Heterogeneity of Smooth Muscle Cell Populations Cultured From Pig Coronary Artery

Hiroyuki Hao, Patricia Ropraz, Vitali Verin, Edoardo Camenzind, Antoine Geinoz, Michael S. Pepper, Giulio Gabbiani, Marie-Luce Bochaton-Piallat

Objective—Heterogeneous smooth muscle cell (SMC) populations have been described in the arteries of several species. We have investigated whether SMC heterogeneity is present in the porcine coronary artery, which is widely used as a model of restenosis.

Methods and Results—By using 2 isolation methods, distinct medial populations were identified: spindle-shaped SMCs (S-SMCs) after enzymatic digestion, with a “hill-and-valley” growth pattern, and rhomboid SMCs (R-SMCs) after explantation, which grow as a monolayer. Moreover, the intimal thickening that was induced after stent implantation yielded a large proportion of R-SMCs. R-SMCs exhibited high proliferative and migratory activities and high urokinase activity and were poorly differentiated compared with S-SMCs. Heparin and transforming growth factor- β2 inhibited proliferation and increased differentiation in both populations, whereas fibroblast growth factor-2 and platelet-derived growth factor-BB had the opposite effect. In addition, S-SMCs treated with fibroblast growth factor-2 or platelet-derived growth factor-BB or placed in coculture with coronary artery endothelial cells acquired a rhomboid phenotype. This change was reversible and was also observed with S-SMC clones, suggesting that it depends on phenotypic modulation rather than on selection.

Conclusions—Our results show that 2 distinct SMC subpopulations can be recovered from the pig coronary artery media. The study of these subpopulations will be useful for understanding the mechanisms of restenosis. (Arterioscler Thromb Vasc Biol. 2002;22:1093-1099.)

Key Words: intimal thickening restenosis endothelial cells myosin smoothelin

Smooth muscle cell (SMC) replication and migration from the media into the intima are essential processes during the development and evolution of atheromatous plaque and restenosis. There is now substantial experimental evidence to support the assumption that SMCs from the arterial wall of several species are phenotypically heterogeneous and that certain subsets of medial SMCs are particularly prone to accumulate within the intima under appropriate stimuli. Two distinct SMC populations have been identified in the rat arterial media: spindle-shaped SMCs (S-SMCs) and epithelioid SMCs, with both exhibiting distinct biological features. Epithelioid SMCs are capable of replicating in the absence of serum and exhibit high migratory activity that is correlated with increased tissue plasminogen activator (tPA) expression. Epithelioid SMCs have been shown to be the predominant component of intimal thickening (IT). Spindle-shaped and epithelioid clones can be recovered from adult rat normal media (NM) and IT, albeit in different proportions according to the origin. These clones, irrespective of their origin, exhibit phenotypic features similar to those of the corresponding whole-cell populations, thus providing evidence that the NM contains cells capable of displaying each of the 2 phenotypes in vitro.

To extend the notion of SMC heterogeneity to other species and to a well-accepted model for human atherosclerosis and restenosis, we have selected the porcine coronary artery for study. We demonstrate that 2 SMC populations are present in this artery and that they exhibit distinct phenotypic features, respond differently to growth factor and heparin treatment or to coculture with coronary artery endothelial cells (ECs), and appear more interchangeable than do corresponding SMC populations described in the rat.

Methods

Cell Culture

Arterial media was dissected from the left anterior descending coronary artery of 8-month-old domestic crossbred pigs obtained from a nearby slaughterhouse. Additional experiments were performed on 3-month-old pigs in which media and IT were studied. IT was induced by implantation of a self-expanding stent (Wallstent, Schneider), as previously described. Fifteen days after injury, IT present between the struts, and the lumen was separated from the stent and the underlying media. Tissue dissection was controlled...
histologically. Cultured medial SMCs did not differ in the 2 groups of animals with the use of the criteria described below. The experiments were performed according to the Swiss Federal Veterinary guidelines and approved by the Ethics Committee of the Geneva Medical Faculty.

SMCs were isolated by 2 methods: enzymatic digestion and tissue explantation. Arterial media was digested as previously described, and SMCs were cultured in DMEM (GIBCO-BRL) containing 10% FCS (HyClone). For tissue explantation, media or 15-day-old IT was cut into 3×3-mm pieces. The luminal or abluminal side of the explants was carefully placed in contact with the culture dish (15 to 20 tissue pieces per 60-mm dish). DMEM plus 10% FCS was added. The times requested for (1) emergence of the first cells from the explant and (2) growth to confluence were recorded. Explanted tissue pieces were removed 2 or 3 days after the first SMC appeared. This was important for maintaining the proportion of different phenotypes (see Results). To quantify the number of explants giving rise to distinct cell types, SMCs were cultured in 24-well plates (1 piece per well) and observed at confluence. Four populations isolated by enzymatic digestion, 4 isolated by tissue explantation from NM, and 1 isolated by tissue explantation from IT were studied between the fifth and eighth passages. In all experiments, SMCs were plated at 4×10^5 cells/cm^2, unless otherwise stated.

SMC cloning was performed by limiting dilution to 7 days after the plating of a primary culture obtained by tissue explantation, as previously described. ECs were isolated from the coronary artery of 8-month-old pigs. Arteries were opened longitudinally, and the endothelium was gently scraped with a surgical blade. Cells were plated into 60-mm culture dishes containing DMEM and bovine aortic EC–conditioned medium (1:1) supplemented with 10% FCS and heparin (100 μg/mL, H-9399, Sigma Chemical Co). ECs started to grow ~10 days after plating and displayed a cobblestone morphology. To eliminate contaminating SMCs, typical EC colonies were trypsinized by using 5-mm-diameter stainless-steel rings and passed into a 24-well plate in medium used for primary culture. ECs were characterized at the third passage and used between the fifth and eighth passages.

Coculture of ECs and SMCs was performed by using the Transwell system (24-mm-diameter Transwell clear polyester membrane, 0.4-μm pore size, Costar). Coronal arterial ECs were plated at 4×10^5 cells/cm^2 into the upper compartment in DMEM plus 10% FCS for 7 days to reach confluence. Whole SMC populations (9×10^5 cells/cm^2) and SMC clones (18×10^5 cells/cm^2) were plated in the lower compartment in serum-free medium (SFM) for 24 hours. Then, EC and SMC populations or clones were placed in coculture in DMEM plus 10% FCS. Medium was changed after 3 days, and cells were counted after 7 days.3

**Cell Proliferation and Migration**

Cell proliferation in 10% FCS, 10% porcine plasma-derived serum (PDS), or SFM was analyzed by cell counting.6,10 Cell migration was analyzed by using a previously described “in vitro wound” model in a confluent culture.5,10 For the collagen gel invasion assay, SMCs were seeded at 1.5×10^5 cells/cm^2 in DMEM plus 10% FCS on the surface of collagen gels.12 After 7 days, cell invasion was assessed by counting the number of cells in 6 randomly selected fields per condition. Each field measured 0.6×0.8 mm and was obtained at focal levels of 50 or 100 μm beneath the surface monolayer. Zymographic and reverse zymographic assays were performed as previously described.6,12

**Immunofluorescence Staining and Western Blotting**

Confluent SMCs were trypsinized, cytocentrifuged onto glass slides, fixed, and stained as previously described6,10 with the use of antibodies recognizing α-smooth muscle actin (α-SM actin),13 desmin (clone D33, Dako), or smoothelin.14 We used 2 affinity-purified rabbit polyclonal IgGs recognizing smooth muscle myosin heavy chains (SMMHCs) with a similar specificity; one was produced in our laboratory,15 and the other is commercially available (Biomedical Technologies).10 ECs were fixed directly in the culture dishes and stained with anti-α-SM actin or anti–CD-31 (Santa Cruz Biotechnology).

Cultured SMCs were processed for SDS-PAGE and immunoblotting as previously described.10

**Influence of Heparin and Growth Factors**

SMCs were incubated in DMEM plus 10% FCS alone or supplemented with one of the following: heparin (200 μg/mL), transforming growth factor (TGF)-β2 (10 ng/mL; gift from Dr D.A. Cox, Novartis, Basel, Switzerland), fibroblast growth factor (FGF)-2 (10 ng/mL; Promega), or platelet-derived growth factor (PDGF)-BB (10 ng/mL; gift from Dr G. Pierce, Amgen, Thousand Oaks, Calif).10 For reversal experiments, cells were treated with FGF-2 and PDGF-BB for 7 days; and then the medium was changed for DMEM plus 10% PDS for 6 days.

**Statistical Analysis**

Results are shown as mean±SEM. Multiple comparisons were performed by ANOVA. Comparisons between treated and control groups were analyzed by independent Student t test. Differences were considered statistically significant at values of P<0.05.

**Results**

**Porcine Coronary Artery Yields 2 Distinct SMC Phenotypes**

SMCs isolated by enzymatic digestion from the NM constantly exhibited a spindle-shaped phenotype with a classic “hill-and-valley” growth pattern at confluence (Figure 1A, a).10 In contrast, SMCs produced by tissue explantation

![Figure 1. Morphological features and proliferative activity of coronary artery SMC populations. A. Phase-contrastphotomicrographs show S-SMCs isolated from the NM by enzymatic digestion (a) and R-SMCs derived from the NM (b) and from IT 15 days after stent implantation (c). Photomicrographs b and c were obtained by tissue explantation. Bar=150 μm. B, S-SMCs continue to grow even after confluence (14 days), whereas R-SMCs stop growing at confluence. At each time point, R-SMCs derived from IT are more numerous than those derived from NM. *P<0.05 and **P<0.01 for R-SMCs vs S-SMCs; P<0.01 at each time point in IT-derived R-SMCs vs NM-derived R-SMCs.](http://atvb.ahajournals.org/doi/10.1161/01.ATV.0000029195.20084.68)
displayed either a spindle-shaped or a rhomboid phenotype (Figure 1A, b), ie, polygonal and flat, but more elongated than the epithelioid phenotype previously described in the rat aorta. Rhomboid SMCs (R-SMCs) formed a monolayer at confluence. The proportion of tissue explants giving rise to the 2 phenotypes depended on the orientation of the explant. When the ab luminal side was placed in contact with the culture dish, it yielded a high proportion of R-SMC population (80.0±9.7%), whereas the luminal side of the media yielded an equal proportion of S-SMCs and R-SMCs (49.2±6.5% and 50.8±6.5%, respectively). In both situations, R-SMCs started to grow from the explants more rapidly than did S-SMCs (at 5 and 10 days, respectively) and reached confluence more rapidly than did S-SMCs (after 6 and 8 days after the appearance of the first cells, respectively). Explants from stent-induced IT produced a high proportion of R-SMC populations (78.2±2.7%, Figure 1A, c) irrespective of the orientation. They started to grow out from the explants after 2 days and reached confluence more rapidly than those derived from NM (within 4 days after the appearance of the first cells). Both phenotypes were stable with repeated passaging.

S-SMC and R-SMC Populations Exhibit Distinct Biological and Biochemical Features

The number of S-SMCs increased progressively even after the cells reached confluence (10 days, Figure 1B). In contrast, the number of NM-derived R-SMCs stopped growing at confluence (10 days, Figure 1B). Compared with S-SMCs, the number of NM-derived R-SMCs was significantly higher during the growing phase (7 days), was similar at confluence (10 days), and was lower after confluence (14 days). The IT-derived R-SMCs exhibited a growth pattern similar to that of the NM-derived R-SMCs, with a higher number of cells at each time point (Figure 1B). [3H]Thymidine incorporation measured after 30 hours was higher in R-SMCs derived from NM (0.074±0.004 and 0.066±0.003 cpm per cell, respectively, corresponding to 184.4±16.4% and 163.2±8.1% of values obtained in S-SMCs) compared with S-SMCs (0.040±0.003 cpm per cell, P<0.01). Thus, R-SMCs, irrespective of their origin, grew more rapidly than do S-SMCs; however, R-SMCs stop growing at confluence, whereas S-SMCs continue to replicate. Cell death evaluated by trypan blue exclusion was minimal in both SMC populations. S-SMCs and R-SMCs, irrespective of their origin, remained quiescent in the presence of 10% PDS or SFM (data not shown).

In vitro migration showed that NM-derived R-SMCs and, to a greater extent, IT-derived R-SMCs displayed a higher migratory activity than did S-SMCs (Figure 2A). The collagen gel invasion assay showed that a high number of R-SMCs invaded the gel (Figure 2B). In contrast, only rare S-SMCs were seen to invade the gel. The maximal depth of invasion was significantly higher for NM- and IT-derived R-SMCs (201.7±18.4 and 178.3±27.3 μm, respectively) than for S-SMCs (95.0±22.4 μm, P<0.05).

The high migratory activity observed in R-SMCs was associated with an increase in urokinase plasminogen activator (uPA) activity, which amounted to 15±1.5-fold in NM-derived R-SMCs and 22±1.8-fold in IT-derived R-SMCs compared with S-SMCs (P<0.001, Figure 2C). tPA and plasminogen activator inhibitor-1 were weakly expressed in the different populations (data not shown).

As previously described, practically all S-SMCs were positive for α-SM actin, 70% expressed SMMHC, and ~15% to 20% expressed desmin and smoothelin. The proportion of R-SMCs positive for each differentiation marker was lower (P<0.01), with the exception of α-SM actin (Table 1); however, the intensity of α-SM actin staining was lower in R-SMCs than in S-SMCs (data not shown). Immunoblotting results were in agreement with immunofluorescence studies (Figure 3).

| TABLE 1. Cytoskeletal Features of S-SMC and R-SMC | % of Positive Cells for |
|--------|--------|--------|--------|--------|
|        | α-SM Actin | SMMHC  | Desmin | Smoothelin |
| S-SMC derived from NM | 99.7±0.3 | 76.3±2.7 | 12.8±1.2 | 21.5±3.1 |
| R-SMC derived from NM | 99.8±0.2 | 37.1±1.5 | 1.5±0.5 | 0 |
| R-SMC derived from IT  | 92.4±0.6 | 35.2±1.7 | 1.4±0.6 | 0 |
S-SMCs clearly expressed the 4 differentiation markers. In R-SMCs, irrespective of their origin, α-SM actin and SMMHC were strongly decreased to 25.7±7.4% and 32.9±3.4%, respectively (P<0.01), compared with S-SMCs. SMMHC types 1 and 2 were expressed in S-SMCs, whereas only SMMHC type 2 was present in R-SMCs. It is alternatively possible that 2 SMMHC-specific antibodies recognize a faster migrating SMMHC type 1, as previously described.16 Desmin and smoothelin were not detected in R-SMCs.

ECs Influence the Phenotype of Coronary Artery SMCs

ECs isolated from porcine coronary artery displayed a more heterogeneous shape than is seen with classic ECs from bovine or porcine aorta.17 However, immunofluorescence staining showed that all coronary artery ECs expressed CD31 and were negative for α-SM actin (data not shown). [3H]Thymidine incorporation in confluent EC monolayers was relatively high (0.025±0.005 cpm per cell), demonstrating that ECs had not reached a quiescent state.

S-SMCs isolated by enzymatic digestion from the NM were cocultured with ECs. SMCs switched from a spindle-shaped to a rhomboid phenotype (Figure 4A, a and b). An additional coculture experiment was performed with the use of 2 SMC clones exhibiting spindle-shaped morphology. These clonal populations switched from a spindle-shaped to a rhomboid phenotype after 7 days of coculture with ECs (Figure 4A, a and b). A further analyze this hypothesis, 2 spindle-shaped clones were cultured in 10% PDS to avoid cell replication, suggesting that ECs treated with FGF-2 and PDGF-BB had an effect on the morphology of R-SMCs (data not shown). However, both strongly inhibited the proliferation of S-SMCs10 as well as R-SMCs, irrespective of their origin (Figure 4B). Immunoblot (Figure 3) showed that heparin increased α-SM actin content in S-SMCs and to a greater extent in R-SMCs (2-fold [P<0.05] and 20-fold [P<0.01], respectively). Heparin increased the desmin content only in S-SMCs (6-fold, P<0.01) and increased smoothelin in both populations (3-fold, P<0.05). It had no effect on SMMHC. TGF-β2 increased α-SM actin content slightly in S-SMCs and more importantly in R-SMCs (1.5-fold [P<0.05] and 5-fold [P<0.01], respectively). It increased desmin content only in S-SMCs (3-fold, P<0.05) and did not affect the expression of smoothelin and SMMHC in either population.

When S-SMCs were treated with either FGF-2 or PDGF-BB, they acquired a rhomboid phenotype and grew as monolayers but did not stop proliferating at confluence (data not shown). This effect was reversed when FGF-2 and PDGF-BB were withdrawn for 7 days, and cells were cultured in 10% PDS to avoid cell replication, suggesting that this change was induced by phenotypic modulation. To further analyze this hypothesis, 2 spindle-shaped clones were treated with FGF-2 and PDGF-BB. Both cytokines induced a switch from a spindle-shaped to a rhomboid morphology in both clonal SMC populations. FGF-2 and PDGF-BB did not modify the morphology of R-SMCs (data not shown). Both increased the proliferation of NM-derived S-SMCs and R-SMCs to a greater extent than was found for IT-derived R-SMCs (Figure 4B). FGF-2 and PDGF-BB increased the number of both types of SMCs invading collagen gels as well.
as the depth of invasion (data not shown). Immunoblots (Figure 3) showed that FGF-2 and PDGF-BB inhibited the expression of H\textsubscript{9251}-SM actin in S-SMCs and R-SMCs; desmin and smoothelin were absent from R-SMCs under control conditions and disappeared after both treatments in S-SMCs. In S-SMCs, FGF-2 and PDGF-BB decreased SMMHC type 2 content to 34.6\textpm{}12.5\% and to 44.7\textpm{}11.5\%, respectively, compared with untreated cells (P<0.01); SMMHC type 1 disappeared. In R-SMCs, FGF-2 and PDGF-BB decreased the SMMHC type 2 content to 64.9\textpm{}10.6\% and 62.2\textpm{}12.7\%, respectively, compared with untreated cells (P<0.05).

**Discussion**

The present study establishes for the first time that normal porcine coronary artery SMCs placed in culture can produce 2 populations with distinct phenotypes. Moreover, R-SMCs are recovered maximally from stent-induced IT, suggesting that this subpopulation is involved in the formation of the lesion, which is analogous to previous findings in the rat.\textsuperscript{5,8,18} SMC clones with a phenotype similar to the phenotype that we described for R-SMCs have been isolated from human arteries\textsuperscript{19,20}; however, the systematic study of human SMC populations is difficult for obvious reasons. Our model allows us to study the biological features of different SMC phenotypes and to test their role in the production and regression of lesions. It is noteworthy that cells capable of generating R-SMCs are allegedly located in the deeper layers of the NM and that we failed to isolate R-SMCs from the IT by enzymatic digestion. We have never obtained cells exhibiting a rhomboid phenotype from the adventitia by either explant (data not shown) or enzymatic digestion.\textsuperscript{11} This is in agreement with previous work suggesting that SMCs are the main component of experimental IT.\textsuperscript{11,21}

Our results show that migratory activity is crucial for isolation of the R-SMC population. Indeed, enzymatic digestion, which allegedly preserves all types of medial SMCs, always results in the production of S-SMCs.\textsuperscript{10} Thus, migratory activity and the capacity to produce uPA represent the main differences between R-SMCs and S-SMCs. It appears that SMCs from different species synthesize different plasminogen activators\textsuperscript{6,22}; the capacity to produce high plasminogen activator activity and to migrate actively furnishes a basis for the role played by R-SMCs in IT and possibly in the development of restenosis. Indeed, the preferential participation in IT formation has already been demonstrated for rat epithelioid SMCs.

Previous work, mainly using the rat, has shown that 2 distinct SMC phenotypes (spindle-shaped and epithelioid) can be obtained by isolating cell populations from different locations within the same vessel or from the same location in animals of different ages.\textsuperscript{4,5,23,24} The production of SMC clones has indicated that these phenotypes cannot be modulated from one into the other.\textsuperscript{5,8,18} As we have shown, this is not the case for porcine coronary SMC populations that can

**TABLE 2. Cytoskeletal Features of Whole and Clonal S-SMC Populations Cocultured With ECs**

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<th>% of Positive Cells for</th>
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<tr>
<td></td>
<td>α-SM Actin</td>
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<tr>
<td>Whole S-SMC population control</td>
<td>99.6\textpm{}0.4</td>
</tr>
<tr>
<td>Coculture with ECs</td>
<td>33.0\textpm{}3.2</td>
</tr>
<tr>
<td>S-SMC clone control</td>
<td>97.3\textpm{}2.7</td>
</tr>
<tr>
<td>Coculture with ECs</td>
<td>16.7\textpm{}2.4</td>
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evolve, at least to some degree, into the other phenotype under the influence of EC products (coculture) or growth factors such as PDGF-BB or FGF-2. It is noteworthy that a bidirectional differentiation has been suggested in human SMC clones.25 It would be of interest to explore whether human epithelioid SMC clones are similarly sensitive to these growth factors. Because S-SMCs are clearly more differentiated than are R-SMCs, the plasticity of pig coronary artery SMCs in culture coincides with the classic concept of contractile and synthetic SMCs, which, by definition, can modulate their phenotypes.26,27

Although porcine coronary artery and rat aortic SMC populations share several similarities, they also show important differences. In both species, S-SMCs exhibit the classic hill-and-valley growth pattern; however, porcine S-SMCs are highly differentiated compared with rat spindle-shaped SMCs, as shown by the expression of SMC-specific differentiation markers, eg, smoothelin. In this respect, they are similar to human SMCs.10 Porcine R-SMCs behave in a manner similar to that of rat epithelioid SMCs in many respects: (1) they grow as a monolayer, (2) they exhibit low expression of differentiation markers, (3) they show high proliferative activity during the growing phase, and (4) they show high migratory activity.4,5 In particular, they can invade a 3D collagen gel (possibly via the production of uPA). However, rat epithelioid SMCs essentially produce tPA.6 Epithelioid SMCs, which have been described in different species, grow in the absence of serum, whereas porcine R-SMCs remain quiescent under these conditions.4,5,18,24 Interestingly, Li et al20 have recently isolated distinct SMC subpopulations from healthy human internal thoracic arteries; these cells exhibit the 2 typical (spindle-shaped and epithelioid) phenotypes. These 2 phenotypes are similar to those we describe in the present study, including the inability of epithelioid cells to grow in the absence of serum. This further supports the similarity between the biological features of porcine and human SMCs.

Previous work with EC-SMC coculture has yielded controversial results. It has been shown that ECs stimulate the proliferation of SMCs, most likely by producing plasminogen activator inhibitor-1, which, in turn, inhibits TGF-β activation, and also that ECs prevent the hill-and-valley growth pattern of SMCs.28 Rat aortic EC conditioned–medium increases the proliferation and decreases the expression of α-SM actin and SMMHC of SMCs from the same vessel.29 It has also been suggested that ECs inhibit SMC growth, particularly when ECs are maintained in a quiescent state.30 None of these studies has taken into account SMC heterogeneity. We have developed a porcine coronary artery coculture model in which ECs and SMCs were isolated from the same vessel. It should be noted that confluent ECs continuously incorporated [3H]thymidine and never reached a quiescent state, suggesting that confluent ECs mimic an injured or dysfunctional endothelium. Under these conditions, ECs induced a switch in S-SMCs from a spindle-shaped to a rhomboid phenotype. This was associated with increased SMC proliferation and a decrease in the expression of differentiation markers, further supporting the concept of a complex interaction between these 2 cell types. Interestingly, in ex vivo experiments using porcine aorta and human saphenous vein, the presence of dysfunctional endothelium resulted in intimal SMC proliferation.31,32 As stated above, PDGF-BB and FGF-2 can also modulate SMC phenotype from spindle-shaped to rhomboid. Heparin33 and TGF-β34 inhibit SMC proliferation and have no influence on the morphology of either population.

In conclusion, our results demonstrate that the media of the porcine coronary artery contains SMCs that can produce 2 phenotypically distinct populations in culture, one of which, the R-SMC population, is a likely candidate for the formation of IT in vivo. Further studies exploring the biological behavior of these populations and of their modulation by pharmacological agents will yield information on the mechanism of restenosis and on the possibilities of controlling its formation.

Acknowledgments

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