History of Discovery

Smooth Muscle Phenotypic Modulation—A Personal Experience

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Abstract—The idea that smooth muscle cells can exist in multiple phenotypic states depending on the functional demands placed upon them has been around for ≈5 decades. However, much of the literature today refers to only recent articles, giving the impression that it is a new idea. At the same time, the current trend is to delve deeper and deeper into transcriptional regulation of smooth muscle genes, and much of the work describing the change in biology of the cells in the different phenotypic states does not appear to be known. This loss of historical perspective regarding the biology of smooth muscle phenotypic modulation is what the current article has tried to mitigate. (Arterioscler Thromb Vasc Biol. 2012;32:1784-1789.)

Key Words: contractile state • synthetic state • smooth muscle differentiation

In 1968, Robert Wissler suggested that the cells of the arterial media are of prime importance in the pathogenesis of atherosclerosis and that they comprise a single cell type. Before this time, most histology textbooks stated that both smooth muscle and fibroblasts existed in the media of medium and large arteries. He further noted that these cells, which maintained vascular tone and thus were contractile, were also responsible for the production of collagen and elastin. Wissler thus proposed that the arterial medial cell could be viewed as a multifunctional mesenchyme, that the migration and proliferation of medial smooth muscle cells are major cellular sources of thickened neointimas, and that many of the so-called lipid-laden foam cells in atherosclerotic lesions may be modified smooth muscle cells.

Smooth Muscle Phenotypic Change

The article by Wissler had a huge impact on the 2 of us. In 1970, we were PhD students studying different aspects of smooth muscle at the University of Melbourne. Gordon was structure (histology) while Julie was function (cell biology and biochemistry). Gordon had noted that during regeneration and repair following injury in vivo, smooth muscle cells in visceral organs, such as the vas deferens and taenia coli, lost their myofilaments and gained large amounts of synthetic organelles (rough endoplasmic reticulum, Golgi, mitochondria, free ribosomes) before proliferating (Figures 1 and 2). Julie had noted that individual visceral smooth muscle cells enzymatically isolated and seeded in primary culture contracted spontaneously at a rate of 1× to 7× per minute, but that after 5 to 7 days in culture changed from spindle/elongated ribbon to become broader and flatter, and then ceased contractions.

This change in shape and behavior nearly always preceded the cell’s commencement of proliferation in the presence of whole blood serum. Gordon examined Julie’s isolated cells by electron microscopy and found that the contractile cells (<5 days in culture) resembled normal mature smooth muscle with cytoplasm full of myofilaments, but that the broadened and flattened cells 7 days in culture exhibited the same synthetic morphology as his regenerating smooth muscle cells in vivo. Thus began our working collaboration of over 40 years—and our personal one resulting in 3 children and 2 (so far) grandchildren.

In the same 1974 article, we showed that if the cells had been seeded densely in primary culture such that a confluent monolayer resulted after ≈2 days of proliferation, then they drew up into hills and valleys. Within a further 2 to 3 days, the cells in the reaggregated hills returned to their original spindle/elongated ribbon shape and recommenced spontaneous, and often synchronous, contraction. Ultrastructurally, they had regained myofilaments and lost many of their synthetic organelles. This showed that the changes in the structure and function of the mature smooth muscle cells in primary culture were reversible. However, if the enzymatically isolated visceral smooth muscle cells were seeded sparsely in primary culture, then they underwent the same change in morphology as the more densely seeded cells on days 5 to 7, took ≈3 weeks and many more cell divisions to achieve confluence, and did not revert to their original phenotype but appeared permanently in a synthetic state.

Our studies also noted that a small percentage (≈0.05%) of visceral smooth muscle cells in the first 2 to 4 days in culture underwent a single division while still contracting strongly, ceasing spontaneous contraction at the beginning of prophase and resuming when the daughter cells were in interphase. These cells were indistinguishable morphologically from other smooth muscle cells in the culture, and like...
them gradually flattened and lost their contractile ability. Division of smooth muscle with a contractile morphology was observed in the developing chicken gizzard by Cobb and Bennett, but most reports showed that the majority of cells observed in mitosis are at least partially modulated toward the synthetic state (Figure 3). Indeed, Poole et al. stated the following: “On looking at cells in the tunica media further and further away from the site of injury, it was seen that there was a continuous gradient in cell morphology from normal smooth muscle cells of the tunica media (which presumably had not been injured) to the cells showing great lack of differentiation nearer to the silk suture. Mitoses were seen among these cells.”

**Distinguishing Synthetic-State Smooth Muscle From Fibroblasts**

The close similarity in the appearance of fibroblasts and synthetic-state smooth muscle cells, both in culture and in developing and regenerating smooth muscle organs, was causing considerable confusion in the literature during the 1960s and 1970s. However, with experience they could still be distinguished. Under phase-contrast microscopy, synthetic-state smooth muscle cells are more phase-dense than fibroblasts and have considerably fewer inclusions in their cytoplasm. The nucleus has a more defined outline and is sausage shaped instead of round or oval as in fibroblasts, and the nucleoli are less phase-dense. Ultrastructurally, synthetic-state smooth muscle cells can be distinguished by the presence of a complete basal lamina, more plasmalemmal vesicles along the plasma membrane, and larger bundles of thin filaments with associated dark bodies.

An easier way to distinguish the 2 cell types was found by our collaborator Ute Groschel-Stewart, who developed antibodies to smooth muscle contractile proteins visualized with fluorescein isothiocyanate. Her antibody to smooth muscle myosin heavy chain did not cross-react with skeletal or cardiac

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**Figure 1.** Smooth muscle cells in the contractile state. Most of their cytoplasm is filled with myofilaments. Transverse section through the rabbit radial artery.

**Figure 2.** Synthetic-state smooth muscle cell from guinea-pig vas deferens, 1 week after transplantation to the anterior eye chamber. Note the large numbers of synthetic organelles and sparse peripheral bundles of myofilaments (Reproduced from Campbell et al).

**Figure 3.** Dividing smooth muscle cell from guinea-pig vas deferens, 1 week after transplantation to the anterior eye chamber. Most of the cytoplasm is filled with synthetic organelles (Reproduced from Campbell et al).
muscle, but strongly reacted with contractile-state smooth muscle myofibrils and only weakly with fibroblasts at room temperature. Reaction of the myosin antibody with smooth muscle was greatly reduced in cells that had undergone phenotypic change to the synthetic state. The major break came with Ute’s development of an antibody to native smooth muscle actin, a feat that had eluded many other researchers. This antibody reacted with smooth muscle cells irrespective of their phenotype, but did not react with fibroblasts or endothelial cells. A definitive way of distinguishing synthetic smooth muscle cells and fibroblasts was now available.

**Phenotypic Change in Vascular Smooth Muscle**

All our early work was done using visceral smooth muscle (vas deferens, taenia coli, ureter, gizzard) that contracted spontaneously in the first few days of primary culture and contained large amounts of myofilaments. If we had begun our studies using vascular smooth muscle cells, which appear morphologically less distinctly smooth muscle than their visceral counterparts, the changes that we so clearly saw may not have been observed. However, when we specifically looked for these changes in vascular smooth muscle cells in culture, we saw them. Our article described a method for the growth of large numbers of enzyme-isolated vascular smooth muscle cells from adult humans, monkey, and rabbit in primary culture and compared the properties of these cells with those that had migrated from explants and those in subculture. Use of Ute Groschel-Stewart’s antibodies not only showed that our cultures were consistently >99% pure smooth muscle but also that the cells that had migrated from explants and those in subculture were already in the synthetic state—loss of the contractile state generally being a prerequisite for migration and proliferation, and a permanent state after multiple rounds of cell division. We drew the conclusion that enzyme-isolated cells were phenotypically more representative of normal, mature smooth muscle cells in the artery wall and should be used instead of explant outgrowth or subcultured cells if one were seeking to discover the initiating events of neointima formation.

This work tended to be in direct conflict with that of Russell Ross, who was using serially subcultured monkey aorta smooth muscle (which had originally grown out of explants over 4 weeks) to investigate the cell biology of atherosclerosis. He showed that these cells, in the ninth subculture or later, synthesize collagen and elastin, but he also maintained that they exhibited a differentiated appearance under the electron microscope with abundant myofilaments and dense bodies. However, we showed that sectioning the cells close to the surface of the culture dish will give an erroneous picture, because that is where large numbers of attachment filaments and stress fibers are located, whereas the rest of cytoplasm contains mainly synthetic organelles. Others variously described the smooth muscle cells that grew from explants of aortic tissue in culture as fibroblast-like or modified smooth muscle cells, with relatively sparse myofilaments generally located at the cell periphery. Indeed, Ross himself stated that he presumed the filaments he saw were myofilaments and that his aortic outgrowth and subcultured cells “markedly resembled smooth muscle cells in vivo that are engaged in the synthesis and secretion of extracellular proteins.”

We thus decided that to resolve our conceptual differences with Ross, we should spend some time in his laboratory. We both won postdoctoral fellowships and worked 1 year (1976) in London, where we continued our collaboration with Ute Groschel-Stewart using her (then) very novel antibodies, 9 months in the Department of Pharmacology at the University of Iowa, Iowa City (where we were married in March 1977), followed by 5 months in Seattle.

During those 5 months, we worked and wrote 2 articles with Ross. The first showed that the change in the phenotype of enzyme-isolated smooth muscle cells occurred in primary culture irrespective of whether whole serum (normal or hyperlipidemic) or platelet-deficient serum was present. It also clearly showed a phenotype-dependent response to serum mitogens, with minimal proliferation occurring until the cells had undergone distinct morphological changes characterized by loss of myofilaments and more extensive synthetic organelles. Some years later, we showed that smooth muscle cells, both densely seeded early in primary culture and those sparsely seeded and proliferating, possess a relatively large number of binding sites for [125] platelet-derived growth factor-BB averaging 126 fmol/10^6 cells with a K_d of 0.53 nmol/L. The second article with Russell Ross was a 61-page invited review in *Physiological Reviews,* “The smooth muscle cell in culture.” It was much more than a review and contained a wealth of original data, showing that smooth muscle cells can alter their structure and function in response to functional demands (eg, injury/repair). It emphasized that there are not 2 phenotypic states, contractile and synthetic, but that smooth muscle can exist in a spectrum of phenotypes, with contractile and irreversibly synthetic at the 2 ends and many forms in between (hence the ability of some smooth muscle cells in culture to divide while still contracting, as described in our 1974 article). This collaborative article went a long way in winning Ross over and in stimulating the use of smooth muscle culture as a tool to investigate developmental and disease processes.

**Reversibility of Phenotypic Change**

In 1981, we showed that reversibility of smooth muscle phenotypic change was dependent on the number of cell doublings of the population before confluence was achieved. Fewer than 5 cell doublings allowed the cells to return to the contractile state (reversibly synthetic), whereas >9 cell doublings rendered them incapable of return (irreversibly synthetic), but not senescent. However, our later studies showed that any return to the contractile state in primary culture was not complete. Although the volume fraction of myofilaments in the cytoplasm of the cells returned to their original levels, as did the level of α-smooth muscle actin mRNA, the level of α-smooth muscle actin protein, once decreased upon phenotypic change, remained low, irrespective of the confluence or low population doublings. This suggests that an increase in the volume fraction of myofilaments is not necessarily caused through an increase in α-smooth muscle actin content, but may be caused by polymerization of cytoplasmic actins or by an increase in myosin or intermediate (10 nm) filaments.

**Lipid Metabolism and Phenotype**

We had returned to Melbourne, Australia, early in 1978, and our first child was born in October 1978, the second in April 1980,
and the third in July 1981. Julie then decided to henceforth publish as Campbell, rather than as Chamley-Campbell (or Chamley). The presence of experts in lipid metabolism at the Baker Medical Research Institute where we worked spurred an interest in determining whether synthetic versus contractile smooth muscle had a propensity to accumulate lipid. Indeed, we showed that a change in smooth muscle phenotype to the synthetic state is accompanied by distinct changes in the cells’ ability to metabolize low-density lipoprotein (LDL), with the rate of 125I-labeled LDL degradation decreasing to about one fifth of the level in contractile state cells. This was not because of changes in the number or affinity of LDL receptors on the cells because saturable binding of LDL was unaltered. The specific activities of lysosomal enzymes acid phosphatase and N-acetyl-β-glucosaminidase increased with change to the synthetic state as did cytochrome c oxidase (mitochondria) and nicotinamide adenine dinucleotide phosphate–dependent cytochrome c reductase (endoplasmic reticulum), but a decrease in the specific activity of the lysosomal enzyme acid cholesterol esterase. Significantly, more 3H-cholesterol oleate was recovered in synthetic than contractile cells after incubation with unlabeled LDL and 3H-sodium oleate. Similar but greatly enhanced differential effects on binding, degradation, and lipid accumulation by synthetic (particularly irreversibly synthetic) versus contractile state cells were shown by incubation in medium containing β-very low-density lipoprotein isolated from hyperlipidemic serum, and the effect was further exacerbated by incubation of the β-very low-density lipoprotein with endothelial cells or macrophages that caused extensive lipid peroxidation. Morphologically, the synthetic-state cells became almost completely filled with lipid droplets, whereas the contractile-state cells were unaffected. Similarly, it was shown that lipid accumulation occurred within the (morphologically) synthetic-state cells of intimates thickenings in the aortae of rabbits fed a 1% cholesterol-enriched diet, whereas there was no accumulation in contractile-state cells of the media.

Collagen/Glycosaminoglycan Synthesis and Phenotype

We also showed that collagen synthesis increased significantly upon change to the synthetic state and was greatest (35-fold) in cells that had undergone >5 cumulative population doublings. Noncollagen protein synthesis also increased but to a much lower extent. The increases in collagen and noncollagen protein were directly related to phenotype and independent of proliferation/quiescence. Type I collagen (as opposed to collagens type III or V) was the predominant collagen synthesized by all phenotypes, with a higher proportion synthesized by synthetic-state cells. Other studies showed that glycosaminoglycan synthesis increased 10-fold in synthetic- versus contractile-state cells, with higher proportions of chondroitin sulfate A/C and dermatan sulfate. Expression of an adhesion molecule for leukocytes, intracellular adhesion molecule-1, occurred only on synthetic-state smooth muscle, with no expression by contractile-state cells even after stimulation with interleukin-1β.

Smooth Muscle Phenotypic Change In Vivo

Using ultrastructural morphometry, we showed that in forming a neointimal thickening after endothelial denudation, smooth muscle cells in the underlying media underwent a change in phenotype before their migration to the intima and proliferation and that the change was reversed once re-endothelialization had occurred. However, if the segment of endothelium experimentally removed from an artery was small such that complete re-endothelialization occurs rapidly, an intimal thickening did not develop even though platelet aggregation and release of platelet-derived growth factor occur within the first 24 hours.

About 10 years later, we showed that 3 days after balloon-catheter injury, the level of heparanase activity in the artery wall is increased by ≈50% and by 140% at 2 weeks, returning to control levels at 6 weeks. Matrix metalloproteinase activity followed a similar pattern. In a subsequent article, both heparan sulfate and chondroitin sulfate were found in close association with smooth muscle cells of the uninjured arterial media as well as being more widely spread within the matrix. Within 6 hours after arterial injury, there was loss of the regular pericellular distribution of both glycosaminoglycans, which was associated with a significant expansion of the extracellular space. This preceded the change in phenotype of the smooth muscle cells as observed with ultrastructural morphometry. The decrease in glycosaminoglycans was greatest at 4 days, after which both rapidly returned around the cells of the media, but the intimal cells failed to produce heparan sulfate as readily as they produced chondroitin sulfate. We also showed that heparan sulfate proteoglycan extracted from the artery wall inhibited the development of a neointima in balloon-injured rabbit carotid arteries when applied to the adventitia in a pluronic gel, as well as inhibiting phenotypic change in vitro. Phosphomannopentaose sulfate (also called PI-88), which inhibits the activity of heparanase, also prevented a change in smooth muscle phenotype and reduced intimal thickening after balloon injury of rat and rabbit arteries.

In 1985, our postdoc Peter Mosse used quantitative morphometry to analyze the volume fraction of synthetic organelles in smooth muscle cells of diffuse intimal thickenings of human carotid arteries adjacent to atherosclerotic plaques taken at endarterectomy. He found enormous phenotypic variability among these cells, but that the vast majority had most of their cytoplasm filled with rough endoplasmic reticulum, free ribosomes, and mitochondria, equating to a 2-fold increase in the volume fraction of synthetic organelles compared with smooth muscle cells of the subjacent media. In a subsequent article, he showed no difference in the volume fraction of synthetic organelles in atherosclerosis-free diffuse intimal thickenings of human tissue taken at autopsy compared with the underlying media. These studies, together with our studies showing lipid accumulation in synthetic but not contractile-state cells, added further to our thesis that change in smooth muscle phenotype was important in atherogenesis.

Macrophages Induce a Change in Smooth Muscle Phenotype

Our next discovery was that living macrophages induce a change in the phenotype of smooth muscle cells in vitro and then stimulate their rate of proliferation beyond confluence to form multilayers. We also showed that macrophages have in their lysosomes a heparan sulfate–degrading endoglycosidase.
that cleaves internal glycosidic bonds and that its action is sufficient to induce a change in the phenotypic expression of smooth muscle cells. Our results further suggested that macrophages possess sulfatases and exoglycosidases, which sequentially release inorganic sulfates and monosaccharide residues from the nonreducing ends of the heparan sulfate fragments released by the endoglycosidase. We, therefore, suggested that macrophages, as well as being a source of lipid-laden foam cells in atherosclerosis, may play other important roles in this disease, such as initiating change in smooth muscle phenotype and influencing their proliferative response and pattern of growth. Our results showing enhanced proliferation were consistent with the findings of others that macrophages produce a potent mitogen resembling platelet-derived growth factor.

How Might Degradation of Heparan Sulfate Trigger a Change in Phenotype?

Cell-associated heparan sulfate proteoglycans occur as membrane-intercalated glycoproteins where the core protein is anchored in the lipid interior of the plasma membrane, and the heparan sulfate chains bind to specific sites on collagen, laminin, and fibronectin. The function of the proteoglycan-mediated interaction is to promote the organization of actin filaments in the attaching cell, which also has the effect of stabilizing cell morphology; thus, removal and destruction of cell-surface heparan sulfate at these sites may initiate a change in smooth muscle phenotype through disorganization of actin filaments with subsequent influences on gene expression. The observation that trypsin (which releases the heparan sulfate proteoglycans from the cell surface) does not by itself induce a change in smooth muscle phenotype suggests that the heparan chains must be completely destroyed or otherwise removed from the vicinity of the cell for this to occur. The ability of free heparin to prevent a change in the phenotype of smooth muscle cells whose extracellular matrix and basal lamina have been degraded and removed during enzymatic isolation supports this view. We showed that smooth muscle cells in the contractile state continuously internalize and degrade their own surface heparan sulfate to free sulfate, and that this occurs via a nonlysosomal pathway. Thus, heparan sulfate internalized from the cell surface may play a role in maintaining the smooth muscle cells in the contractile state. In other cell systems, it is known that surface heparan sulfate is regularly internalized and degraded, whereas fractions enriched in the rare 2-O-sulfate glucuronate units are not fully degraded but transported to the nucleus where they influence gene expression.

However, over the years the factors reported to control smooth muscle phenotype have been many and complex. We have shown that T lymphocytes, T-lymphocyte–conditioned medium, and a T-lymphocyte–derived cytokine, interferon-γ, are potent inducers of smooth muscle phenotypic change. Activation of RhoA (a key regulator of the actin cytoskeleton) by sphingosine-1-phosphate enhanced the expression of contractile proteins α-smooth muscle actin, smooth muscle myosin heavy chain, and SM-2, whereas inhibition of RhoA induced a more extreme synthetic phenotype including increased expression of vimentin. Transient transfection of synthetic-state cells with the constitutively active RhoA (Val4RhoA) caused a reduction in cell size and reorganization of cytoskeletal proteins to resemble that of the contractile phenotype. Others have reported that oxidized phospholipids or unsaturated lysophosphatidic acids (which activate Rho) induce phenotypic change. Platelet-derived growth factor-BB was reported to induce smooth muscle phenotypic change in vitro, specifically through destabilization of α-actin mRNA, with interleukin-1β playing a synergistic role. Various other factors have been reported.

Perspectives

Over the past 20 or so years, many other researchers have contributed to our knowledge of smooth muscle phenotype. In particular, they have investigated the complex mechanisms that regulate transcription of smooth muscle gene expression and control smooth muscle differentiation and phenotypic modulation, and identified a number of important marker genes. They have also shown that smooth muscle phenotypic modulation involves the activation of micro RNAs and embryonic stem cell pluripotency genes and that epigenetic mechanisms play an important role. These are important and exciting discoveries that have greatly advanced the field.

However, as is often the case in science, the originators of the ideas on which much of this work depends appear to have been forgotten. Rarely is there acknowledgment of the studies of Wissler and others who first observed the multifunctional mesenchyme nature of smooth muscle or of ours that described its phenotypic modulation in response to functional demands, cataloged its changes in structure and function, examined its controlling factors, and highlighted its relevance to disease processes such as atherosclerosis. Unfortunately, once articles, particularly major reviews, appear that lack historical perspective of discovery, the wheel becomes reinvented, and others adopt the same citations, albeit in otherwise excellent articles.

This loss of historical perspective regarding smooth muscle phenotypic modulation is what the current article has tried to mitigate. It also serves as a reminder that the basic cell biology related to phenotypic change should not be forgotten among studies of transcriptional regulators.

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Disclosures

None.

References

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