Cultured Porcine Coronary Artery Smooth Muscle Cells
A New Model With Advanced Differentiation

Thomas Christen, Marie-Luce Bochaton-Piallat, Pascal Neuville, Sander Rensen, Mireille Redard, Guillaume van Eys, Giulio Gabbiani

Abstract—Arterial intimal thickening after endothelial injury induced in rodents has proven to be a relatively unreliable model of restenosis for testing clinically useful compounds. The same has been found for cultured rat or rabbit vascular smooth muscle cells (SMCs). To test alternative possibilities, we have studied several differentiation features of porcine coronary artery SMCs, cultured up to the 5th passage after enzymatic digestion of the media. The effects of heparin, transforming growth factor (TGF)-β1 or TGF-β2, and all-trans-retinoic acid (tRA) on proliferation, migration, and differentiation of these cells also were examined. Porcine arterial SMCs in culture not only express high levels of α-smooth muscle (SM) actin but, contrary to rodent SMCs, also maintain an appreciable expression of SM myosin heavy chain isoforms 1 and 2, desmin, and smoothelin, a recently described late differentiation marker of vascular SMCs. We demonstrate for the first time that smoothelin is colocalized with α-SM actin in these cells. Finally, we show that in the porcine model, heparin is more potent than TGF-β1 or TGF-β2 and tRA in terms of inhibition of proliferation and migration and of increasing the expression of differentiation markers. This model should be a useful complement to in vivo studies of SMC differentiation and of pathological situations such as restenosis and atheromatosis. (Circ Res. 1999;85:99-107.)

Key Words: atheromatosis □ restenosis □ actin □ smoothelin □ myosin

The arterial intimal thickenings (IT) induced in the rat or rabbit after endothelial lesion are presently the most-studied models for atheroma formation and/or restenosis and have been useful for defining several biological features of smooth muscle cells (SMCs). However, these models have many important limitations (for review, see Reference 1), which could explain the clinical failure of substances that proved to be efficient inhibitors of IT formation in these animal models.

The biological features of SMCs in culture also have been systematically studied using cells derived from rat or rabbit arteries,2–7 but these, too, have shown limitations similar to those observed in vivo experiments. Among the models in large animals, the pig coronary artery IT has been used more and more.8,9 Pigs may develop spontaneously coronary atheromatosis with age, and the induction of typical plaques is easily achieved by a cholesterol-rich diet.10,11 Furthermore, angioplasty and other interventions can be performed in porcine coronary arteries with the same instruments as in humans. Although pig aortic and coronary SMCs have already been studied in vitro, no systematic description of their differentiation features during culture has been published.

The present study describes the characterization of differentiation features and several biological properties of cultured porcine left anterior descending (LAD) coronary artery SMCs. We show that, contrary to rat or rabbit arterial SMCs,2–7 porcine SMCs maintain in culture a high level of differentiation marker expression, including the hitherto unreported expression of smoothelin, a recently described marker of late SMC differentiation. We describe the replicative and motile properties of these cells as well as the influence on these activities of heparin, TGF-β1 or TGF-β2, and tRA. Our findings suggest that porcine coronary artery SMCs retain a much higher level of differentiation and behave differently in vitro from the majority of SMCs previously studied and thus may represent a better model for the study of agents influencing SMC behavior in vitro and in vivo.

Materials and Methods

Cell Culture

Coronary arteries from domestic crossbred pigs (Sus scrofa domesticus) were obtained from a nearby slaughterhouse at the age of ~8 months. The 40 mm of the LAD distal to the branching of the left circumflex coronary artery was chosen for dissection and enzymatic
digestion. The vessel was opened by a longitudinal cut. The endothelium was gently scraped with a surgical blade, and the media was then stripped from the underlying adventitia with two ophthalmological tweezers and finely minced. The material of two coronary arteries was pooled and digested for 60 minutes at 37°C in 6 mL of DMEM (Gibco BRL, Life Technologies) with 2% HEPES (Life Technologies) containing 40 mg of collagenase (Sigma-blend collagenase, type F, 1,800 U/mg; Sigma), 4.2 mg of elastase from hog pancreas (9.7 U/mg; Fluka), and 12 mg of soybean trypsin inhibitor (1 mg inhibiting 2.1 mg trypsin; Worthington). The enzymatic activity was stopped by FCS (Seromed, 10% final concentration; Biochrom KG), and the suspension was filtered through a sterile gauze to discard remnants. The filtrates of two preparations were pooled and plated on a 100-mm culture dish (Falcon, Becton-Dickinson) in DMEM without FCS. Two hours later, FCS was added up to a final concentration of 10%. The medium was changed after 24 hours, and the primary culture was confluent in ~6 days. Medium was always changed every 3 days. The plating efficiency (ratio of attached to plated cells) at 24 hours was found always to be ~70%. These cells were passed by a 1:2 split ratio and studied up to the 6th passage.

Cell Treatment
SMCs at the 5th passage were plated at a density of 60 cells/mm² in 100-mm culture dishes containing DMEM supplemented with 10% FCS. In a first series of experiments, medium was changed 24 hours after plating, and SMCs were incubated alone or in combination with one of the following: 200 μg/mL heparin (H-9399; Sigma), 10 mg/mL TGF-β1 (Sigma), 10 mg/mL TGF-β2 (gift of Dr D.A. Cox, Novartis, Basel, Switzerland), or 10−3 mol/L triA (Sigma). In a second series of experiments, medium was removed when cells reached subconfluence, and the cells were then incubated in DMEM supplemented with Monomed (a defined serum-free medium containing insulin, transferrin, sodium selenite, 2-mercaptoethanol, 2-aminooethanol, sodium pyruvate, glutamine, and a BSA–oleic acid complex; Commonwealth Serum Laboratories, Melbourne, Australia), containing the above-described agents. Vehicles (0.1% BSA in 4 mmol/L HCl for TGF-β1 or TGF-β2 and DMSO [Fluka] for triA) were added in control cells at the same concentration as for treated cells. Medium was changed after 3 days, and cells were treated for 6 days.

Cell Proliferation and [3H]-Thymidine Incorporation
For evaluation of proliferation, SMCs were plated in DMEM supplemented with 10% FCS as described above and were counted after 7 days using a hemocytometer. The results were calculated, as the ratio of counted to seeded cells.

For [3H]-thymidine incorporation, SMCs were plated at a density of 60 cells/mm² in DMEM supplemented with 10% FCS. At subconfluence, cells were synchronized for 3 days in DMEM containing Monomed. Fresh medium plus 10% FCS alone or containing one of the above-mentioned agents and 0.1 μCi/mL [3H]-thymidine (specific activity, 5 Ci × mmol−1 × L−1; Amersham) was then added for 24 hours. [3H]-thymidine incorporation was evaluated by trichloracetic acid and perchloric acid precipitation and counting in a liquid scintillation counter (Beckman Instruments).

Cell Migration
For evaluation of migratory capacity, SMCs were plated at a density of 60 cells/mm² in DMEM in the presence of 10% FCS. Confluent cultures were scratched with a silicon-coated stick to obtain a 0.8-mm-wide in vitro wound and photographed in phase contrast using a Zeiss Axiosvert 35 photomicroscope (Carl Zeiss, Oberkochen, Germany). Fresh medium plus FCS alone or containing one of the above-described agents was added. After 24 hours, nuclear staining with propidium iodide (0.05 mg/mL; Fluka) was performed, and migrating cells invading the empty space were counted using a Zeiss Axiosvert 35 photomicroscope and a KS400 interactive image-analy-sis system (Carl Zeiss Vision). Six randomly preselected fields (length, 2.5 mm) were analyzed per condition. Results were calculated as the total number of migrated cells per field.

Immunohistochemical Techniques
Immunohistochemical stainings were performed on 4-μm-thick sections from formaldehyde-fixed paraffin-embedded blocks. Sections were deparaffinized and immersed in methanol containing 0.5% H2O2 for 10 minutes. Sections were then incubated for 1 hour at room temperature with the following first antibodies: mouse monoclonal immunoglobulin (IgG)2a specific for α-smooth muscle (SM) actin at a dilution of 1:250; mouse monoclonal IgG1 anti-desmin at a dilution of 1:10 (Clone D33; Dako), affinity-purified rabbit polyclonal IgG specific for SM myosin heavy chain (MHC) at a dilution of 1:7; and undiluted mouse monoclonal IgG1 specific for smoothelin.13 Immunoreactivity of SMMHC and smoothelin staining was intensified by three microwave treatments for 5 minutes in citrate buffer (10 mmol/L, pH 6.0) before using the first antibody. Sections were incubated 30 minutes at room temperature with a secondary biotinylated goat anti-mouse or anti-rabbit antibody at a dilution of 1:250 (Dako). The presence of the specific protein was evaluated by means of the streptavidin-biotin-complex peroxidase method (Dako). Development of peroxidase activity was done with 3-aminophenazol (Sigma). Slides were counterstained with hematoxylin, mounted in Eukitt (Kindler, Freiburg, Germany), and observed using a Zeiss Axioshot photomicroscope (Carl Zeiss) equipped with a Plan-Neofluar ×20/0.5 objective (Carl Zeiss). Pictures were acquired with a high-sensitivity Coolview color camera (Photonic Science, London, UK). Images were processed with Adobe Photoshop 5.0 (Adobe System, Mountain View, Calif) and printed with a digital Fujifilm Pictrography 4000 printer (Fujifilm, Tokyo, Japan).

For immunofluorescence, SMCs were cultured in the presence of 10% FCS, and simple or double stainings were performed directly in the culture dishes or on SMCs cytocentrifuged on glass slides.6 Cells were then fixed in methanol for 5 minutes at −20°C and stained with anti-α-SM actin, in ethanol for 30 seconds at room temperature and stained with anti-SMMHC or anti-desmin, or in methanol/acetone (1:1, vol/vol) for 5 minutes at −20°C and stained with anti-smoothelin. Anti-mouse IgG2a, anti-mouse IgG1, and anti-rabbit IgG coupled with either FITC or TRITC were used as secondary antibodies. Cell counts on cytocentrifuged cells were performed using an Axioshot photomicroscope (Carl Zeiss) at ×40 magnification for at least three different fields for each sample. Each experiment was repeated at least three times. Photographs were taken with Tmax 400 film (Eastman Kodak, Rochester, NY) by using oil immersion Plan-Apochromat ×40/1.3 and ×63/1.4 objectives (Carl Zeiss).

Confocal Laser Scan Microscopy (CLSM) Analysis
Cells were grown in DMEM supplemented with 10% FCS on Labtec slides (Miles Scientific, Naperville, IL), stimulated for 3 days with heparin at a concentration of 200 μg/mL, fixed in methanol/acetone (1:1, vol/vol) for 5 minutes at −20°C, and stained with anti-α-SM actin, anti-vimentin, or anti-smoothelin, followed by the above-described secondary antibodies. Cell specimens were observed with a Zeiss confocal laser scan fluorescence-inverted microscope (LSM 410; Carl Zeiss) equipped with two lasers used simultaneously: a helium-neon laser (excitation wavelength at 543 nm) and an argon laser (excitation wavelength at 488 nm). The excitation spectra were separated by a dichroic beam splitter of 488/543 nm at 512 nm. The emission of the two fluorochromes were separated by a 560-nm dichroic beam splitter. Two detectors were used in parallel and were preceded with a 590- to 610-nm (rhodamine channel) or a 510- to 525-nm (fluorescein channel) narrow-band barrier filter. The partial superposition of the emission spectra of the two fluorochromes was negligible.14 Specimens were observed through an oil immersion Plan-Neofluar ×63/1.4 objective. Between 20 and 40 optical sections of 512×512 pixels separated by 0.25 μm were performed in the z-axis. A three-dimensional image corresponding to the projection of all optical sections in one plane was reconstructed using the Imaris program.
Protein Extraction, Electrophoresis, and Western Blotting

Freshly isolated cells and confluent culture dishes after trypsinization and cell samples were washed in PBS supplemented with protease inhibitors: 10 mmol/L EGTA (Acros Organics), 1 mmol/L TAME (Fluka), 1 mmol/L PMSF (Fluka), 4 mg/100 mL aprotinin (Trasylo; Bayer AG), 0.5 mmol/L benzamidine (Sigma); and 1 mmol/L DFP (Fluka). Cells were resuspended in sample buffer containing all of the aforementioned inhibitors plus 1% SDS (Bio-Rad), 1% DTT (ICN), and 10% glycerol (Fluka), 80 mmol/L Tris-HCl at pH 6.8 (Gibco), and bromophenol blue (Merck), sonicated (Sonifier; Branson Sonic Power Co, Danbury, Conn), and boiled for 3 minutes. Protein concentration was determined according to Bradford.17

Western blotting, 2 to 20 μg of total protein was electrophoresed and transferred to a nitrocellulose filter (Protran; Schleicher and Schuell, Dassel, Germany). Filters were incubated with either anti–α-SM actin antibody at a dilution of 1:500, anti-desmin at a dilution of 1:500, or anti-smoothelin antibody at a dilution of 1:5 followed by a goat anti-mouse IgG at a dilution of 1:1000 (Biomedical Technologies Inc). This antibody does not recognize nonmuscle isoform B18 (Robert B. Low, personal communication, 1998). A goat anti-rabbit IgG at a dilution of 1:10 000 was used as second antibody. Enhanced chemiluminescence was used for detection (Amersham). Quantification of results was accomplished as described above.

α-SM Actin cDNA Probe, RNA Extraction, and Northern Blotting

Because the sequence of the porcine α-SM actin mRNA is not known, we selected a conserved sequence among mouse, rat, rabbit, and human in the 3’ untranslated region (3’UTR). This region is specific for a given isoform19 but may not be species specific. Two primers selected at the extremities of this conserved sequence were used for polymerase chain reaction (PCR).

For practical reasons, PCR amplification was performed on an α-SM actin cDNA already cloned in a plasmid containing parts of the 3’UTR of the gene and yielded a DNA fragment of 121 bp. Two microliters of this plasmid was amplified in 30 cycles (94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds). PCR was performed in 1× reaction buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl [pH 9], and 0.1% Triton X-100) containing 25 pmol/μL sense and reverse primer, 25 mmol/L MgCl2 (Promega), 200 μmol/L each dNTP (Promega), and 1 U Taq DNA polymerase (Promega). All PCR reactions began with a hot start. PCR products were then electrophoresed on 2% to 4% agarose gel. DNA was stained with ethidium bromide (Sigma) and visualized in UV light. Elution and purification of specific DNA fragments on gels were performed with a JetSorb gel extraction kit (Genomed, Research Triangle Park, NC) according to manufacturer information. Ligation and cloning of specific insert were accomplished by using PCR 2.1 Vector (Invitrogen). Identity and orientation of insert in recombinant plasmid were confirmed by sequencing (T7 sequencing kit; Pharmacia Biotech, Dübendorf, Switzerland) using a sequencing gel electrophoresis apparatus (model S2001; Gibco).

For yet-unexplained reasons, direct extraction of total RNA from the media of coronary arteries resulted in an important degree of RNA degradation. Therefore, total RNA was extracted from freshly isolated cells of the media according to manufacturer instructions as for confluent culture dishes. For Northern blot hybridization, 10 to 20 μg of total RNA was denatured with glyoxal, separated by...
electrophoresis on 1% agarose gel and blotted on Hybond N-filters (Amersham). After UV fixation, filters were stained with methylene blue to verify correct loading and transfer. Filters were then processed for hybridization by random priming (Megaprime DNA labeling; Amersham) of specific cDNA probes for α-SM actin. Prehybridization and hybridization were performed at 50°C for 3 and 15 hours, respectively, in 5× SSC, 5× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.1% SDS, and 100 μg/mL salmon sperm DNA. After hybridization, filters were washed twice for 15 minutes at 50°C in 4× SSC and 0.1% SDS. They were then exposed to Kodak X-Omat SO-282 (Eastman Kodak) at ~70°C for 24 hours to 3 days. Autoradiographic signals were digitized and processed as described above. α-SM actin mRNA levels were calculated taking into account differences in loading between lanes as assessed by scanning the 18S band of the methylene blue–stained membrane.

Statistical Analysis
Results are shown as mean ± SEM. For statistical evaluation, the results were analyzed by Student t test. Differences were considered statistically significant at values of P<0.05.

Results
Expression of SMC Differentiation Markers
In Vivo
The porcine LAD has a mean diameter of ~4 mm just after branching away from the left circumflex artery, similar to the corresponding human vessel. Van Gieson elastin staining showed the typical architecture of a muscular artery with interrupted elastic fibers within the media (Figure 1a). Elastic fibers were abundant in the internal portion of the adventitia.

For immunohistochemical analysis of the coronary arteries, we chose a set of four well-accepted SMC differentiation markers: α-SM actin, desmin, SMMHC, and smoothelin, a protein recently described in arterial tissue, probably the most specific marker of well-differentiated SMCs.15,21 Almost all SMCs in the media expressed α-SM actin and SMMHC (Figure 1b and 1c) whereas a small proportion of cells in the media appeared negative for desmin and smoothelin (Figure 1d and 1e). Endothelial and adventitial cells were negative for all markers. All markers showed a similar staining distribution in proximal compared with distal segments of the same vessel (data not shown).

Features of SMCs In Vitro
Enzymatic digestion was preferred to explant method because it allows to include an SMC population as representative as possible of the in vivo setting.3 During primary culture and after subsequent passages, SMCs exhibited the typical spindle-shaped morphology with a hills-and-valleys pattern at confluence. SMCs displayed typical stress fibers after staining with anti–α-SM actin antibody (Figure 2a). The SMMHC staining demonstrated the classical interrupted distribution at the cell periphery (Figure 2b). The anti-desmin antibody showed a pattern irradiating from the nucleus to the cell surface (Figure 2c). The distribution of smoothelin has remained controversial up to now because this protein was not expressed spontaneously in cultured cells from conventional sources.15,22 In our SMCs, a stress fiber–like organization of smoothelin was observed (Figure 2d) with a strong intensity in the center of the cell and decreasing gradually toward the cell periphery.

Figure 2. Morphological and cytoskeletal features of porcine coronary artery SMCs in vitro. Immunofluorescence staining of porcine SMCs at the 5th passage for α-SM actin (a), SMMHC with typical interrupted distribution of the protein (b), desmin (c), and smoothelin (d). Note the stress fiber pattern of smoothelin distribution. Bar=5 μm for panels a, c, and d; 5 μm for panel b.

CLSM analysis performed on cultured porcine SMCs showed that smoothelin was colocalized with α-SM actin (Figure 3a) and not with vimentin (Figure 3b). Colocalized pixels represented 85% of the total pixel number in the α-SM actin/smoothelin double staining and only 3% in the vimentin/smoothelin double staining.

Immunofluorescence staining performed on cytoscintification fuded SMCs showed that in accordance with in vivo observations, almost all freshly isolated SMCs (0 hours) were positive for α-SM actin and SMMHC (Figure 4). About 80% of the cells were positive for desmin and smoothelin. After 7 days of primary culture (P0), the proportion of positive cells decreased to ~70% for α-SM actin (P<0.01), to 40% for desmin (P<0.01), and to 35% for smoothelin (P<0.01). The proportion of SMMHC-expressing cells remained unchanged. The intensity of staining however clearly decreased in about half of SMMHC-positive SMCs; it remained at this level up to the 5th passage (P5). After one passage (P1), the percentage of α-SM actin– and smoothelin-positive SMCs remained at a level similar to that of primary culture whereas the percentage of desmin-positive SMCs decreased to ~30% (P1 versus P0, P<0.05). At the 5th passage, the percentage of
The number of \( \alpha \)-SM actin–positive cells represented only 15% of the value at 0 hours and was clearly detectable up to the 9th passage (data not shown). In passaged rat or rabbit arterial SMCs, no expression of SMMHC 2 protein has been reported.\(^{24}\text{--}^{27}\) The expression of nonmuscle MHC isoform B remained unchanged up to the 5th passage (data not shown). Confluence at the 5th passage did not affect the level of \( \alpha \)-SM actin expression (data not shown), again different from previously reported rat data.\(^{3,28}\) Thus, porcine coronary artery SMCs remain relatively well differentiated in culture.

The expression of \( \alpha \)-SM actin mRNA remained constant from freshly isolated cells up to the 5th passage (n = 4 experiments for each condition; data not shown). Moreover the probe gave a specific signal also with rat and human SMC mRNA (data not shown).

\( \alpha \)-SM actin–expressing cells increased to a level similar to that of 0 hours (P5 versus 0 and P1, \( P < 0.01 \)). The proportion of desmin-positive cells also increased to a level similar to that of primary culture (P5 versus P1, \( P < 0.05 \)). The percentage of smoothelin-positive cells further decreased to \( \approx 15\% \) (P5 versus P0 and P1, \( P < 0.01 \)). These results are different from those previously described for adult rat aortic SMCs in which percentages of \( \alpha \)-SM actin–, SMMHC–, and desmin–positive cells represent only \( \approx 80\%, \approx 45\%, \) and 30%, respectively, at P0 and 70%, 3%, and 0%, respectively, at P5.\(^3,6,23\) Thus, porcine coronary artery SMCs maintain a high degree of differentiation in vitro.

Immunoblotting results were in agreement with those of immunofluorescence studies (Figure 5). In primary culture, the content of \( \alpha \)-SM actin was decreased to \( \approx 80\% \) (\( P < 0.01 \)), that of SMMHC to 50% (\( P < 0.01 \)), that of desmin to 60% (\( P < 0.05 \)), and that of smoothelin to 40% (\( P < 0.01 \)) compared with 0 hours. Values further decreased at the 1st passage, to \( \approx 60\% \) for \( \alpha \)-SM actin and 30% for SMMHC (\( P < 0.05 \)) whereas desmin and smoothelin values did not change. At the 5th passage, \( \alpha \)-SM actin content increased and reached a value close to that of primary culture (P5 versus P1, \( P < 0.05 \)). The SMMHC and desmin contents were maintained at the levels observed at the 1st passage, ie, 40% and 60% of the value at 0 hours, respectively (P1 versus P5, not significant, P5 versus 0 hours, \( P < 0.05 \)). Smoothelin expression at the 5th passage represented 10% of the value at 0 hours and was significantly smaller than in primary culture (\( P < 0.05 \)). All studied proteins, including desmin and smoothelin, remained clearly detectable up to the 9th passage (data not shown). In rat aortic SMCs, the decrease of \( \alpha \)-SM actin, SMMHC, and desmin content during culture has been shown to be more pronounced than that observed in the pig model: at the 5th passage, \( \alpha \)-SM actin content is only \( \approx 15\% \) of the value at 0 hours whereas SMMHC and desmin proteins are not detectable.\(^3,6,23\)

**Figure 4.** Percentage of porcine coronary artery SMCs expressing differentiation markers in vitro. Histogram showing the percentage of \( \alpha \)-SM actin–, SMMHC–, desmin–, and smoothelin–positive cells just after enzymatic digestion (0 hr), in primary culture (P0), after 1 passage (P1), and after 5 passages (P5). The number of \( \alpha \)-SM actin–positive cells decreases initially in culture and then increases at P5 to the value observed at 0 hours. The proportion of SMMHC-expressing cells does not change during subculture, whereas the percentage of desmin-positive cells and of smoothelin-positive cells decreases progressively during subculture. n = 3 experiments for each condition. \( ^{*} P < 0.05, \quad {^{**}} P < 0.01 \) compared with 0 hours.

**Figure 5.** Expression of differentiation markers in porcine coronary artery SMCs in vitro. A, SDS-PAGE of total protein extracts (a through d) and immunoblots (e through h) showing \( \alpha \)-SM actin, SMMHC, desmin, and smoothelin expression in SMCs just after enzymatic digestion (a and e), in primary culture (b and f), after 1 passage (c and g), and after 5 passages (d and h). B, Densitometric scanning of immunoblots with values normalized to the pixel values measured in freshly isolated cells (0 hr). \( \alpha \)-SM actin, SMMHC, and desmin content decreases in primary culture (P0) to a level that is maintained at P1 and P5. Smoothelin content progressively decreases up to P5. n = 3 experiments for each condition. \( ^{*} P < 0.05, \quad {^{**}} P < 0.01 \) compared with 0 hours.
Several laboratories, including ours, have shown that heparin, TGF-β1 or TGF-β2, and tRA are able to modulate cultured rat arterial SMC phenotype. Heparin, as well as TGF-β1 or TGF-β2, inhibits proliferation and induces differentiation of rat arterial SMCs, albeit through different mechanisms. tRA also is capable of affecting differentiation of these cells. To test whether porcine SMC features can be modulated in culture by these agents, we exposed cells to heparin (200 μg/mL), TGF-β1 (10 ng/mL), TGF-β2 (10 ng/mL), or tRA (10⁻⁶ mol/L) for 6 days.

When compared with control cells, the proliferation of SMCs stimulated during 7 days with 10% FCS was inhibited by 50% (P<0.01) in heparin-treated cells and 30% (P<0.01) in cells growing in TGF-β1 or TGF-β2–supplemented medium (Figure 6A). tRA had no effect on cell proliferation. The [³H]-thymidine incorporation rate, measured in synchronized SMCs 24 hours after addition of the different agents, was decreased to 40% (P<0.01) for heparin-treated cells and to only 60%, 75%, and 80% (P<0.01) for cells treated with TGF-β1, TGF-β2, and tRA, respectively (Figure 6B) when compared with control cells.

Migratory activity was assessed by measuring the capability of SMCs to invade an in vitro wound when treated with FCS alone or supplemented with one of the different agents (Figure 7). After 24 hours of treatment, heparin decreased migration to ~40% and TGF-β1 to ~60% when compared with control; TGF-β2 and tRA had no significant effect under our conditions.

Heparin increased the protein expression of SMMHC 2, desmin, and smoothelin by 3.5-fold (P<0.05), 4-fold (P<0.01), and 3-fold (P<0.05), respectively, whereas levels of α-SM actin and SMMHC 1 were not significantly changed (Figure 8). TGF-β1 caused only a slight but significant decrease in SMMHC 2 (P<0.01) and a 2-fold increase in the protein expression of desmin (P<0.01). The expression of all other markers tested did not change after treatment with TGF-β1. The only effect of TGF-β2 was to cause a slight but significant decrease in SMMHC 2 (P<0.01). tRA decreased only the expression of desmin by 30% (P<0.01).

The effects of the different agents on SMCs were also tested in absence of serum. As expected, SMCs did not grow in Monomed.12 The migration of cells was inhibited by treatment with heparin and not with the other agents. Serum withdrawal itself had no effect on α-SM actin expression but increased the expression of SMMHC 2 and to a lesser extent of desmin and smoothelin when compared with the 10% FCS condition, as assessed by Western blot analysis (data not shown). The expression of all differentiation markers was not affected by any of the above-mentioned agents compared with the Monomed condition (data not shown).

**Discussion**

Our results show that SMCs cultured from porcine coronary artery retain a higher degree of differentiation than has been described in previous studies of SMCs cultured from animals.
Cytoskeletal proteins are well-accepted markers of the differentiation state of SMCs. Usually, a constellation of markers is necessary to assess SMC differentiation, and we have followed the same criterion for cultured porcine coronary artery SMCs. As in the rat model, almost all porcine SMCs express α-SM actin and SMMHC in vivo; but, differently from rat aortic SMCs where desmin is found only in half of the cells, most porcine coronary SMCs express this marker. Smoothelin, which cannot be studied in the rat with the presently available antibody, is present in practically all porcine SMCs, as previously described in human arteries. The expression of α-SM actin, desmin, and SMMHC is maintained at much higher levels in porcine SMCs compared with rat and rabbit SMCs when using such criteria as number of positive cells and protein or mRNA contents. In newborn rat arterial SMCs, the only situation in which SMMHC is expressed at a relatively high level, only 43% of the cells are positive at the 5th passage. In the present study, we additionally have been able to discriminate between SMMHC 1 and 2 protein expression. Both isoforms are markers of an advanced state of SMC differentiation. SMMHC 2 appears only at birth. At the 5th passage, >90% of the cells were positive for SMMHC 1 and 2 whereas only a very small percentage has been shown to be positive in adult rat arterial SMCs. Moreover, SMMHC 2 protein was expressed in passaged porcine SMCs, although it has been shown that its expression at the protein level is strictly limited to primary culture of adult rat arterial SMCs. It is noteworthy that ≈50% of porcine coronary SMCs expressed desmin up to the 9th passage, whereas in rat aortic SMCs, desmin is not detectable in similar conditions. In general, the relationships between α-SM actin mRNA and protein expression correspond more closely in porcine coronary artery SMCs than they do for rat and rabbit. This suggests a tighter coupling between transcription and translation than is found in other species.

Smoothelin is a recently described cytoskeletal protein specific for SMCs; during development, it appears later than α-SM actin, desmin, and SMMHC. Smoothelin expression disappears during culture in all primary and passaged SMCs tested to date. Porcine coronary SMCs, however, maintain expression of smoothelin protein up to the 9th passage. Two smoothelin isoforms have been described: a small isoform of 59 kDa and a large isoform specific to vascular SMCs, to which the molecular mass of 94 kDa had been assigned previously. We have systematically tested cultured SMCs and arterial tissues, including those samples used for previous studies, and conclude that the molecular mass of the large isoform is 115 kDa, as assessed by electrophoretic migration.

The expression of smoothelin in cultured porcine SMCs allowed us to establish by CLSM that at least in cultured SMCs, smoothelin is colocalized with α-SM actin and not with intermediate filaments. In transfection experiments, smoothelin displayed a stress fiber–like pattern, an intermediate filament–like pattern, or both, depending on the cell type used. It is noteworthy in those regards that a putative actin-binding site has been suggested in the sequence of the smoothelin gene. Additional work using CLSM in tissues

such as the rat and rabbit, and that they maintain these properties even after several passages in culture. Thus, porcine cells represent a new model for examining the properties of well-differentiated SMCs, as well as regulation of the expression of differentiation markers such as SMMHC and smoothelin and their isoforms.

Porcine coronary arteries are very similar to human vessels in size and histology. Of all species examined to date, the pig is most similar to the human in its cardiovascular morphology and physiology, as well as its susceptibility to atherosclerosis. Human arterial SMCs also have been reported to replicate relatively more slowly and to maintain a high degree of differentiation in terms of markers such as SMMHC compared with rodent SMCs. The fact that cultured porcine coronary artery SMCs also remain highly differentiated further suggests that the porcine culture model will be of importance in terms of improving our understanding of human vascular disease. It should be stressed that no well-established data on differentiation features of human vascular SMCs are present in the literature. The scarcity of data may be due to difficulties in obtaining standardized SMC populations from human donors.
and assessing the binding capacity of smoothelin to α-SM actin will furnish more information on the possible interaction of the two proteins.

We have tested the effects of heparin, TGF-β1 or TGF-β2, and tRA that are known to modulate SMC differentiation, proliferation, and migration. Heparin, used at a concentration that has shown maximal effects on rat SMC proliferation, inhibited proliferation and increased differentiation marker expression only in the presence of serum whereas migration was inhibited by heparin also in the absence of serum. Similar findings have been described for rat and rabbit SMCs. Heparin did not affect the expression of α-SM actin and SMMHC 1 but increased the expression of SMMHC 2, desmin, and smoothelin, suggesting that a low level of protein expression is a prerequisite for heparin activity.

Both TGF-β isoforms reduced proliferation in porcine coronary SMCs, but migration was inhibited by only TGF-β1. It is noteworthy that proliferation of rat SMCs can be inhibited or stimulated by TGF-β according to the concentration per cell used. Both TGF-β isoforms had no inducing effect on α-SM actin or SMMHC and in fact decreased expression of SMMHC 2. This finding is different from what Hautmann et al have shown in rat arterial SMCs, for both α-SM actin and SMMHC. However, these investigators used a lower dose of TGF-β and different growth conditions than we used in the present study. Interestingly, TGF-β increased desmin expression in porcine SMCs, a marker that is rarely modulated in other models.

tRA is considered to be a potential inducer of SMC differentiation. However, proliferation, migration, and differentiation marker expression were not changed in porcine SMCs when treated with tRA. Nevertheless tRA, similarly to heparin and TGF-β, reduced serum-stimulated [3H]-thymidine incorporation. tRA inhibits rat arterial SMC proliferation, and [3H]-thymidine incorporation is decreased in only SMC whole populations and clones with an epithelioid phenotype. Moreover, IT development after endothelial injury is reduced in rats fed with a tRA-supplemented diet. On the contrary, tRA stimulates migration of cultured rat SMCs as well as the expression of tissue-type plasminogen activator, the main enzyme responsible for the degradation of extracellular matrix in this cell type. Interestingly, in cultured adult rat aortic SMCs and in the present model, α-SM actin expression was not affected by tRA. Taken together, these findings suggest that tRA acts mainly on poorly differentiated SMCs.

In conclusion, we have defined several biological and differentiation features of cultured porcine coronary artery SMCs. We have shown that they differ importantly from those established previously for rat and rabbit SMCs in terms of maintaining a more advanced degree of differentiation; in this respect, they appear to exhibit features reminiscent of those observed in human arterial SMCs. Our results indicate the potential value of these cells in the analysis of differentiation marker expression in SMCs. Porcine cells derived from the normal media or from the intimal thickening induced by angioplasty and/or stenting may provide further insight into the mechanisms involved in those processes as well as on the action of substances capable of influencing the biological behavior of SMCs in vitro and possibly in vivo.

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References


22. Christen et al Porcine Coronary Smooth Muscle Cells


Cultured Porcine Coronary Artery Smooth Muscle Cells: A New Model With Advanced Differentiation
Thomas Christen, Marie-Luce Bochaton-Piallat, Pascal Neuville, Sander Rensen, Mireille Redard, Guillaume van Eys and Giulio Gabbiani

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