varying function of
transcript abundance, this method transforms all values to an arbitrary but common scale ("normalized values"), so that differences between 2 such normalized values approximate log10 ratios of the corresponding transcript levels. Figure II (available online at http://atvb.ahajournals.org) illustrates this normalization on 2 typical exposures.

The t test identifies 235 genes as differentially expressed between the 4 media and 5 plaque samples with a probability value \( P < 0.01 \). Could this large number of genes have arisen by a chance alignment of genetic, biological, or technical factors unrelated to media/plaque status? To address this, we performed the following permutation analysis. We arbitrarily split the samples into 2 groups A and B and counted genes differentially expressed between A and B at the same significance level, restricting analysis to the 126 possible partitions that collocate all replicates (typically 4) of 4 of the samples into A and all replicates of the other 5 into B. Figure 2A shows the number of splits having a designated number of media samples in group A. Figure 2B plots the number of genes in each split exhibiting significant \( (P < 0.01) \) t test probability values, stratified on the number of media samples in group A. The great majority of partitions have many fewer "significant" genes (median 63) than the 235 found in the pure media/plaque partition; only 5 have more, all "unbal-

Figure 2. Permutation analysis. A, The number of partitions of the 9 samples having the given number of media samples in group A, the 4-sample side of the partition. B, A “box and whisker” plot for the partitions identified in A, of the number of significant \( (P < 0.01) \) genes in each partition, stratified by the number of media samples in group A. Boxes enclose the middle 50% of data values, horizontal lines mark medians, whiskers extend to the most extreme values within 1.5 times the height of the box from the box, and circles mark more extreme points. Most counts are well below that for the “pure” media/plaque partition, with lowest counts arising from “mixed” partitions, where media and plaque are equally represented in each group. C and D plot the same information for the 40 partitions separating the media and plaque samples of the 2 patients having both.

Figure 3. A dendrogram demonstrating average-link hierarchical clustering of the prefiltered data set of 164 genes is presented. Seventeen media arrays and 18 plaque arrays went into this analysis. The actual filter numbers are described in the workbook for the normalized data set available in supplementary information. Two main branches exist, 1 consisting primarily of media samples, the other mainly of plaque samples.

To identify the most predictive subset of genes, we used a procedure called leave one out cross validation (LOOCV35). Leaving out each filter in turn, we calculated t test probability values for each of the 4046 genes for media versus plaque and retained those genes that appeared among the top 200 genes in at least 75% of the LOO iterations. A heat map of the 164 genes thus identified is located in Figure III (available online...
at http://atvb.ahajournals.org). Additionally, they were used to perform an unsupervised hierarchical clustering of the 35 array hybridizations. The resulting dendrogram (Figure 3) confirms important general characteristics of the data set. In particular, most of the replicates are near neighbors in the dendrogram, and the main branch separates most of the media cell strains from most of the plaque strains. The appearance of some of the technical replicates of plaque arrays in the "media cluster" and vice versa is not totally unexpected. It is perhaps more surprising that the majority (14 of 17) of the arrays from the 2 patients having paired media and plaque samples were correctly separated. The overall separation was 80% accurate, with 3 of the 7 errors being misclassifications of paired samples; not a bad result for this unsophisticated algorithm.

The data were analyzed by a number of additional methods including SAM; see supplemental materials for the entire raw and normalized datasets (available online at http://atvb.ahajournals.org). SAM identified 322 genes with a q-value (loosely, "false discovery rate") of less than 5%. After eliminating duplicated genes and those lacking a unigene Cluster number, and selecting a t test cutoff of $P<0.01$, there were 208 genes.

208 genes of 4046 assayed is a large difference especially considering that these differences are detectable under base-line culture conditions. Table 1 identifies some of these genes. We identified several different functional subsets of genes including proteases and protease inhibitors, extracellular matrix (ECM) and matrix modifying genes, transcription factors, cytoskeletal genes, and membrane adhesive genes. Table 2 lists the ECM and ECM-modifying genes. Versican is included in the table to divide the upregulated medial genes from the plaque upregulated genes. With a probability value of 0.08, versican did not meet our test of significance, although we have used it previously to define distinct phenotypes in rat SMCs. There are also large groups of transcription factors and signaling molecules. The complete gene list can be found in Table II (available online at http://atvb.ahajournals.org).

**Quantitative Polymerase Chain Reaction (QPCR) Validation**

A QPCR assay was used to measure 14 selected RNAs. The selected genes were chosen based on several criteria including demonstration of a significant fold change, expression at low, medium or high relative normalized intensities, and probability values ranging up to 0.05. Table 3 lists the results of the QPCR assay compared with the array expression analysis. Supplemental Table III (available online at http://atvb.ahajournals.org) lists the primers that were used to generate control templates for 15 genes (14 test and 1 for normalization). Of the 14 genes assayed, 9 (or 64%) were differentially expressed with the same direction of change as measured by the expression array; that number increased to 73% after eliminating the 3 most weakly expressed genes. Overall, results by QPCR support our permutation analysis and suggest that a high percentage of the genes with $P<0.01$ are differentially expressed.

**Discussion**

Somatic mutations, loss of heterozygosity, DNA methylation, and histone acetylation, all potential mechanisms for establishing a genetically distinct set of plaque SMCs, have been described in previous studies of cells derived from the plaque. The most important additional evidence
from the present study is the identification of a pattern of gene expression that consistently distinguished 5 different isolates of plaque SMCs from medial cells. The existence of this pattern implies that there is some common somatically heritable mechanism underlying differences between the plaque and medial cells. These data cannot distinguish between hypotheses attributing the expression phenotype to a genetic change as expected in neoplasia or to a nonneoplastic hypothesis, eg, a differentiation event with a change in phenotype as a consequence of epigenetic modifications.9,42,43

Microarray reports that distinguish genetically different cell types are common in the neoplasia literature. A classic example is the acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) array analysis, where approximately 10% of the genes on the array were differentially expressed.12 Thus, our 208 genes, or 5% of those queried is an impressive phenotypic signature. The existence of distinctive smooth muscle subsets is not surprising, as it has been recognized for over a decade that vascular SMCs in vivo display a diversity of phenotypes.15–18 This heterogeneity appears to increase when SMCs are cultured in vitro.25,44–48

Our data suggests the existence of an intrinsic difference in vivo between plaque and medial SMC. This hypothesis is strengthened by the effort we made to minimize selective pressures during the isolation and culturing procedures. Nonetheless, it is important to consider the possibility that some very early event after putting cells into culture, for example, a difference in ability to survive enzymatic digestion, could have selected for 2 cell types.

We think it is important not to over-interpret the specific sets of genes identified here as evidence for important mechanisms contributing to plaque progression in vivo. An underlying difference may result in quite distinct expression patterns in vitro and in vivo. For example, plaque SMCs in vitro have elevated transcripts for a number of enzymes that influence the amount and type of modification to glycosaminoglycan structures (GAGs) of membrane and matrix proteins. These include key enzymes (Table III) involved in synthesis and modification of GAGs implicated in lipoprotein

<table>
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<th>SAM</th>
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<th>Gene Name</th>
<th>Ratio</th>
<th>t Test</th>
<th>UG Cluster</th>
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<td>3</td>
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Comparison of extracellular matrix and matrix-modifying genes in plaque and media SMCs by SAM and t-test rank. The M/P ratio was determined from the unlogged normalized intensities.
accumulation and presentation of growth factors in plaque. It would not be surprising, however, if the differences seen in vitro were specific for the ways the 2 cell types adapt to culture conditions, that is, by controlling the availability of growth factors.

Perhaps more relevant to a mechanism that might account for selection of a subset of cells is the upregulation of glutathione-S-transferase (GST) in media compared with plaque SMC. Reduced glutathione plays a critical role in providing intracellular protection from a broad variety of toxic agents. Studies of a null genotype (GSTMI) showed that the loss of activity is associated with an elevation in the levels of DNA adducts. Other studies have shown that DNA adducts are detectable in atherosclerotic plaque lesions, and adduct levels were significantly correlated with known atherogenetic risk factors. It would thus be interesting to know if plaque SMCs are more sensitive to oxidative damage. Our finding that the extracellular form of superoxide dismutase (sESOD) is downregulated in plaque cells suggests that plaque cells may respond differently to oxidative stress.

In summary, the consistency of these patterns in 5 independent plaque isolates in culture implies that differences exist at the somatic genetic level. It remains to be determined whether this phenotype is present in the cells before they become plaque SMC, or whether it is the result of epigenetic adaptation to the atherosclerotic environment. Moreover, we should consider the accumulating body of evidence that arterial SMCs can arise from several distinct embryological sources, diverse populations of stem cells in the adult. Future experiments will need to address the question of the origin of the plaque SMCs.

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References


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