Rat Aortic Smooth Muscle Cells Isolated From Different Layers and at Different Times After Endothelial Denudation Show Distinct Biological Features In Vitro

Augusto Orlandi, H. Paul Ehrlich, Patricia Ropraz, Luigi G. Spagnoli, Giulio Gabbiani

Abstract Endothelial denudation by balloon injury of the rat aorta induces the development of a neointima as a consequence of the migration and proliferation of smooth muscle cells (SMCs). Initially, intimal SMCs show a dedifferentiated phenotype, which reverts to a normal differentiated phenotype after endothelial cells have resurfaced the vessel lumen. We investigated in vitro the proliferative and phenotypic features of SMCs from different layers of rat aorta isolated 15 and 60 days after endothelial denudation. Freshly isolated intimal cells 15 days after balloon injury (IT-15) appeared rounded and showed a decreased content of α-smooth muscle actin, smooth muscle myosin, and desmin compared with intimal cells isolated 60 days after balloon injury (IT-60). No morphological and cytoskeletal differences were observed among freshly isolated IT-60 cells and other medial populations, which included medial SMCs that underlie the intimal thickening. In culture, IT-15 cells showed increased proliferative activity both in monolayers and in free-floating collagen lattices. Decreased expression of α-smooth muscle actin and smooth muscle myosin was documented in IT-15 cells compared with IT-60 cells and other medial SMC populations in monolayer. Moreover, IT-15 cells suspended in collagen lattices were poor at contracting these collagen lattices compared with IT-60 and control SMCs. IT-60 cells were equivalent to control SMCs at lattice contraction except for a temporary delay at day 1. Cells from the media underlying the intimal thickening isolated 15 and 60 days after balloon injury proliferated less, had an increased content of α-smooth muscle actin, and had a greater percentage of α-smooth muscle actin mRNA per total actin mRNA compared with IT-60 and control SMCs. Our model appears suitable to investigate the adaptation of differently derived SMC populations to various stimuli and factors involved in SMC phenotypic modulation.

Key Words • atheromatosis • smooth muscle proliferation • actin isoforms • myosin • desmin • collagen lattice contraction

Intimal thickening after endothelial denudation has been widely used as a model for the development of the atheromatous plaque and has furnished useful information on smooth muscle cell (SMC) susceptibility to microenvironmental stimuli. Only a small proportion of medial SMCs are activated to migrate and replicate after endothelial denudation; this may reflect a heterogeneity of SMC phenotypic features that has been described by means of several other criteria. It has been shown that cultured SMCs from the normal media and from the experimental intimal thickening show different growth patterns. SMCs from the intimal thickening, collected 15 days after endothelial lesion, proliferate more actively than do medial cells in the presence of the same amounts of serum; moreover, contrary to medial cells, SMCs are capable of growing in the absence of serum factors. This behavior is also seen in normal medial SMCs derived from 18-month-old rats but not in fibroblasts cultured from the same animals. Thus, the growth pattern of SMCs in vitro may be different if they are collected under different conditions.

In this work we compare the behavior of rat cultured SMCs from experimental intimal thickenings 15 days after deendothelialization, when SMCs are actively replicating, and 60 days after deendothelialization, when endothelial continuity has been restored and SMCs are arrested in their replicative activity. We also studied the behavior of cultured SMCs from the media underlying the intimal thickening at the same time intervals. Our findings indicate that there are differences in proliferating activity, differentiation features, and capacity to contract a collagen gel among these SMCs and, in particular, between SMCs from intimal thickening 15 and 60 days after deendothelialization. These findings support the possibility that SMCs display a heterogeneity of phenotypic and functional features in vitro, which apparently depend on the particular in vivo situation of the cells at the time of isolation.

Methods

Animals

One hundred male Wistar rats, weighing 270 to 290 g, were used for the experiments. Rats were preanesthetized with enflurane (Ethrane, Abbott Laboratories) for 60 seconds and anesthetized with pentobarbital sodium (Nembutal sodium,
Abbott, 35 mg/kg body wt IP), and the endothelium of the thoracic aorta was removed by the method of Baumgartner and Studer, 14 with minor modifications. To verify the degree of aortic denudation, six rats randomly selected received 1 mL IV of Evans blue (Merck; 1% solution in 0.9% NaCl) immediately after the catheter-induced lesion and were killed 30 minutes later by cervical dislocation. The surfaces of balloon-looned thoracic aortas appeared completely blue. Fifteen and 60 days later, rats were killed as described above, and their aortic tunicae mediae were dissected under sterile conditions, immersed in a saline solution, and then opened longitudinally. An intimal thickening (IT) was generally present in a longitudinal ventral band, as previously reported. 2 Those aortas exhibiting only focal or slight IT were discarded.

Cell Isolation and Culture

The aortas were placed in culture dishes, and the ITs (15 and 60 days after balloon-induced lesions) were isolated from the underlying media with fine forceps under a dissecting microscope; the tunica media underlying the intimal thickening (UM) and the tunica media of the remaining thoracic aortic wall (RM) were carefully isolated from the adventitia with fine forceps and a scalpel. When necessary, the endothelial layer was gently scraped with a scalpel blade. As a control, the aortic tunicae mediae of untreated rats were isolated and digested enzymatically as previously reported. 15 To control the quality of our procedure, aortic samples of IT, UM, and RM were randomly selected, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined by light microscopy. The isolation always appeared complete. For each enzymatic digestion, IT and UM samples of four rats with endothelial lesions and the tunicae mediae of three control rats were finely minced and digested at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing collagenase (Clostridium histolyticum, type I, 405 U/mg, Sigma), 400 U/mL elastase (hog pancreas, Fluka), 10.6 U/mL trypsin (Fluka), 10 mmol/L ethylene glycol-bis(β-aminoethyl) ether-N,N,N',N'-tetraacetic acid (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF, Merck), and 7.8% benzamidine (Sigma) and sonicated. The results were expressed as the ratio between counts per minute and the number of cells. Small aliquots of cell suspensions were employed to evaluate the cell size by flow cytometric analysis using a FACScan (Beckton Dickinson Immunocytometry System). All procedures were repeated in two independent experiments.

Immunofluorescence Staining

Cells were washed in DMEM, counted with a hemocytometer, and diluted to a final concentration of 10^6 cells/mL. Samples (100 μL) were cytocentrifuged with a Shandon cytocentrifuge (Shandon Scientific Co) for 5 minutes at 125 g. Cells were fixed in methanol for 5 minutes at 20°C and stained with a monoclonal IgG2a antibody specific for α-smooth muscle actin (α-SM actin) 20 combined with a mouse monoclonal IgG1 antibody specific for human desmin (M760, Dakopatts) or with one of two rabbit polyclonal affinity-purified IgG: anti-smooth muscle myosin heavy chain (SM myosin) or anti-human von Willebrand factor (Sigma). As control, diluted rat (1:10) and rabbit (1:20) immunoglobulins were used. Tetramethyl rhodamine-labeled goat anti-mouse IgG (Nordic Immunology Laboratory) and anti-mouse IgG2a (Nordic), fluorescein isothiocyanate-labeled anti-mouse IgG1 (Nordic), and anti-rabbit IgG (Nordic) were used as second antibodies. Counting of approximately 1000 cells for each population was performed in triplicate with a Zeiss Vidas (Carl Zeiss) interactive image analysis system connected to a high-sensitivity AVT-Horn FMC-4005-type camera (AVT-Horn) with an Axiphot (Zeiss) photomicroscope.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), cell samples remaining after DNA measurements were mixed with 5× concentrated buffer containing 5% SDS (Bio-Rad Laboratories AG), 5% dithiothreitol (Fluka), 5 mmol/L PMSF, and 5 mmol/L sodium-p-tosyl-L-arginine methyl ester (Sigma) in 0.1 mol/L Tris HCl, pH 6.8, 21 boiled for 3 minutes, and centrifuged at 5000 rpm for 5 minutes. Protein content was determined according to the method of Bradford. 22 All procedures were repeated three times. Proteins (40 μg) were electrophoresed on a 5% to 20% gradient gel and stained with Coomassie brilliant blue (R 250, Fluka) in methanol, acetic acid, and water (45:10:45, vol/vol/vol). For quantification of total actin and myosin, gels were scanned with a computerized laser beam densitometer (Genopti SA) as previously described. 23

For Western blotting, 2 to 15 μg of proteins was electrophoresed on a 5% to 20% gradient gel. Separated proteins were transferred to nitrocellulose filters (0.45 μm, Schleicher & Schuell), which were incubated with anti-α-SM actin (1:500) followed by a goat anti-mouse IgG (1:100) or with anti-SM myosin (1:200) followed by a goat anti-rabbit IgG (1:100) as previously described. 23 Enhanced chemiluminescence was used for detection (Amersham). Quantification of films was performed by scanning as described above. Density values were normalized to the α-SM actin content of control SMCs.

RNA Extraction and Northern Blot Hybridization

To evaluate the mRNA expression in SMC cultured populations, cells were scraped from Petri dishes with a rubber policeman in a sterile solution of 4.5 mol/L guanidine isothiocyanate, 50 mmol/L EDTA (Fluka), 25 mmol/L sodium citrate (Fluka), 0.1 mol/L β-mercaptoethanol (Fluka), and 2% N-lauroylsarcosine (Sigma). Cells were homogenized by use of a
syringe with a 23-gauge needle, and RNA was purified by ultracentrifugation through a cushion of 5.7 mol/L CsCl according to the method of Chirgwin et al.25 The RNA pellets were resuspended in 10 mmol/L Tris-HCl (pH 7.4)/0.5% SDS and 1 mmol/L EDTA and extracted two times with saturated phenol/chloroform and once with chloroform/isomyl alcohol (24:1, vol/vol). The RNA was precipitated with ethanol, resuspended in sterile water, and stored at −70°C. Five to 15 µg of total RNA was denatured with glyoxal, electrophoresed in 1% agarose gel, and transferred overnight to a nitrocellulose membrane (Hybond-N, Amersham). The next day, the membrane was baked for 2 hours at 80°C under vacuum and stained with 0.04% methylene blue in 0.5 mol/L sodium acetate to verify correct loading and transfer. Hybridization was performed with a riboprobe26 derived from the coding region of the rat α-SM actin mRNA (total actin probe, pRNAαA-C) or with a synthetic oligonucleotide derived from the 3′ untranslated region of rat α-SM actin mRNA, with [γ-32P]ATP as previously reported.27 Hybridizations were performed according to Maniatis et al.29 Films were exposed to X-Omat AR films (Kodak) at −70°C between intensifying screens, developed, and analyzed by means of computerized densitometric scanning as described above.

Collagen Lattice Contraction

Control SMCs, IT-15 cells, and IT-60 cells were grown in monolayer culture and used at the fifth passage. The collagen for these experiments was purified from bovine tendon by limited pepsin digestion. Briefly, tendons were dissected from bovine hooves and cut into small pieces. The tissue (1 g/100 mL) was swollen overnight in 0.5 mol/L acetic acid with stirring. All procedures were performed at 4°C. The swollen tissue was homogenized, pepsin (Sigma) was added at 10 mg/100 mL, and the mixture was stirred for 2 days. The digest was centrifuged at 10 000g for 30 minutes, and the insoluble pellet was discarded. Sodium chloride was added 10% wt/vol to the supernatant, and the mixture was stirred overnight. The precipitated collagen was collected by centrifugation and dissolved in 1.0 mol/L NaCl/50 mmol/L Tris HCl, pH 7.5. The collagen solution was cleared of particulate matter by centrifugation and exhaustively dialyzed against 1.0 mmol/L HCl. The clear viscous solution was frozen, lyophilized, weighed, dissolved in 1.0 mL of 15.0 mg/mL HCl and stored at 4°C. The SMCs were freed from monolayer culture by trypsinization and counted. Aliquots of 200 000 cells in 3.0 mL of DMEM with 10% FCS were rapidly mixed with 1.0 mL of collagen solution, and the mixture was poured into a 60-mm Petri dish. The dish was placed in a 37°C incubator, in which the collagen polymerized in less than 90 seconds, trapping cells within the newly formed matrix. The cell-populated collagen lattices (PCLs) were detached from the dish 1 hour after their manufacture. Their diameter was measured on a regular basis for 14 days with a ruler to the nearest 0.5 mm.

Statistical Analysis

Results were expressed as arithmetical means of single experiments ±SEM. For statistical evaluation, the results were analyzed with Mann-Whitney, Student’s t, and nonparametric χ2 tests. The differences were considered statistically significant at values of P<.05.

Results

Morphological Findings

IT cells freshly isolated 15 days after endothelial injury (IT-15) appeared as individual cells, in contrast to cells recovered 60 days after endothelial denudation (IT-60, see below), RM cells both at 15 and 60 days, and control SMCs. IT-15 cells rapidly adhered and proliferated, reaching confluence in 4 to 5 days. During this proliferative phase, IT-15 cells appeared characteristically epithelioid, with a tendency to grow in small groups. When confluent, they conserved their epithelioid appearance and grew in a single layer (Fig 1a), as previously reported.8 These morphological characteristics persisted through the fifth passage.

Immediately after enzymatic digestion and plating, IT-60, RM, and control SMCs plated as clusters of 5 to 20 cells. They remained quiescent for 24 to 48 hours and then started to proliferate, becoming confluent in 7 to 8 days. At confluence, they were spindle-shaped and exhibited a characteristic “hill-and-valley” appearance30 (Fig 1b and 1d). UM-15 and UM-60 cells when seeded were also prevalently organized in small clusters; they started to proliferate later than RM and control SMCs and reached confluence only after 10 to 12 days. After two or three passages they remained subconfluent (particularly UM-15, Fig 1c) and showed an accumulation of extracellular material in the space between cells.

When subconfluent, IT-15 cells appeared smaller in situ compared with RM and the other medial cell populations. Analysis of cell size by flow cytometry showed that the volume of IT-15 cells was 81.2±5.0% compared with that of IT-60 cells (P<.05). IT-15 cells were also smaller than all medial populations (data not shown).

Cell Proliferation

To examine proliferative activity, passaged cells (passage 5) were cultured for 4 days in the absence or in the presence of serum. Seeding efficiency (percentage of number of cells attached per number of cells plated 15 hours after plating) was similar in all groups (mean, 88.7±4.3%). In the absence of serum, only IT-15 cells showed an increase of cell number (1.89±0.12).

As indicated in Fig 2, in the presence of serum, IT-15 cells proliferated more than all other groups (P<.01). IT-60 cell proliferation rate did not differ from those of RM and control SMCs. UM-15 cells proliferated less compared with RM-15 (P<.03) and control SMCs (P<.01); the same was true for UM-60 compared with IT-60, RM, and control SMCs (P<.03, P<.01, and P<.001, respectively). No differences were demonstrated between UM-15 and UM-60 cells.

[3H]thymidine incorporation experiments correlated with the growth curves of SMC populations (Fig 2). The IT-15 incorporation rate was at least double that of other SMC populations, including control SMCs (P<.01). No differences were present among IT-60, RM, and control SMCs. UM-15 and UM-60 incorporation levels were lower than corresponding RM and control SMCs (P<.01). In the absence of serum (ie, medium containing selenium and transferrin), the [3H]thymidine incorporation was low and similar in all SMC populations with the exception of IT-15 cells, in which the incorporation was about 25% higher than in the other populations.

Cytoskeletal Features

Table 1 summarizes the number of α-SM actin–, SM myosin–, and desmin–positive centrifuged cells in all situations studied. Freshly isolated IT-15 cells showed a markedly reduced percentage of α-SM actin–, SM my-
FIG 1. Photomicrographs showing that after 7 days of subculture, passage 5 intimal thickening cells at day 15 (a) appear epithelioid and grow in a monolayer. Intimal thickening at day 60 (b) and control medial smooth muscle cells (d) appear spindle-shaped and form "hills and valleys." Cultured underlying medial cells at day 15 (c) remain subconfluent. Original magnification ×200.

osin–, and desmin-positive cells compared with UM, RM, and control SMCs (P<.01), confirming the biochemical data previously reported.3,6 Freshly isolated IT-60 cells presented features similar to those of UM, RM, and control SMCs.

Table 2 summarizes the percentage of SMCs positive for α-SM actin and SM myosin in the different cultured populations at the fifth passage. IT-15 cells showed a decrease of α-SM actin–positive cells and an almost total disappearance of SM myosin–positive cells compared with the corresponding freshly isolated SMCs. IT-60 cells showed a percentage of α-SM actin– and SM myosin–positive cells similar to that of RM and control SMCs. Interestingly, UM-15 and UM-60 cultured cells showed a percentage of α-SM actin–positive cells similar to that of RM and control SMCs and a slightly but significantly increased SM myosin–positive cell percentage compared with the same cells (P<.01 in both cases). Finally, no desmin-positive cells were detected in all cultured aortic SMC populations, as previously reported for passage 5 cultured medial SMCs.27

Actin and Myosin Content in the Different SMC Cultured Populations

The densitometric analysis of SDS-PAGE (Fig 3) revealed that the percentage of total actin per total protein in IT-15 cells (8.4±0.3%) was reduced compared with other populations (P<.01). The percentage of total actin per total protein was similar in IT-60, RM, UM, and control SMCs. The percentage of myosin per total protein in IT-15 (1.6±0.5%) was not significantly different from IT-60, RM, and control SMCs. Both IT-15 and IT-60 myosin contents per total protein were slightly but significantly reduced compared with corresponding UM populations (P<.05 and P<.03, respectively). No significant differences were present between myosin percentages per total protein of UM, RM, and control SMCs.

The protein content per cell of cultured IT-15 cells was less than that of IT-60 (68.2±3.0%, P<.02) and of other populations (P<.01). No significant differences were present between the protein contents of IT-60 and medial populations. Cultured IT-15 cells showed a decrease of both actin and myosin content per cell compared with IT-60 and other SMC populations. This marked decrease of actin per IT-15 cell (around 40% compared with IT-60 cells) is explained in part by the reduction of protein per cell (around 25% of IT-60 value) and in part by the reduction in the percentage of total actin per total protein (around 10% compared with IT-60 cells). The decrease of myosin per IT-15 cell (around 40% of IT-60 value) reflects in part the reduction of protein content per cell (around 25% of IT-60 value). Finally, UM-15 and UM-60 cells showed an increased myosin content per cell compared with other
Actin Isoform mRNA Expression

RNA from the different postconfluent cultured populations was hybridized with the total actin probe pRAoaA-C. As previously described, two bands were recognized: a 2.1-kb band corresponding to cytoplasmic actin mRNA and a 1.7-kb band corresponding to α-SM actin mRNA. In our experiments (Fig 4), the band corresponding to α-SM actin mRNA was always larger than that corresponding to cytoplasmic actin mRNAs because SMCs were collected after confluence.\textsuperscript{28} Densitometric analysis of Northern blots (Fig 4) demonstrated that the expression of α-SM actin mRNA of IT-15 cultured cells (52.4±0.9% of total actin mRNA) did not differ significantly from that of control SMCs (50.8±3.1% of total actin mRNA) or from IT-60 and RM cells; it was slightly but significantly lower compared with the percentage of α-SM actin mRNA of UM-15 and UM-60 populations (82.4±1.5% and 75.8±3.6%, respectively; \textit{P}<.01). However, the analysis of integrated densities after hybridization with the total actin probe indicated that IT-15 cells expressed total and α-SM actin mRNA levels similar to those of IT-60 cells and control SMCs. The total actin mRNA of UM-15 and UM-60 was not significantly different compared with other populations. The hybridization with the specific oligonucleotide for α-SM actin confirmed the results obtained with the total actin probe (data not shown). Since the expression of α-SM actin protein was always lower in IT-15 than in IT-60 (see above), we assume that this decrease in α-SM actin expression takes place at the level of mRNA translation rather than at the level of transcription as previously observed.\textsuperscript{29,31}

Cell-Populated Collagen Lattice Contraction

SMCs were able to contract collagen lattices. There were differences between the three SMC populations tested in the model (Fig 5). At day 1, collagen lattices containing control SMCs (C PCLs) had contracted to 363±97 mm$^2$, which was a fivefold decrease in lattice size. Collagen lattices containing IT-60 and IT-15 cells (IT-15 PCL and IT-60 PCL) decreased in size to only 1206±205 mm$^2$ and 1276±156 mm$^2$, respectively. The IT-60 and IT-15 PCLs were identical in size at day 1 and had contracted significantly less than the C PCL (\textit{P}<.001). At day 2, C PCLs were reduced to 262±38 mm$^2$. This represents a sevenfold decrease in lattice size. IT-15 PCL had decreased to only 1061±186 mm$^2$ during the same period. This small change represents a less than onefold reduction in lattice size. With IT-60 PCL at day 2, lattice size was 722±145 mm$^2$, which was a 2.5-fold decrease in lattice size. Between day 1 and day 2, a significant difference had developed between IT-60 and IT-15 PCL (\textit{P}<.001). The IT-60 contracted

### Table 1. Percentage of α-SM Actin-, SM Myosin-, and Desmin-Positive SMCs Freshly Isolated From Different Layers of the Aorta and at Different Times After Endothelial Denudation

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<tr>
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<th>Percentage of SMCs Positive to</th>
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<tbody>
<tr>
<td></td>
<td>α-SM Actin</td>
</tr>
<tr>
<td>Control media</td>
<td>88.2±3.9</td>
</tr>
<tr>
<td>Intimal thickening at 15 days</td>
<td>37.6±1.6</td>
</tr>
<tr>
<td>Media under intimal thickening at 15 days</td>
<td>85.2±1.0</td>
</tr>
<tr>
<td>Remaining media at 15 days</td>
<td>87.9±4.1</td>
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<tr>
<td>Intimal thickening at 60 days</td>
<td>86.2±4.7</td>
</tr>
<tr>
<td>Media under intimal thickening at 60 days</td>
<td>87.9±1.9</td>
</tr>
<tr>
<td>Remaining media at 60 days</td>
<td>86.0±5.0</td>
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SM indicates smooth muscle; SMCs, smooth muscle cells. Values are mean±SEM.
TABLE 2. Percentage of α-SM Actin- and SM Myosin-Positive SMCs Cultured up to the Fifth Passage From Different Layers of the Aorta and at Different Times After Endothelial Denudation

<table>
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<tr>
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<th>Percentage of SMCs Positive to</th>
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<tbody>
<tr>
<td></td>
<td>α-SM Actin</td>
</tr>
<tr>
<td>Control media</td>
<td>74.1±3.4</td>
</tr>
<tr>
<td>Intimal thickening at 15 days</td>
<td>19.7±3.0</td>
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<tr>
<td>Media under intimal thickening at 15 days</td>
<td>75.6±4.6</td>
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<tr>
<td>Remaining media at 15 days</td>
<td>74.7±3.7</td>
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<tr>
<td>Intimal thickening at 60 days</td>
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<tr>
<td>Media under intimal thickening at 60 days</td>
<td>77.9±2.8</td>
</tr>
<tr>
<td>Remaining media at 60 days</td>
<td>74.9±3.5</td>
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SM indicates smooth muscle; SMCs, smooth muscle cells. Values are mean±SEM.

faster than the IT-15 PCL. At day 2, significant differences developed between all three groups (P<.001).

By day 5, IT-15 PCL showed significantly less lattice contraction than IT-60 PCL and C PCL (P<.001). By day 7, the areas of IT-60 PCL and C PCL were identical. The IT-15 PCL showed significantly less contraction than the other groups. At day 14, C PCL and IT-60 PCL were still identical. IT-15 PCL were significantly larger than the other groups (P<.001). Minimal lattice contraction had occurred between days 7 and 14.

Discussion

The mechanisms regulating SMC motility and replication are considered essential for the understanding of atheromatous plaque development (for review see References 1 and 32 through 36). They have been extensively studied in culture and in vivo, mainly via the model of arterial deendothelialization. SMC activation may depend not only on several local factors but also on intrinsic properties of these cells, which apparently vary with different situations. Previous work from our and other laboratories has shown that SMCs from normal and injured arteries may differ in their growth properties and phenotypic expressions.

Fig 4. Autoradiogram of Northern blots of total mRNA from different confluent populations cultured at the fifth passage hybridized with the total actin probe. The 1.7-kb band corresponds to α-smooth muscle (SM) actin mRNA, and the 2.1-kb band corresponds to cytoplasmic actins. Densitometric scanning of autoradiograms reveals that intimal thickening cells at day 15 (a) express cytoplasmic actin and α-SM actin mRNA levels similar to intimal thickening cells at day 60 (d), remaining aorta medial cells at day 15 (c), remaining aorta medial cells at day 60 (f), and control smooth muscle cells (SMCs) (g). The percentage of α-SM actin mRNA of underlying medial cells at day 15 (b) and underlying medial cells at day 60 (e) is increased compared with other SMC populations (P<.005).

Fig 5. Graph showing contraction of cell-populated collagen lattices (PCLs) over a 14-day period. Cell-populated polymerized collagen gels were cast with 200 000 cells from control smooth muscle cells (SMC), intimal thickening cells at day 15 (IT-15), or intimal thickening cells at day 60 (IT-60) populations, 5 mg of collagen, Dulbecco’s modified Eagle’s medium plus final concentration 10% fetal calf serum in a total volume of 4.0 mL in a 60-mm Petri dish. The measured internal area of the dish was 1693 mm², which was the initial area of each lattice. The measured diameter of each lattice was recorded on the days indicated and its area calculated. For each point on the graph: days 1 and 2, n=16; day 5, n=12; day 7, n=8; and days 9 and 14, n=4. IT-15 PCLs were significantly different from control PCLs at all times (P<.01). IT-60 PCLs were significantly different from C PCLs at days 1 and 2 only (P<.001). IT-15 PCLs were significantly different from IT-60 PCLs at all times but day 1 (P<.001).
features in vitro. Thus, SMC populations cultured from newborn, young adult, and old rats have different proliferation rates and different cytoskeletal composition.13 Surprisingly, SMCs cultured from old rats replicate more actively and are more dedifferentiated than those from newborn or young adult animals. Moreover, SMCs cultured from old rats show serum-independent growth, probably mediated through autocrine platelet-derived growth factor–like activity.12 A similar phenomenon has been reported for a population of SMCs derived from 13- to 18-day-old rats46 and for SMCs isolated from the IT that developed 15 days after carotid denudation.8 Thus, evidence exists supporting the possibility that SMCs have heterogeneous features in different situations in vivo and that these features can be maintained, at least in part, in culture. Whether this behavior corresponds to an in vivo modulation of SMC features or to a selection of certain cells after endothelial denudation is not clear at present. It is noteworthy, however, that SMC clones with different properties can be isolated from the rat aorta of normal animals at different ages.13

Our experiments were performed to verify whether, after endothelial denudation, SMC activation (1) affects, in addition to intimal thickening, cells of the underlying media and (2) persists in intimal cells 60 days after injury, when endothelial continuity has been reestablished. Our results indicate that the replicative and phenotypic properties of cultured IT-15 cells clearly differ from those of the UM (and of course from those of the control media). It is noteworthy that UM-15 and UM-60 cells show in vitro a low replicative activity compared with both intimal and control SMCs and do not easily reach confluence, similar to classic old cells,46 as if SMCs with high replicative features had migrated into the IT. Moreover, IT-15 cells do not contract a collagen gel as well as control SMCs do. The features of IT-15 cells also differ from those of IT-60 cells, the properties of the latter being essentially similar to those of control SMCs with the exception of a slight but significant decrease in their capacity to initially contract a collagen gel compared with control SMCs, suggesting that intimal SMCs at 60 days have not fully reverted to normal.” The switch from an activated to a “normal” phenotype in vivo observed in IT-60 cells compared with that of IT-15 may be due to a cell modulation during this period or to a loss (eg, by apoptosis) of a particular SMC subpopulation. Further studies are needed to clarify this point, but it is well established that IT cell density greatly decreases between 15 and 60 days after deendothelialization.6,40

Our experiments with PCL are in agreement with the results concerning cell proliferation and cytoskeletal features of the different SMC populations. All SMC populations contracted collagen lattices as expected.47,48 IT-15 cells, however, showed a significantly lower contractile activity than IT-60 and control SMCs.

It has recently been shown that a proportion of clones deriving from medial SMCs from old rats show dedifferentiated features, contrary to clones derived from young adult or newborn animals13; this correlates with dedifferentiated features of cultured populations of SMCs from old rats.13 Further experiments that involve cloning SMCs from intimal thickening or the underlying media at different intervals after endothelial denudation could answer the question as to whether these clones behave similarly to those produced from medial cells of young and old animals.

The switching of the in vitro behavior of SMCs isolated 60 days after deendothelialization compared with those isolated 15 days after deendothelialization and the fact that medial UM cells at 15 and 60 days show a slow replicative phenotype and serum dependence speak against the possibility that an exogenous oncogenic agent (eg, a virus) is responsible for the activation of SMCs recovered from intimal thickening 15 days after injury. However, this possibility should be taken into account and has been suggested for rapidly growing cultured rodent cells.49 Transformation of cells by many agents is capable of activating a gene producing a platelet-derived growth factor–like molecule.50-52 In any event, it is noteworthy that accelerated growth, serum independence, changes in cytoskeletal composition, and the collagen-contracting activity of cells isolated 15 days after endothelial denudation do not revert in vitro, whereas intimal thickening SMCs revert in vivo to a quiescent and differentiated state at 60 days when reendothelialization has been completed. When placed in culture at this time, they behave almost like control SMCs. Further studies on the mechanisms of this in vivo modulation may be important for understanding the factors that play a role in the control of SMC mitotic and differentiating activities.

In conclusion, it appears that irrespective of the mechanism, a stimulus such as endothelial denudation exerted in vivo alters the capacity of SMCs that have migrated to the intima to react, after having been placed in culture, to standard amounts of growth factors present in serum compared with quiescent cells of the normal media. This modification of reactivity is not only limited to SMCs that proliferate and migrate into the intima but is also present during a limited period of time, after which they reacquire the physiological capacities of control medial SMCs, suggesting that the expression of different phenotypic features by cultured SMCs under the influence of standard amounts of serum factors depends on their situation at the time of isolation.

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