Abstract—Early morphological and biochemical studies indicated that vascular smooth muscle cells (VSMCs) exhibited two distinct phenotypes and that a change from the contractile to the synthetic phenotype was a prerequisite for progression of vascular disease. More recently, it has become evident that these phenotypes probably represent the extremes of a spectrum of phenotypes that may coexist in the vessel wall, which are dictated by their environment and functional requirements and which reflect differing patterns of gene expression. Therefore, knowledge of the key factors that regulate these patterns of gene expression is likely to lead to the ability to manipulate VSMC phenotype. However, before such factors can be identified, the relationship between VSMC gene expression and VSMC phenotype must first be established. We therefore undertook a differential screen of cDNA from VSMCs in vitro to provide a bank of gene markers that could be used under a variety of circumstances to define VSMC phenotype in terms of the pattern of genes expressed. Using this approach, we have found that the pattern of gene expression that occurs during neointima formation in the balloon-injured rat carotid artery is very similar to that seen at a specific period in the developing aorta of the early neonate and is characterized by coexpression of genes for both contractile and matrix proteins. Furthermore, recent studies have shown that VSMCs isolated at different stages of aortic development can stably maintain different phenotypic characteristics in cell culture. The use of these cells in transfection experiments with SM-specific promoter–chloramphenicol acetyltransferase reporter constructs may enable us to determine what regulates the pattern of gene expression in different VSMC phenotypes. Such studies may ultimately lead to the identification of transcription factors responsible for determining VSMC phenotype and may therefore provide targets for therapy aimed at manipulating VSMC gene expression in vascular disease. (Arterioscler Thromb Vasc Biol. 1998;18:333-338.)

Key Words: vascular smooth muscle • phenotype • differentiation • development • intima
repertoire of genes that can be expressed by adult VSMCs in vitro. Our ultimate aim was to define the patterns of VSMC gene expression that characterize the phenotypic changes that occur in vivo and to determine the mechanisms that regulate them.

**Isolation of VSMC Gene Markers**

We used the well-defined phenotypic modulation of adult rat VSMCs in cell culture as a model and hypothesized that the differences in gene expression between cell populations in culture would reflect similar differences in VSMC populations in vivo. Using differential screening of a cDNA library that had been constructed to contain markers of both the “contractile” and “synthetic” phenotypes of adult rat VSMCs in culture, we isolated and characterized 12 cDNAs. Ten were preferentially expressed in differentiated aortic medial VSMCs: α- and γ-SM actins, SM22α, phospholamban, aquaporin-1, SM–myosin heavy chain (SM-MHC), elastin, osteoglycin, and ubiquitin; and two were preferentially expressed in VSMCs that had proliferated in culture: matrix Gla protein (MGP) and osteopontin. The protein products of these genes fall broadly into two categories: those associated with contractile function, namely, α- and γ-SM actins, SM-MHC, calponin, SM22α, polyubiquitin, and phospholamban; and those associated with the extracellular matrix: elastin, osteoglycin, MGP, and osteopontin. Importantly, these two groups of genes reflect the two major functions of VSMCs, namely, (1) contraction and (2) the synthesis and maintenance of the extracellular components of the vascular wall.

As well as providing a bank of markers for the analysis of VSMC gene expression in vivo, the differential cDNA screen also identified genes that had not been previously associated with the vasculature, thereby providing new insights into the role of VSMCs. For example, osteopontin and MGP were initially discovered in developing bone, and subsequent studies have now shown that both genes may also play a significant role in vascular calcification. Also, the high expression of aquaporin-1 in VSMCs implies a hitherto-unrecognized need for VSMCs to transport water rapidly across the cell membrane.

**Expression of VSMC Markers in Human Disease**

Using in situ hybridization to study expression of these genes in human vessels, we found marked heterogeneity of gene expression and, by implication, VSMC phenotype in atherosclerotic plaques (the Table). In particular, we found high expression of some contractile genes in a subset of VSMCs of the fibrous cap of some lesions and conversely, marked downregulation of the same genes in apparently “contractile” VSMCs in the media adjacent to atherosclerotic plaques. The same contractile genes were also downregulated in the medial VSMCs of transplanted vessels with chronic vascular rejection. Furthermore, we also found marked heterogeneity of gene expression within VSMC populations. For example, calponin was expressed exclusively in medial VSMCs but not in intimal VSMCs, either in diffuse thickenings of normal vessels or advanced atheromatous plaques, whereas SM22α was highly expressed by medial VSMCs, hardly at all by VSMCs in diffuse intimal thickenings, but at high levels in some intimal cells of the fibrous cap in some advanced lesions. Additionally, SM22α remained highly expressed in most VSMCs of the thinned media associated with advanced atherosclerotic lesions, whereas calponin was downregulated in the same cells. In contrast, genes for the matrix proteins osteoglycin and MGP were both expressed by intimal VSMCs in early, diffuse, intimal thickenings and advanced atherosclerotic plaques, and both were constitutively expressed by contractile medial VSMCs in normal vessels. These observations argued for a far greater complexity of VSMC gene regulation in vivo than had been predicted from cell culture experiments and questioned the validity of VSMC culture as a model for VSMC behavior in vivo. These findings also posed a number of questions concerning the regulation of VSMC phenotype during disease development and progression. Since the proliferative and phenotypic response of VSMCs in cell culture had traditionally been thought to reflect similar changes in the VSMC response...
to mitogenic stimulation in vivo, we studied VSMC gene expression during neointima formation after balloon-induced intimal injury in the rat carotid artery, a relatively “pure” model of the VSMC response to injury.

Expression of VSMC Markers After Balloon Injury of the Rat Carotid Artery

Balloon injury of the rat carotid artery induces medial VSMCs to enter the cell cycle and migrate into the intima, where they continue to proliferate for as long as 14 days to create a neointima. Previous morphological and biochemical studies based on α-SM actin protein content had indicated that intimal cells and a proportion of the medial cells “dedifferentiated” as they migrated into the intima and proliferated and did not show evidence of redifferentiation until ≈60 days after injury. These data therefore predicted a general downregulation of contractile genes in the early period after balloon injury. However, contrary to expectations, we found that proliferating neointimal cells 7 days after injury were expressing high levels of mRNA coding for contraction-associated proteins, such as calponin, SM22α, and phospholamban, as well as for matrix proteins such as osteoglycin, MGP, osteopontin, and elastin (Figure) (C.M.S., unpublished observations, 1997). This pattern of expression therefore reflected simultaneous upregulation of the genes for both the “contractile” and “synthetic” phenotypes and was inconsistent with the notion that neointimal cells were dedifferentiating. Interestingly, two other studies have also reported high expression of H19 and members of the myocyte specific enhancer binding factor (MEF2) family of transcription factors, both potential regulators of VSMC differentiation, in the neointima after balloon injury. Indeed, in each of these studies including our own, expression of differentiation markers was considerably higher in the neointima than in the surrounding medial VSMCs. Taken together, these data suggest that only low levels of expression of contractile genes are required to maintain the differentiated phenotype in quiescent, adult, medial VSMCs, possibly reflecting low turnover of contractile proteins, whereas proliferating neointimal cells responding to vascular injury simultaneously upregulate the genes required for both contraction and vascular repair. Firulli et al interpreted the higher expression of MEF2 factors in the neointima than the media to indicate that different transcriptional mechanisms may be involved in the initial synthesis of contractile proteins and their subsequent maintenance.

Since the processes involved in repairing mature vessels, namely, VSMC proliferation, VSMC differentiation, and matrix synthesis, are closely similar to those required for new vessel formation, it has been suggested that the VSMCs involved in vessel repair might reexpress a fetal or neonatal phenotype. Consistent with this proposal, many studies have noted that cultured neointimal and neonatal VSMCs share similarities in morphology and expression of genes for matrix proteins and some differentiation factors, such as H19. Therefore, having defined the pattern of VSMC gene expression after vascular injury, we went on to study the expression of the same genes during vascular development.

Expression of VSMC Markers During Development of the Neonatal Aorta

Until recently, little was known about the molecular mechanisms controlling VSMC lineage determination and differentiation. However, recent studies of gene expression during the early stages of vascular development and sequence analysis of SMC-specific gene promoters have started to shed light on these complex processes. The initial commitment of aortic precursor cells to an SMC lineage appears to occur in the mouse fetus between days 9 and 10 post coitum, as indicated by the earliest detection of SM-specific genes. After this commitment, the cells are proliferative and express detectable levels of SMC differentiation markers, such as SM-MHC, SM22α, and calponin. The next major change occurs in the early neonatal period, when VSMC proliferation decreases abruptly and growth continues via VSMC hypertrophy and elaboration of extracellular matrix. During this stage, VSMCs produce the matrix proteins that create the three-dimensional vessel wall structure, and the cells take on the morphological characteristics of the mature, contractile, adult phenotype. When we analyzed the pattern of gene expression during neonatal aortic development, we found upregulation of SMC differentiation markers such as calponin, SM22α, phospholamban, and polyubiquitin after birth, which began at approximately week 2 and reached a maximum between weeks 4 and 12. Expression of these genes was then reduced to basal “adult” levels thereafter, unless the vessel was injured as discussed above. During this same period (weeks 4 to 12), there was also a marked and transient upregulation of matrix protein gene expression, particularly elastin (C.M.S., unpublished observations, 1997). Thus, there is a period during early postnatal vascular development when cells that are already committed to a VSMC lineage express high levels of genes coding for contractile proteins and high levels of genes for matrix proteins. In terms of gene expression, they are phenotypically intermediate between contractile and synthetic.

Overall, our studies indicate that VSMCs can exhibit a spectrum of gene expression and that the genes expressed during vascular repair are similar to those expressed during the early phase of postnatal development. To that extent, the
VSMCs involved in vascular repair have not dedifferentiated to a fetal phenotype but rather are exhibiting an intermediate phenotype characteristic of the maturation phase of vascular development, which occurs well beyond the stage of VSMC determination. We did not set out to answer the question of the origin of intimal cells, and our data are consistent with both an ability of adult VSMCs to “regress” to a more immature phenotype and the selective emergence of a population of undifferentiated cells.

The accumulated data have led us to postulate at least three phases of VSMC-specific transcriptional activity: the first, during early intrauterine development, when VSMC-specific transcription factors first drive expression of VSMC-specific proteins (the determination phase); the second, during postnatal vascular growth, when the cells express a combination of contractile and matrix protein genes (the maturation phase); and the third, when vascular growth has ceased and VSMC gene expression is reduced to levels required predominantly to maintain contractile function (the contractile phase).

This gene-based elaboration of the original phenotype-modulation hypothesis is almost certainly an oversimplification of the mechanisms controlling VSMC phenotype but provides a paradigm that can be tested experimentally. Our hypothesis necessarily implies three different, probably overlapping, transcriptional programs: the first, involving transcription factors responsible for the first expression of VSMC-specific genes, the determination program; the second, which regulates the combined postnatal expression of contractile and matrix genes, the maturation program; and the third, which maintains expression of appropriate contractile proteins, the contractile program. Our paradigm therefore implies that adult VSMCs reactivate aspects of the maturation program after vascular injury.

Isolation of VSMC Cultures With Stably Different Phenotypes

If our paradigm is correct, then we should be able to recreate the different phases of gene expression in cell culture either by applying the appropriate extracellular stimuli or by isolating cells in which the transcriptional program is stably expressed. Numerous studies of cells cultured from different species have demonstrated that cytokines, matrix components, and mechanical stimuli can influence VSMC phenotype and behavior. Also, it has previously been noted that VSMCs can exhibit different stable phenotypes in cell culture depending, for example, on their origin or the age of the animal from which they were isolated. Thus, cells isolated from fetal rats have a greater proliferative capacity than do adult cells in culture, and cells isolated from neonatal animals express higher levels of matrix proteins. Of particular interest to us has been the recent observation by Gabbiani’s group, that VSMCs isolated from early neonatal rats maintain high levels of α-SM actin through several passages in cell culture, whereas equivalent adult cells, as expected, rapidly downregulate α-SM actin expression when placed in culture. In preliminary experiments using Northern blot analysis of mRNA from these neonatal cells, we have found that they also maintain high expression of a number of other VSMC differentiation markers, including γ-SM actin, ubiquitin, calponin, and SM-MHC (C.M.S. et al, unpublished observations, 1997). These data therefore support the concept of distinct, stable differences in gene expression in VSMCs derived from different sources that can be maintained and therefore studied in cell culture.

Identification of Phenotype-Specific Transcription Factors

It has long been known that VSMC phenotype can also be influenced by local environmental influences, particularly in cell culture. Since the expressed repertoire of genes responsible for a particular phenotype must ultimately be determined by the activity of transcription factor binding, singly or in combination, to specific promoter and/or repressor regions of VSMC-specific genes, studies have been aimed at identifying key regulatory elements in the promoters of VSMC-specific genes. This has been achieved by linking regions of SM-specific promoters to easily quantifiable reporter proteins, such as chloramphenicol acetyltransferase, and transfecting the constructs into VSMCs that are then stimulated by the agent of interest. Once a regulatory element has been identified, corresponding synthetic oligonucleotides can be used to identify regulatory factors from nuclear extracts by electrophoretic mobility shift assays. Such techniques have recently demonstrated that angiotensin II–induced expression of α-SM actin is mediated by the interaction between the homeodomain transcription factor MHox and two CArG domains contained within the first 155 bp of the α-actin promoter. Part of this region of the promoter also mediates vasopressin-induced α-SM actin expression. Importantly, a new transforming growth factor-β-responsive element (ie, TCE) has been identified in a number of SM-specific promoters that, along with specific CArG boxes, mediates the effects of transforming growth factor-β on VSMC gene expression.

The aforementioned experiments demonstrate the feasibility of identifying the transcription factors and regulatory elements responsible for mediating environmental influences on VSMC gene expression and therefore phenotype. However, the observation that cells derived from neonatal and adult vessels express stably different phenotypes under similar environmental conditions suggests intrinsic differences in their respective programs of gene expression. Evidence from a number of studies showing that SM-specific promoter regions have differential activity in different VSMC isolates supports this concept. For example, Firulli et al have shown that a chloramphenicol acetyltransferase reporter construct with four MEF2 binding sites is differentially activated in different isolates of adult rat VSMCs under similar culture conditions, and in preliminary experiments in our own laboratory, we have found stable and reproducible differences in expression of two SM22α promoter–chloramphenicol acetyltransferase constructs when comparing fetal, neonatal, and adult VSMCs, suggesting that different regions of the promoter are active/inactive in these different cell types (Z. Lawal et al, unpublished observations, 1997). It is hoped that such studies on the activity of VSMC-specific promoters in cells exhibiting different stable phenotypes in vitro will lead to identification of the factors that dictate cell phenotype.

Implications for Human Disease

Although the aforementioned studies will provide important insights into the regulation of VSMC-specific genes, they are
based on rodent VSMCs, which may differ significantly from human VSMCs. In particular, many studies of VSMC heterogeneity have highlighted species differences in the type and extent of heterogeneity, while our own studies have shown major differences in gene expression between rodent and human VSMCs. Furthermore, it is most researchers’ experience that human VSMCs are more difficult to establish in cell culture than are rat cells. Nevertheless, a limited number of studies have shown that primary human cell cultures derived from medial cells can be established and maintained over a number of passages. In comparison, cells derived from atherosclerotic plaques proliferate poorly, senesce early, and readily undergo apoptosis. Thus, as with rodent cells, stable differences in phenotype between human cells derived from different sources can be identified. Further studies analyzing expression of VSMC differentiation markers in human cells are now required so that our knowledge of human cells will equal that of rodent cells.

Why Is VSMC Heterogeneity Important?
The VSMC is the only cell populating the normal vascular media wherein it is uniquely responsible for maintaining vascular tone. The contractile phenotype is therefore essential for hemodynamic stability. Except in unusual circumstances when the adventitia may be involved, the VSMC is also the only vascular cell capable of repairing the injured vessel wall by migrating, proliferating, and elaborating an appropriate extracellular matrix. It is therefore equally essential that the VSMC can also adopt a phenotype capable of these functions. These diverse roles argue for heterogeneity or plasticity of VSMC phenotype. The ability of VSMCs to modulate to a phenotype capable of, among other things, secreting and responding to various growth factors, was considered a key pathogenic factor capable of, among other things, secreting and responding to diverse roles argue for heterogeneity or plasticity of VSMC phenotype. These studies have shown that primary human cell cultures derived from atherosclerotic plaques proliferate poorly, senesce early, and readily undergo apoptosis. Thus, as with rodent cells, stable differences in phenotype between human cells derived from different sources can be identified. Further studies analyzing expression of VSMC differentiation markers in human cells are now required so that our knowledge of human cells will equal that of rodent cells.

Summary
There is evidence for marked heterogeneity of VSMC phenotype in rodents and humans. However, the contractile and synthetic phenotypes initially described in cell culture probably represent the extremes of a continuous spectrum of VSMC phenotypes that can occur in vivo. We have postulated that different VSMC phenotypes are determined by different programs of gene expression. By studying the genes expressed by VSMCs and determining the intrinsic and extrinsic factors that regulate their expression, both during normal development and disease, it is hoped to derive a better definition of phenotype based on the “program” of gene expression. This approach offers the best opportunity for a rational approach to therapeutic modulation of VSMC phenotype in the future.

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References
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