Cultured Arterial Smooth Muscle Cells Maintain Distinct Phenotypes When Implanted Into Carotid Artery

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Abstract—Cultured arterial smooth muscle cells (SMCs) with distinct phenotypic features have been described by several laboratories; however, it is not presently known whether this phenotypic heterogeneity can be maintained within an in vivo environment. To answer this question, we have seeded into the intima of denuded rat carotid artery 2 SMC populations with well-established distinct biological features, ie, spindle-shaped, not growing in the absence of serum, and well differentiated versus epithelioid, growing in the absence of serum, and relatively undifferentiated, derived from the aortic media of newborn rats (aged 4 days) and old rats (aged >18 months), respectively. We show that these 2 populations maintain their distinct biochemical features (ie, expression of α-smooth muscle actin, smooth muscle myosin heavy chains, and cellular retinol binding protein-1) in the in vivo environment. The old rat media-derived SMCs continue to produce cellular retinol binding protein-1 but little α-smooth muscle actin and smooth muscle myosin heavy chains, whereas the newborn rat media-derived SMCs continue to express α-smooth muscle actin and smooth muscle myosin heavy chains but no cellular retinol binding protein-1. Our results reinforce the notion of arterial SMC phenotypic heterogeneity and suggest that in our model, heterogeneity is controlled genetically and not by the local environment. (Arterioscler Thromb Vasc Biol. 2001;21:949-954.)

Key Words: α-smooth muscle actin ■ smooth muscle myosin ■ restenosis ■ intimal thickening ■ atherosclerosis

Atheromatous plaque formation and restenosis are characterized by the accumulation of smooth muscle cells (SMCs) within the intima. This phenomenon results from the combined action of several cytokines, growth factors, and extracellular matrix components. It has been proposed that SMCs from the arterial wall are phenotypically heterogeneous and that a subset of medial SMCs is particularly prone to migrate into the intima and to proliferate under appropriate stimuli. The heterogeneity of arterial SMCs has been established in vitro by isolating cell populations from different locations or from the same location in animals of different ages. Two main phenotypes have been described in the rat: a spindle-shaped phenotype, with the classic “hills and valleys” growth pattern typically obtained from the adult media, and an epithelioid phenotype typically isolated from the intimal thickening (IT) produced 15 days after endothelial injury. The same phenotypes, spindle-shaped and epithelioid, have been recovered when SMCs are cultured from the media of newborn rats (aged 4 days) and old rats (aged >18 months), respectively. The epithelioid cells are capable of replicating in the absence of serum, exhibit a high migratory activity, and are less differentiated compared with spindle-shaped cells. Moreover, the level of SMC cytoskeletal protein expression decreases with the age of the donor. We have found that cellular retinol binding protein (CRBP)-1 is specifically expressed by the epithelioid cell populations. Epithelioid and spindle-shaped clones can be recovered, albeit in different proportions, from both the normal media and the IT 15 days after endothelial injury, thus providing evidence that in normal conditions, the arterial media contains cells capable of developing in vitro the 2 phenotypes.

Because nearly all results suggesting SMC heterogeneity have been obtained in vitro, we wondered whether the distinct SMC phenotypes are permanent or are temporarily preserved by culture conditions. We tested the hypothesis that differences observed in culture are retained when SMCs are seeded back into an in vivo environment. For this purpose, using a previously developed model, we have implanted aortic SMCs cultured from newborn and old rats in injured rat carotid arteries and studied the expression of 3 differentiation markers, ie, α-smooth muscle (SM) actin, SM myosin heavy chains (MHCs), and CRBP-1, at different time points after seeding. Our results show that the specific phenotype of SMCs observed in vitro is maintained when they are placed back into an in vivo environment.

Methods

Cell Culture and Labeling
Aortic SMCs were isolated by enzymatic digestion from newborn (7-day-old) and old (≥18-month-old) Fischer 344 rats (Simonsen...
Laboratories, Gilroy, Calif) as previously described. Cells were grown in DMEM (GIBCO) supplemented with 10% FCS (HyClone) and used between passages 5 and 10. Cells were trypsinized and resuspended in DMEM. They were incubated for 5 minutes with PKH-26 (Sigma), a fluorescent cell linker that stably incorporates into membrane lipid bilayer and has excitation and emission wavelengths similar to those of rhodamine.

In Vivo Experimental Procedures
Thirty male Fischer 344 rats (aged 3 months) were subjected to left carotid balloon injury. Then, 5×10⁴ PKH-26–labeled aortic SMCs cultured from newborn and old rats in 50 μl culture medium were infused into the injured carotid segment as previously described. Right carotid arteries were used as a control.

Immunohistochemistry and Immunofluorescence
Seven and 14 days after endothelial injury, carotid arteries were fixed with 4% neutral buffered formalin and embedded in paraffin or snap-frozen in precooled liquid isopentane and embedded in OCT (Miles Laboratories).

Paraffin-embedded sections were stained with the following antibodies: (1) a mouse monoclonal IgG2a recognizing α-SM actin, (2) 2 affinity-purified rabbit polyclonal IgGs recognizing SMMHC types 1 and 2, with 1 produced in our laboratory and 1 obtained from Biomedical Technologies Inc, and (3) an affinity-purified rabbit polyclonal IgG specific for CRBP-1 produced and tested in our laboratory. Samples (6 animals per condition) were observed by use of a Zeiss Axiophot photomicroscope (Carl Zeiss) with an oil-immersion Plan-Neofluar ×40/1.3 objective. Images were acquired with a color high-sensitivity Coolview camera (Carl Zeiss). To evaluate the surface and cell density of IT, 3 randomly selected fields per animal (n=5) in hematoxylin–methylene blue–stained transversal sections were analyzed by using KS400 software (Kontron System, Carl Zeiss). Results are given as mean±SEM. The Mann-Whitney test was used for statistical analysis. For illustrations, images were processed with Adobe Photoshop 5.0 (Adobe System) and printed with a digital Fujifilm Pictrography 4000 printer (Fujifilm).

To localize the seeded cells, PKH-26 and SMMHC were simultaneously detected on OCT-embedded specimens. Unmounted cryostat sections were observed with a Zeiss Axiophot photomicroscope (Carl Zeiss) equipped for epi-illumination and with specific filters for rhodamine and fluorescein; an oil-immersion Plan-Neofluar ×40/1.4 objective was used. Images of PKH-26–labeled SMCs were acquired in the rhodamine channel as described above. Then the same sections were fixed in acetone at −20°C for 5 minutes and air-dried. They were incubated with anti-SMMHC, followed by a goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Southern Biotechnology Associates). Preparations were mounted in buffered polyvinyl alcohol. Images of the SMMHC staining were acquired in the fluorescein channel and overlaid with those of the PKH-26 labeling using Adobe Photoshop 5.0.

SDS-PAGE and Immunoblotting
ITs stripped from the media were immediately frozen in liquid nitrogen and crushed. Tissues and cultured SMCs were processed for SDS-PAGE and for immunoblotting for α-SM actin, SMMHC types 1 and 2, and CRBP-1, as previously described.

Results
The phenotype of SMCs isolated from Fischer rat aorta was characterized by evaluating the expression of α-SM actin and SMMHC. At the fifth passage, the percentage of α-SM actin–positive and SMMHC–positive cells was higher in SMCs cultured from newborn rats (96% and 50%, respectively) than in SMCs cultured from old rats (48% and 1%, respectively). These values were similar to those previously observed in aortic SMCs isolated from age-matched Wistar rats. Thus, cultured Fischer rat aortic SMCs exhibit a differentiation level that decreases with the age of the donor.

When SMCs were seeded into injured rat carotid artery, a typical IT developed, allegedly composed of seeded and endogenous cells. The ITs that developed after the seeding of newborn rat– and old rat–derived SMCs were, as expected, significantly larger at 14 days than at 7 days (38.540±1742 and 27.942±3014 μm², respectively, for newborn rats, P<0.001; 40603±1887 and 23021±1935 μm², respectively, for old rats, P<0.001) and showed a significant decrease in cell density at 14 days compared with 7 days (68±3 cells per 10⁴ μm² and 76±4 cells per 10⁴ μm², respectively, for newborn rats, P<0.05; 59±2 cells per 10⁴ μm² and 84±6 cells per 10⁴ μm², respectively, for old rats, P<0.05). At all time points studied, no differences in the surface and cell density of the neointima were observed between ITs that developed after the seeding of SMCs cultured from newborn and old rats.

As expected, in the normal carotid artery media, practically all SMCs were positive for α-SM actin and SMMHC (data not shown). When SMCs cultured from newborn rats were implanted into injured carotid arteries, the 7-day-old and the 14-day-old ITs were characterized by a uniform and strong staining for α-SM actin and SMMHC (≥70% to 100% of positive cells for each time point, Figure 1a through 1d). In contrast, at the same time points, the seeding of old rat–derived SMCs gave rise to an IT that reacted very weakly for α-SM actin and was practically negative for SMMHC (<10% of positive cells for each time point, Figure 1e through 1h). However, a small proportion of IT cells, particularly when they were situated close to the lumen, contained α-SM actin and SMMHC at 7 and, more important, at 14 days (Figure 1e through 1h). At 14 days, α-SM actin and SMMHC stainings were negative in the luminal layers of the media (Figure 1g and 1h), suggesting the penetration of implanted SMCs in these layers. Thus, arterial SMCs cultured from rats at different ages and exhibiting a distinct degree of differentiation appear to maintain their phenotype when they are seeded into injured rat carotid artery.

Seeded newborn rat– and old rat–derived SMCs were identified by the presence of PKH-26, a stable lipophilic cell membrane linker. PKH-26 and SMMHC were detected on the same section. At 3 days, ITs containing newborn rat– or old rat–derived SMCs were composed only of seeded cells (data not shown). At 7 days, the external portion of IT formed after seeded of the 2 cell types was positive for PKH-26 (red staining, Figure 2a and 2b), whereas the luminal portion was practically negative. As expected, in IT formed by newborn rat–derived SMCs, PKH-26–positive cells expressed SMMHC (data not shown), whereas in IT composed of old rat–derived SMCs, PKH-26–positive SMCs did not express SMMHC (Figure 2a). Similar results were obtained at 14 days, except that PKH-26 staining (red staining, Figure 2b) was also visible in the most superficial layer of the media. Here again, SMMHC was present in PKH-26–positive cells (including those visible in the most internal layer of the media) in IT containing newborn rat–derived SMCs and absent in IT containing old rat–derived SMCs (Figure 2b). This last result corresponds with the findings illustrated in Figure 1g and 1h and indicates that seeded SMCs are capable of crossing the internal elastic lamina. Moreover, in 14-day-old IT containing old rat–derived SMCs, some of the PKH-
Negative cells were positive for SMMHC (Figure 2c), supporting their medial derivation. Immunoblot analyses showed that the expression of α-SM actin and SMMHC was higher in aortic SMCs cultured from newborn rats than in those derived from old rats (Figure 3A and 3B). The 2 isoforms of SMMHC, types 1 and 2, were present in newborn rat–derived SMCs, whereas no SMMHC was detectable in old rat–derived SMCs (Figure 3A and 3B). At 20 days, seeding of newborn rat–derived SMCs induced an IT containing α-SM actin and SMMHC; however, SMMHC appeared as a single band that corresponded to SMMHC type 1 (Figure 3C). In arteries seeded with old rat–derived SMCs, IT showed weak expression of α-SM actin and was negative for SMMHC (Figure 3D). Normal media and 20-day-old IT of balloon catheter–injured carotid artery without seeded cells exhibited a significant expression of α-SM actin and SMMHC (Figure 3E and 3F), in accordance with the previous observations that at this time IT, SMCs have ceased replication and have acquired a significant degree of differentiation.

We have previously reported that CRBP-1 is expressed in cultured epithelioid SMCs (including those derived from old rat aorta) but not in spindle-shaped SMCs, and have suggested that it could represent a marker of epithelioid cells in vitro and in vivo. Therefore, it was of interest to investigate whether CRBP-1 is maintained in IT composed of old rat–derived SMCs. Immunohistochemistry using CRBP-1 antibody showed that 7-day-old IT induced after the seeding of newborn rat–derived SMCs was practically negative (Figure 4a), whereas IT induced after the seeding of old rat–derived SMCs was strongly positive (Figure 4b). Positive cells could irregularly be seen only close to the luminal surface. The medial SMC underlying the IT expressed low levels of CRBP-1 in both situations (Figure 4a and 4b). Western blots using the same antibody and performed at 21 days showed that CRBP-1 was expressed in IT composed of...
 Discussion

The concept of arterial SMC heterogeneity has gained wide acceptance in the last years. The distinct phenotypes of arterial SMCs have been mainly identified in vitro, suggesting that specific features of SMC populations arise and are maintained in the particular environment of cell culture. Hence, it was of interest to investigate whether in vitro SMC phenotypes are preserved when SMCs are placed into injured carotid arteries, as determined by the expression of cytoskeletal proteins and CRBP-1.

old rat–derived SMCs and absent in IT composed of newborn rat–derived SMCs (Figure 4c). Thus, newborn rat– and old rat–derived aortic SMCs essentially maintain their respective phenotypes when they are implanted into injured carotid arteries, as determined by the expression of cytoskeletal proteins and CRBP-1.

Figure 4. Immunolocalization of CRBP-1 in 7-day-old IT induced after seeding with newborn rat–derived (a) and old rat–derived (b) aortic SMCs into the injured carotid artery and immunoblots (c) showing CRBP-1 expression in 20-day-old IT induced after seeding with newborn rat–derived (A) and old rat–derived (B) aortic SMCs. At 7 days, IT produced after seeding of newborn rat–derived SMCs is practically negative for CRBP-1 (b). Similar results are obtained at 20 days, as shown by immunoblots (c). Bar=50 μm (a and b).

Spindle-shaped and epithelioid rat SMC populations have been cultured from tissues collected during different developmental or experimental situations. Moreover, we have shown that spindle-shaped and epithelioid SMC clones can be recovered from normal media and/or IT after balloon-induced endothelial injury, albeit in different proportions. We have decided to use as an experimental model for spindle-shaped cells newborn rat–derived aortic SMCs and for epithelioid cells old rat–derived aortic SMCs, because they have been described and/or confirmed by several laboratories and can be discriminated in vitro by the expression level of 2 well-known differentiation markers, α-SM actin and SMMHC. α-SM actin is highly expressed in SMCs isolated from newborn rats compared with SMCs derived from old rats. SMMHC isoforms 1 and 2 are markers of an advanced stage of SMC differentiation and are present in cultured newborn rat–derived SMCs, whereas they are not detectable in old rat–derived SMCs. Conversely, CRBP-1 has been shown to be expressed in old rat–derived SMCs and to be not detectable in newborn rat–derived SMCs. By means of immunohistochemistry and Western blots with specific antibodies, we have demonstrated that the differentiation level of both cultured SMC populations does not change when cells are implanted for 20 days into the injured carotid artery. We cannot exclude the possibility that in vitro conditions permanently altered the genotype of the 2 cell populations, eg, by DNA methylation. However, seeded newborn rat–derived SMCs express SMMHC isoform 1 but lose SMMHC isoform 2, suggesting that seeded cells are somehow influenced by the intimal environment. Moreover, differences between the 2 implanted populations could eventually disappear if seeded cells are allowed to reside in the neointima for a long time.

The model that we have used has been designed for the utilization of cultured arterial SMCs as vehicles for gene therapy. Arterial SMCs, transfected with a gene of interest, are seeded onto the luminal surface of a balloon-injured carotid artery and give rise to a typical IT. This model is attractive, insomuch as it is, to our knowledge, the only way to place SMCs back into a vascular environment. We have also observed that old rat–derived SMCs, clearly distinguished from endogenous SMCs by the presence of PKH-26 and the absence of α-SM actin and SMMHC, are capable of invading the underlying media and, here again, retain their specific phenotype.

When media-derived SMC populations are placed in culture, they tend to proliferate indefinitely in the presence of serum. When the same cells are seeded into the denuded carotid artery, they stop replicating and continue to express the transfected gene over time. Interestingly, the old rat–derived SMCs, which in vitro exhibit a high replicative activity and grow without serum factors, give rise to an IT similar in size to the IT containing seeded newborn rat–derived SMCs, which in culture require serum to replicate. We have no explanation for this phenomenon. This observation suggests that the microenvironment of the neointima can influence the replicative activity and the serum dependence of SMCs exhibiting different phenotypes.

PKH-26–positive SMCs were always more numerous in the deep portion of IT, in accordance with previous results showing that seeded SMCs, tracked by the expression of a stable transfected human gene, have essentially the same location. PKH-26 is lost as cells divide multiple times, and this could in part explain its absence at the luminal...
portion of IT. Moreover, after the seeding of old rat–derived SMCs, a proportion of PKH-26–negative SMCs expressed α-SM actin and SMMHC (Figure 2c), supporting their medial derivation.

Walker et al first described the 2 main SMC phenotypes, spindle-shaped and epithelioid, obtained from the normal carotid media and from the IT induced 15 days after endothelial injury with use of a balloon catheter, respectively. Other laboratories, including ours, have further extended these data by demonstrating that SMCs with an in vitro epithelioid phenotype can be isolated from different sources, including the normal media.2,7,9,11,21 The epithelioid phenotype is mainly characterized by its ability to proliferate in the absence of serum.5,7,21 A high migratory activity that is correlated with an increased tissue plasminogen activator expression21,39 and a low differentiation level (decreased α-SM actin and SMMHC).10,22,23 Recently, Frid et al23,40 have shown that distinct in vitro SMC populations isolated from the bovine pulmonary artery characterized by their morphological and expression of differentiation specific markers can be recognized in vivo. Moreover, we have found that CRBP-1, a protein involved in retinoic acid metabolism, is a specific marker of the epithelioid phenotype.22,23 After endothelial injury, CRBP-1 transiently appears in a subpopulation of medial SMC and in the IT and disappears allegedly by apoptosis41 when the IT is reendothelialized.23 It is noteworthy that in our experimental conditions, CRBP-1 expression persists in old rat–derived SMCs, which populate IT, further demonstrating that CRBP-1 is a reliable phenotypic marker of epithelioid SMCs.

Previous work from other laboratories and ours has suggested that a specialized subset of medial SMCs is prone to proliferate and migrate from the media into the intima and is responsible for IT formation.7,9,11,12,14–16,21 In vitro conditions appear to help in establishing and maintaining subpopulations and clones with specific phenotypes.8,21 The present observation that each of the SMC-specific phenotypes is maintained when cells are placed back into the carotid artery extends the notion of phenotype stability to an in vivo situation. On the other hand, it appears likely that during the in vivo formation and evolution of IT after endothelial injury, several phenomena (not necessarily operating in vitro) intervene to regulate the selection and then the disappearance of SMC subsets, particularly those exhibiting the epithelioid phenotype.

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