Characteristics of Coronary Smooth Muscle Cells and Adventitial Fibroblasts

Sachin Patel, MD; Yi Shi, MD, PhD; Rodica Niculescu, DVM; Eugene H. Chung; Jack L. Martin, MD; Andrew Zalewski, MD.

Background—Recent findings suggesting the involvement of adventitial cells in coronary repair have raised questions regarding the phenotypic “plasticity” of medial smooth muscle cells (SMCs). Accordingly, the aims of the present study were to examine the characteristics of coronary medial and adventitial cells and to compare the responses of coronary and noncoronary SMCs to stimulation.

Methods and Results—Enzymatically isolated coronary SMCs (human and porcine) were distinct from noncoronary SMCs, showing poor adhesion and spreading, as well as lower proliferation, collagen synthesis, and LDL degradation. Several extracellular matrix components (Matrigel, collagen I and IV, laminin, vitronectin, fibronectin) or growth factors (epidermal growth factor, platelet-derived growth factor-BB, insulin growth factor-1, interleukin-1α) failed to augment the adhesion or proliferation of coronary SMCs to the levels observed in noncoronary SMCs. Unlike coronary SMCs, coronary fibroblasts demonstrated high adhesion, proliferation, collagen synthesis, and avid LDL metabolism. Limited responses of coronary SMCs were associated with sustained expression of differentiation markers (α-smooth muscle actin, h-caldesmon, and smooth muscle myosin heavy chain), whereas noncoronary SMCs showed marked phenotypic heterogeneity.

Conclusions—Coronary SMCs appeared to maintain highly differentiated phenotype in response to stimulation, whereas coronary adventitial fibroblasts demonstrated several characteristics that are essential during vascular repair. Coronary SMCs, however, were distinct from noncoronary medial cells, which displayed greater phenotypic heterogeneity and versatility in culture. We postulate that the mechanism of vascular repair may differ among vascular beds, pointing to the importance of coronary artery–specific investigations in vascular biology. (Circulation. 2000;101:524-532.)

Key Words: remodeling ■ arteries ■ muscle, smooth ■ cells

Several mechanical, hemodynamic, or infectious factors contribute to the activation of resident vascular cells, resulting in the formation of intimal lesions.1,2 This process leads to changes in the cellular composition of the vessel wall, which accompany a broad spectrum of cardiovascular disorders.3-5 Although intimal cells share some similarities with medial smooth muscle (SM) cells (SMCs), they also display several distinct characteristics regarding morphology, gene expression, and synthetic properties (for a review, see Schwartz et al). Several concepts have emerged regarding the mechanisms underlying the expansion of vascular cells in various disease states.6-8 The first paradigm is based on the assumption that the cells involved in vascular repair are primarily of SM origin. The most widely accepted view implies the “plasticity” of virtually all medial SMCs, which modulate their phenotype in response to stimulation. This has been based on the observed changes in cultured SMCs rapidly losing their contractile features and becoming “synthetic” cells.6 A second paradigm, which is based on more recent studies, however, suggests heterogeneity of the arterial media, with only a fraction of SMCs capable of a rapid response to stimulation. These “stem” SMCs, isolated from the rat aorta or carotid artery, differ in regard to morphology, gene expression, and behavior in culture compared with the remaining medial cells.9,10 The third paradigm implies that highly reactive “nonmuscle” cells, being able to proliferate, migrate, and synthesize extracellular matrix proteins, are also involved in the repair of the vessel wall. Although they are sparsely distributed within vascular media (eg, carotid artery, pulmonary artery, and saphenous vein), nonmuscle fibroblasts are abundant in the adventitia.11-13 After severe coronary injury (porcine model), nonmuscle cells of adventitial origin proliferate and migrate to the luminal region during neointimal formation.14,15 They also acquire α-SM actin (myofibroblast formation) and are capable of the synthesis of several extracellular matrix proteins.16-18

Although these concepts remain the subject of ongoing debate, they are not mutually exclusive, because regional
differences in the mechanisms of arterial repair may arise from the diverse lineages of vascular cells. In particular, the coronary vasculature demonstrates unique development, which differs from that of the aorta and its major tributaries. The results of the present study suggest that coronary SMCs are less responsive to stimulation and exhibit lower synthetic capability than other vascular SMCs. When the cellular constituents of the coronary arteries were analyzed, adventitial cells demonstrated particularly dynamic phenotypic characteristics. These findings suggest there are functional differences in the cellular constituents of coronary and noncoronary vascular beds, which may influence the mechanisms of vascular repair and lesion formation.

Methods

Cell Isolation and Culture

Porcine arteries (epicardial coronary arteries, iliac artery, thoracic aorta) were harvested and opened, and their endothelial layers were removed. Human epicardial coronary arteries and samples of thoracic aorta, which were obtained from the recipients of heart transplantation, were prepared in an identical manner. For coronary arteries, the adventitia and media were separated with the assistance of surgical loops (magnification 2×). The separation of adventitial and medial layers was confirmed through immunostaining with α-SM actin antibody of random samples (not shown). For other vascular beds, the adventitial layer was stripped off, and only the media was used for cell isolation. The tissues were minced into small pieces and repeatedly incubated with collagenase type II (1 mg/mL; Worthington) and elastase (0.5 mg/mL; Sigma) for 30 minutes at 37°C with rocking. The cells were collected after passage of the digestion solution through a filter (pore size 70 μm; Becton Dickinson). After the addition of 10% heat-inactivated FBS and centrifugation, the cells were suspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. The cells from all digestions were pooled, and the viability was estimated in random samples through trypan blue exclusion or MTT assay. Freshly isolated cells were plated at 20,000 cells/well in 10% FBS and was stopped by the addition of 100 μg/mL CuCl2. Oxidation was measured by monitoring the change of the diene absorption (234 nm) and was stopped by the addition of 100 μmol/L EDTA and 40 μmol/L butylated hydroxytoluene. LDL was iodinated with Na125I (2 mCi; DuPont-New England Nuclear) in DMEM containing 10% FBS and ascorbic acid (25 μg/mL) for 100 U/mL tannic acid and BSA (100 μg/mL). Newly synthesized LDL was calculated as the difference between the radioactivity values of the samples treated, with or without collagenases (dpm/cell). The data represent mean±SD of 6 to 9 values derived from 3 experiments.

Protein Synthesis

For overall protein synthesis, cells were labeled with 35S-methionine (50 μCi/mL; DuPont-New England Nuclear) for 40 hours (primary cells) or 24 hours (early passages). The conditioned media and 2 washes were collected and subjected to TCA (10%) precipitation. After being washed twice with 5% TCA, radioactivity was determined with a beta scintillation counter (Wallac). Protein synthesis was expressed as dpm per cell number. Data represent mean±SD of 6 values derived from 3 experiments.

For collagen synthesis, subconfluent primary cells (200,000 cells) and confluent cells (passages 2 to 6) were labeled with 3H-proline (10 μCi/mL; DuPont-New England Nuclear) in DMEM containing 10% FBS and ascorbic acid (25 μg/mL) for 40 hours (primary cells) or 24 hours (confluent cells), respectively. The conditioned media and 2 washes were precipitated with 10% TCA at 4°C overnight. After centrifugation, the pellets were washed twice with 5% TCA and once with absolute ethanol. The pellets were dried and then hydrolyzed overnight with 0.45 mL of 0.2 mol/L NaOH. Radiolabeled collagen was quantified according to the collagenase digestion method. Briefly, hydrolyzed samples were divided into 2 aliquots (0.2 mL each) and neutralized by the addition of 0.2 mL of 0.08 N HCl. After the addition of N-ethylmaleimide (1.25 μmol/L; Calbiochem), samples, with a final volume of 0.5 mL were then incubated at 37°C for 5 hours. The control samples were treated without the addition of collagenases. The reaction was stopped by the addition of 0.5 mL of 20% TCA containing 0.5% tannic acid and BSA (100 μg/mL). Newly synthesized collagen was calculated as the difference between the radioactivity values of the samples treated, with or without collagenases (dpm/cell). The data represent mean±SD of 6 to 9 values derived from 3 experiments.
Immunostaining
Vascular cells were plated onto coverslips with or without Matrigel coating. Coronary SMCs were grown on coverslips coated with and without Matrigel. At different time points, the coverslips were fixed with HistoChoice for 30 minutes and air dried. The coverslips were stained with Vectastain Elite ABC system (Vector Laboratories). They were incubated with primary antibodies for 1 hour at room temperature, followed by biotinylated secondary horse anti-mouse antibodies (1:2000; Vector Laboratories). Table 1 lists specific antibodies, the concentrations used, and their sources. Negative controls included either the omission of primary antibody or its replacement with irrelevant mouse IgG (125 ng/mL). The immunostains were visualized with the use of a diaminobenzidine tetrahydrochloride substrate kit (Vector Laboratories) followed by hematoxylin counterstaining.

Table 1. Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>α-SM actin</td>
<td>1A4</td>
<td>1:50</td>
<td>Sigma</td>
</tr>
<tr>
<td>h-Caldesmon</td>
<td>hCD</td>
<td>1:200</td>
<td>Sigma</td>
</tr>
<tr>
<td>SM MHC</td>
<td>hSM-V</td>
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<td>Sigma</td>
</tr>
<tr>
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<td>3F8</td>
<td>1:200</td>
<td>Seikagaku</td>
</tr>
<tr>
<td>SM2 MHC</td>
<td>1G12</td>
<td>1:800</td>
<td>Seikagaku</td>
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Statistical Analysis
Data are expressed as mean±SD values. One-way ANOVA was used to compare the multigroup variables. If the F test results were significant, Bonferroni’s analysis was carried out to determine the differences among subgroups. A value of \( P<0.05 \) was required to reject the null hypothesis.

Results
Cell Morphology, Adhesion, and Growth
Enzymatic digestion yielded >80% viable cells regardless of vascular bed (trypan blue exclusion and MTT assay). As expected, medial cells isolated from the aorta and iliac artery displayed a spindle-shaped morphology, followed by a typical “hill-and-valley” pattern at confluence. In contrast, coronary SMCs demonstrated distinct morphology in culture (Figure 1). Coronary adventitial cells isolated from the same vessels, however, were spindle shaped after plating and showed a hill-and-valley configuration at confluence, thus displaying morphology similar to that of noncoronary SMCs (Figure 1).

Porcine SMCs derived from noncoronary vascular media showed rapid adhesion to plastic, which was comparable to that of coronary adventitial fibroblasts (Figure 2A).
contrasted with coronary medial SMCs, which showed significantly lower adhesion \((P < 0.01)\). Because cell attachment is dependent on the surrounding extracellular matrix proteins, we attempted to increase coronary SM adhesion through the coating of culture surfaces with several matrix proteins (Matrigel, collagen types I and IV, laminin, vitronectin, fibronectin). As shown in Figure 2B, different extracellular matrix proteins, the use of EGF in the culture medium, and the prolongation of adhesion time to 48 hours produced only minimal improvement in the adhesion of coronary SMCs. Each column represents mean \(\pm\) SD (n = 6 to 9).

As expected, porcine noncoronary SMCs exhibited rapid growth in the presence of 10% FBS, with exponential growth beginning at 2 days (Figure 3A). These cells continued to replicate after being subcultured for >10 passages and exhibited similar growth on plastic or Matrigel-coated surfaces. In contrast, the growth response of porcine coronary SMCs was markedly slower, although coronary adventitial fibroblasts isolated from the same vessels displayed a dynamic growth in culture (Figure 3A). These differences were not species dependent, inasmuch as human coronary SMCs also demonstrated lower growth capabilities (Figure 3B). To determine whether specific growth factors are required for coronary SMC growth, several growth factors (EGF, PDGF-BB, IGF-1, or IL-1α) were added to culture medium containing 10% FBS. Notwithstanding this stimulation, coronary SMCs continued to demonstrate limited replication in culture (Figure 3C). To exclude the possibility that their slower growth rate was attributable to differences in cell adhesion, we attempted to increase adhesion through the coating of culture surfaces with several matrix proteins.
growth was due to low density, porcine coronary SMCs were also plated at high density (100,000 cells/well), which failed to increase coronary SMC growth or their ability to reach confluence after 30 days in culture (not shown).

Metabolic Labeling, Collagen Synthesis, and LDL Metabolism

To compare metabolic activity in different vascular cells, overall protein synthesis was assessed with 35S-methionine labeling. No major differences were observed in total protein synthesis, which was consistent with similar viability of primary porcine cultures (Figure 4A, P=NS). Because “synthetic” phenotype of SMCs is marked by an increase in extracellular matrix synthesis, de novo collagen synthesis was measured with the use of 14C-proline incorporation, followed by collagenase digestion. Noncoronary SMCs from the aorta and iliac artery showed comparable collagen synthesis, whereas coronary medial SMCs produced significantly less collagen (Figure 4B, P<0.001). Coronary adventitial fibroblasts from the same coronary arteries also exhibited a higher ability to synthesize collagen than coronary SMCs (P<0.001).

The differences among vascular cells raised the question regarding regional LDL metabolism. To this end, the ability of vascular cells to degrade LDL was determined. Coronary SMCs demonstrated significantly lower degradation of both native LDL (P<0.01) and oxLDL (Figure 5A, P<0.001). Coronary adventitial fibroblasts showed the most avid metabolic processing of modified LDL, which exceeded values observed in the coronary and noncoronary SMCs (P<0.001). Differential LDL degradation was not species dependent, because primary cultures of human vascular cells demonstrated a similar pattern (Figure 5B). The avid degradation of LDL by adventitial cells was maintained even in late passages (passages 2 to 7; not shown).

Expression of SM Differentiation Markers

To examine whether these characteristics of vascular cells are related to their differentiation, several markers of SM differentiation were assessed in primary cultures. There was pronounced heterogeneity of α-SM actin expression in noncoronary SMCs (Table 2). As illustrated in Figure 6, α-SM actin ranged from negative to strongly positive in subconfluent cells. The markers of “late” SM differentiation, such as...
h-caldesmon and SM-myosin heavy chain (MHC), also displayed heterogenous distribution in noncoronary SMCs (Figure 6). Likewise, a similar pattern was observed with SM1- and SM2-MHC antibodies (not shown). In contrast, isolated coronary SMCs demonstrated uniform expression of α-SM actin (Table 2). As shown in Figure 7, they lacked heterogenous distribution of cytoskeletal markers (α-SM actin, h-caldesmon, and SM-MHC) compared with noncoronary SMCs. This highly differentiated phenotype was present in cells cultured in 10% FBS for 1, 3, 5, and 10 days. Figure 7 also illustrates the defective spreading of coronary SMCs, which did not change in the presence of several components of extracellular matrix (eg, vitronectin, collagen types I and IV, or fibronectin; not shown).

The vast majority of coronary adventitial fibroblasts were devoid of α-SM actin after isolation (Figure 8). This was followed by a dynamic upregulation in α-SM actin immuno-reactivity in coronary adventitial fibroblasts, which became almost uniformly positive for α-SM actin at 5 days, thereby acquiring the characteristics of myofibroblasts.

**Discussion**

Two major conclusions can be derived from the results of the present study. First, isolated coronary SMCs exhibit a highly differentiated phenotype, whereas noncoronary SMCs display greater phenotypic heterogeneity and more exuberant responses in culture (eg, proliferation, collagen synthesis, LDL degradation). Second, although coronary SMCs are less responsive than other SMCs, coronary adventitial nonmuscle cells demonstrate several phenotypic characteristics that enable them to react to pathophysiological stimuli.

**Coronary Medial SMCs**

Different morphology and gene expression, as well as distinct proliferative and migratory capabilities, have been previously identified among noncoronary SMCs. Whether the selective expansion of certain subtypes of SMCs or their differential survival in culture accounted for phenotypic versatility of noncoronary SMCs remains to be determined. Nevertheless, coronary SMCs, isolated and cultured in an identical manner, exhibited marked differences. Although differentiation and growth can be dissociated in culture, coronary SMCs continued to express differentiation markers and failed to replicate in the presence of several growth factors (Figure 3). To account for possible species differences, we also examined human coronary SMCs, which showed similar morphology and proliferative activity as porcine coronary SMCs. It should be underscored, however, that our findings do not necessarily contradict previous reports describing the growth of α-SM actin–positive cells from human coronary arteries. The particularly great ability of adventitial fibroblasts to migrate, to overgrow medial SMCs, and to become myofibroblasts (Figure 8) raises questions as to the origin of cells previously thought to be SMCs.

The exact mechanism underlying differences between coronary and noncoronary SMCs remains to be determined. The development of coronary vessels in situ from the coelomic mesothelium, rather than from ectodermal or mesodermal portions of the aorta, may confer different

<table>
<thead>
<tr>
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<th>IA-SMCs</th>
<th>CA-SMCs</th>
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<tbody>
<tr>
<td>1</td>
<td>76±3</td>
<td>29±2</td>
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<td>67±14</td>
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<td>61±6</td>
<td>82±12</td>
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</tr>
<tr>
<td>7–10</td>
<td>100±0</td>
<td>96±2</td>
<td>99±1</td>
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Enzymatically isolated porcine SMCs (primary cultures) were grown on coverslips in 10% FBS. The time refers to days in culture after plating. Noncoronary SMCs reached confluence at 7 to 10 days, whereas coronary SMCs remained subconfluent. Results are presented as mean±SD (n=10 fields, >500 cells counted).
cellular properties. In fact, cell type–specific transcriptional regulation of differentiation was recently emphasized by Owens et al. The extrinsic signals to be considered include extracellular matrix components (eg, laminin, heparan sulfate glycosaminoglycan) that modulate SMC differentiation. It is less likely, however, that regional variations in the composition of vascular extracellular matrix in situ were responsible for the observed differences after enzymatic cell isolation. We also emphasize that the effects of several other factors, which are absent in culture conditions, may promote dedifferentiation of coronary SM in vivo; these factors include age-, gender-, and environment-dependent phenomena (arterial retention of LDL or advanced glycosylation end products), which await clarifications in studies involving the coronary vasculature.

Coronary Adventitial Nonmuscle Cells

The stimulation of coronary adventitial nonmuscle cells resulted in phenotypic changes typically attributed to synthetic SMCs. The expression of α-SM actin by adventitial nonmuscle cells exemplified the formation of myofibroblasts (Figure 8). The selective expansion of a small number of SMCs in adventitial samples was unlikely, inasmuch as coronary SMCs demonstrated poor attachment and growth (Figures 2 and 3). As shown in the present study, adventitial nonmuscle cells displayed several properties necessary for arterial repair (ie, high adhesion, proliferation, and collagen synthesis). Recently, they have also been shown to possess increased matrix-degrading activities and migration compared with coronary SMCs, which constitutively express TIMP-1 and -2. Notwithstanding these findings, we emphasize that although the conditions for activation of nonmuscle cells likely exist after acute mechanical medial injury in humans, the migration of the cells during chronic medial damage is more difficult to determine. The cytotoxic effects of modified LDL during atherogenesis, however, often result in medial thinning, which may enable nonmuscle cell translocation and their involvement in lesion formation. The avid degradation of oxLDL by activated coronary nonmuscle cells (Figure 5) suggested a high expression of scavenger receptor and the possibility of their involvement in foam cell formation on reaching the intima.

Clinical Implications

Disappointing results of several clinical studies that target coronary restenosis have exemplified a low predictive value of pharmacological testing in animal models of noncoronary

Figure 7. Photomicrographs demonstrating uniform expression of cytoskeletal markers in coronary SMCs. Enzymatically isolated porcine coronary SMCs were grown in 10% FBS. They exhibited uniform and sustained expression of all SM cell markers (3 to 5 days after plating) (magnification ×490). N/C indicates negative control (mouse IgG instead of primary antibody).

Figure 8. Photomicrographs showing cytoskeletal changes in coronary adventitial fibroblasts. Enzymatically isolated porcine coronary adventitial cells were grown in 10% FBS. They were initially devoid of α-SM actin but began to acquire α-SM actin in culture (3 to 5 days) and became uniformly positive for α-SM actin at 10 days (magnification ×490).
arterial injury. The presented results and previous observations in vivo indicate greater susceptibility of coronary adventitial fibroblasts to mitogenic stimuli compared with coronary SMCs. Whether the suggested involvement of adventitial cells will affect the outcomes of antihyperplastic interventions applied in vivo (eg, brachytherapy or pharmacological approaches) remains to be determined. Interestingly, however, the adventitial delivery of some agents appears to be more effective than endoluminal administration. We postulate that a better understanding of the unique characteristics usually attributed to synthetic SMCs. We collagen. In contrast, adventitial nonmuscle cells displayed with a limited ability to adhere, proliferate, and synthesize specifically at a reduction in coronary hyperplastic responses.

In conclusion, in the present study, we examined the characteristics of coronary medial SMCs and adventitial nonmuscle cells. Coronary SMCs, which differed from non-coronary SMCs, showed a highly differentiated phenotype with a limited ability to adhere, proliferate, and synthesize collagen. In contrast, adventitial nonmuscle cells displayed characteristics usually attributed to synthetic SMCs. We postulate that the mechanisms of vascular repair and lesion formation may differ among vascular beds, which points to the importance of future coronary artery-specific investigations in vascular biology.

Note Added in Proof
Since the submission of this manuscript, Christen et al have also reported the presence of a highly differentiated phenotype of coronary SMCs and the unique response of these cells in culture.

Acknowledgments
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