Age Influences the Replicative Activity and the Differentiation Features of Cultured Rat Aortic Smooth Muscle Cell Populations and Clones

Marie-Luce Bochaton-Piallat, Françoise Gabbiani, Patricia Ropraz, Giulio Gabbiani

The replicative activity and the differentiation features of aortic smooth muscle cells (SMCs) cultured as whole populations or clones from newborn (4-day-old), young adult (6-week-old), and old (18-month-old) rats were studied by means of cell counting, [3H]thymidine incorporation, and measurement of the expression of cytoskeletal proteins and mRNAs. In whole populations at the fifth passage, replicative activity increased and differentiation features (ie, expression of α-smooth muscle actin, desmin, and smooth muscle myosin heavy chains) decreased with increasing age of the donor animal. SMC clones derived from newborn or young adult rats showed more differentiated cytoskeletal features than their parental populations; however, most SMC clones from old rats showed dedifferentiated features similar to those observed in their parental populations. Our results suggest that (1) SMCs of the rat aortic media behave as a heterogeneous population; (2) cultured whole SMC populations behave differently from clones as far as their replicative activity and differentiation features are concerned; and (3) SMCs derived from old rats, whether grown as whole populations or as clones, dedifferentiate more substantially and replicate more actively than corresponding cultures from newborn or young adult rats when submitted to the same amount of serum growth factors; these differences may play a role in arterial development as well as in the formation and evolution of the atheromatous plaque. (Arterioscler Thromb. 1993;13:1449-1455.)

Key Words • actin isoforms • desmin • smooth muscle myosin • atheromatosis • aging

One of the major features of atheromatous plaque development is the increase of the intimal cell population, which is mainly due to medial smooth muscle cell (SMC) migration and proliferation (for review see References 1 to 3). The early observations of Benditt and Benditt4 suggest that the initial SMC proliferative event must involve a small proportion of the media population, possibly one cell. This may be due to an exogenous or endogenous stimulus affecting a single (or few) cell(s) but may also depend on the fact that SMCs are heterogeneous in their replicative potentiality. It has also been shown that human and experimental atheromatous SMCs show dedifferentiated features, at least as far as their morphology (for review see Reference 5) and cytoskeletal equipment6-9 are concerned, and the above considerations apply to the acquisition of such features by SMCs. The possible heterogeneity of SMCs has been little explored until now because of the lack of experimental models. A suitable model could be based on the production of SMC clones. Ideally, these clones should be produced from human SMCs; practically, however, clones derived from SMCs of an experimental animal are relatively easy to obtain and may furnish new, useful information on the general biology of arterial SMCs.

We have previously reported10 that clones of rat aortic SMCs from newborn rats show distinct differentiation features compared with those of their parental population. Here we show that clones as well as whole populations of aortic SMCs from rats at different ages that have been exposed to the same amounts of serum growth factors exhibit distinct differentiation and replicative features, replication being higher and differentiation lower in SMCs from old rats. This suggests that, at least in the rat, age is important in determining the response of SMCs to factors possibly playing a role in several normal and pathological phenomena involving the arterial wall, including the formation of the atheromatous plaque.

Methods

Cell Culture and SMC Cloning

The thoracic aortic media of 4-day-, 6-week-, and 18-month-old Wistar rats was carefully dissected and digested enzymatically, as previously described.10 SMCs were then plated on 100-mm plastic Petri dishes at a density of 2×10⁴ cells/cm² in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Seromed Biochem, Berlin, FRG). Cell populations were always brought to the fifth passage in the same medium and the same-sized dishes. All experiments were done at least in triplicate for each age group. For cloning, cells were trypsinized 48 hours or 7 days after plating and were cloned by limiting dilution (0.75 cells per well) in DMEM containing 20% FCS.10 Isolated cells could be...
obtained if SMCs were recovered after plating, which was not possible if SMCs were used immediately after the enzymatic digestion. Clonal growth was visually controlled during the first 10 through 15 days after plating, and wells allowing the slightest suspicion of the initial presence of two cells were discarded. Confluent cloned cells were expanded into 24-well and six-well culture dishes; they were then plated on either 60-mm or 100-mm Petri dishes in the same medium. The total number of clones was obtained from three experiments for each age group. For immunohistochemical and biochemical studies, SMCs from whole-cell and clonal populations at the fifth passage were plated at a density of 5 x 10^3 cells/cm^2 and harvested after 7 days of culture, unless otherwise stated.

Fibroblasts were obtained from explants of 6-week- and 18-month-old rat dermis and subcutaneous tissue and were cultured in minimum essential medium (GIBCO) in the presence of 10% FCS.

**Cell Sorting**

Flow cytometric analysis was performed on freshly digested SMCs. Briefly, cells were fixed with cold 70% ethanol overnight and then stained with 50 μg/mL propidium iodide (Fluka, Buchs, Switzerland) containing 100 U/mL RNase A (Calbiochem, San Diego, Calif). The samples were then analyzed using an FACScan (Beckton Dickinson, Lincoln Park, NJ).

**Cell Proliferation and [3H]Thymidine Incorporation**

For the measure of cell proliferation, whole-cell populations of aortic SMCs were used at the fifth passage and were seeded at a concentration of 2 x 10^4 cells/cm^2. Cells were trypsinized and counted at day 4 by using an FACScan (Beckton Dickinson). Rat fibroblasts at the fifth passage were seeded at a concentration of 2 x 10^4 cells/cm^2 and counted 4 days later as described above. For [3H]thymidine incorporation, SMCs were plated at a concentration of 7 x 10^3 cells/cm^2 and were synchronized for 4 days in DMEM supplemented with 10 μg/mL selenium (Collaborative Research, Lexington, Mass) and 5 μg/mL transferrin (GIBCO). Then, fresh medium plus 20% FCS and 0.1 μCi/mL [3H]thymidine (5 Ci/mmol/L specific activity; Amersham, Buckinghamshire, UK) were added for 20 hours. Thymidine incorporation was determined by trichloroacetic acid precipitation and by counting in a Beckman scintillation counter (Beckman Instruments, Fullerton, Calif). Whole cell and clonal populations at the fifth passage were also plated at a concentration of 7 x 10^3 cells/cm^2 in DMEM supplemented with 2% plasma-derived serum (PDS). After 4 days of culture, cells were counted as described above.

**Immunofluorescence Staining**

Double immunofluorescence was performed directly in the Petri dish; we also examined cytocentrifuged SMCs. A mouse monoclonal immunoglobulin (lg) 2a15 specific for α-smooth muscle (SM) actin (anti-α-SM-1) and two affinity-purified rabbit polyclonal IgGs specific for desmin16 and for SM myosin heavy chains 17 were used as previously described. SM myosin heavy chain antibody specifically recognizes the SM myosin heavy chains of rabbit, human, and rat aorta myosins (Reynolds 10 and A. Chiavatego, PhD, and G. Gabbiani, MD, unpublished data, January 1993). Cell counts were performed using a Zeiss VIDAS (Carl Zeiss, Oberkochen, FRG) interactive image analysis system, a high-sensitivity AVT-Horn CCD camera, type FMC-4005 (AVT-Horn, Aalen, FRG), and a Zeiss Axiopt (Carl Zeiss) photomicroscope. Pictures were taken on Tmax black and white film (Eastman Kodak, Rochester, NY) by using a plan apochromate ×63/1.40 objective. Results are expressed as mean±SEM. Statistical evaluation of differences for immunofluorescence-stained cell counts and for biochemical experiments was performed by means of Student's t test.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting**

Cells were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as previously described. For immunoblotting, between 2 and 10 μg protein was electrophoresed on 5% to 20% gradient gel and then transferred to nitrocellulose paper that was incubated with anti-α-SM-1. Enhanced chemiluminescence was used for detection (Amersham). For quantification, SDS-PAGE and films were scanned with a computerized laser beam densitometer (Genoiff, Geneva, Switzerland).

**[35S]Methionine Incorporation and Two-Dimensional PAGE**

At 6 days of culture, medium was removed and SMCs were pulsed with 100 μCi [35S]methionine (1000 Ci/mmol/L specific activity; Amersham) in methionine-deficient DMEM with 20% FCS for 16 hours. SMCs were then processed for further analysis by two-dimensional PAGE using pH 4 to 6.5 preblended ampholines (Pharmacia, Uppsala, Sweden). The films were scanned as described above.

**RNA Extraction and Northern Blot Hybridization**

RNA isolation was performed according to Chirgwin et al. In all experiments, between 3 and 6 μg total RNA was denatured with glyoxal, electrophoresed in 1% agarose gel, and transferred overnight to nylon membranes (Hybond-N, Amersham). The membranes were then processed for hybridization either with a probe7 recognizing total actin mRNA (pRAoaA-C) or a 29-mer synthetic oligonucleotide10 derived from the rat α-SM actin 3′-untranslated region and specific for rat α-SM actin mRNA. The films were scanned as described above.

**Results**

**Replicative and Cytoskeletal Features of SMC Populations**

As previously described, the percent of aortic SMCs in the G0-G1 phase freshly isolated from newborn rats was 48.6% and in the S-G2 phase it was 51.4%. No polyplloid cells were present. In contrast, the percent of SMCs in the G0-G1 phase was, as expected, 90.9% in young adult rats and remained 84.5% in old rats. In both populations, no SMCs were seen in the S-G2 phase, but a proportion of tetraploid cells was detected (9.1% in young adult and 15.5% in old rats).

No remarkable differences were noted in the morphology of newborn, young adult, and old rat SMC...
Cytoskeletal features of newborn, young adult, and old rat SMC populations were characterized first by means of immunofluorescence. In SMCs freshly isolated from newborn and young adult rat aortas, the percentages of \( \alpha \)-SM actin-, desmin-, and SM myosin–positive cells (Table 1) were similar to those previously described.\(^{10} \) In newborn rat SMCs, the percentage of \( \alpha \)-SM actin–positive cells remained unchanged at the fifth passage compared with freshly isolated cells, whereas in young adult rat SMCs it decreased significantly. The percentages of desmin– and SM myosin–positive cells decreased less importantly in newborn rat SMCs than in young adult rat SMCs. SMCs freshly isolated from old rat aorta showed percentages of \( \alpha \)-SM actin–, desmin–, and SM myosin–positive cells similar to those observed in newborn and young adult rat SMCs. At the fifth passage, the percentage of \( \alpha \)-SM actin–positive cells was lower than newborn and young adult rat SMCs; desmin– and SM myosin–positive cells had disappeared.

Densitometric scanning of SDS-PAGE (Fig 2) showed that the proportion of total actin per total protein was lower (\( P<.001 \)), as expected,\(^{10,23} \) in freshly isolated SMCs from newborn rats (7.6±0.3%) compared with SMCs from young adult and old rats; the values of these two last groups were similar (11.2±0.3% and 12.7±0.7%, respectively). At the fifth passage the total actin content was 12.7±0.3% in newborn rat SMCs but was significantly (\( P<.001 \)) decreased in young adult and old rat SMCs (9.2±0.1% and 7.8±0.1%, respectively). The difference between young adult and old rats was also significant (\( P<.01 \)).

Table 1. Cytoskeletal Features of Whole SMC Populations Cultured From Newborn, Young Adult, and Old Rat Aortas

<table>
<thead>
<tr>
<th>Condition</th>
<th>( \alpha )-SM Actin</th>
<th>Desmin</th>
<th>SM Myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Newborn</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Hours</td>
<td>92.2±1.1</td>
<td>60.4±2.1</td>
<td>87.4±2.4</td>
</tr>
<tr>
<td>Passage 5</td>
<td>92.5±1.0</td>
<td>2.8±0.4</td>
<td>43.1±2.6</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Hours</td>
<td>91.4±1.0</td>
<td>57.4±2.0</td>
<td>86.2±0.8</td>
</tr>
<tr>
<td>Passage 5</td>
<td>68.9±1.5</td>
<td>0</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td><strong>Old</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Hours</td>
<td>92.0±3.9</td>
<td>51.0±3.4</td>
<td>86.1±0.6</td>
</tr>
<tr>
<td>Passage 5</td>
<td>38.1±2.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

SMC indicates smooth muscle cell; SM, smooth muscle.
The a-SM actin mRNA, expressed as a percentage of total actin mRNA (total probe), was always predominant (more than 90%) in freshly isolated SMCs regardless of donor age; it was still predominant in passaged newborn rat SMCs (84.0±3.3%), but became minor in young adult (45.8±4.6%) and old rat SMCs. Immunoblotting (5 μg total protein loading) showed that the a-SM actin content was lower in freshly isolated SMCs compared with young adult and old rat SMCs but was higher in passaged newborn rat SMCs compared with young adult and old rat SMCs. However, lower in freshly isolated SMCs from newborn rats compared with young adult and old rat SMCs. At the fifth passage, the a-SM actin content decreased with the age of the donor animal (the arrowhead points to actin).

Replicative and Cytoskeletal Features of SMC Clones

Cloning efficiency of clones derived from newborn and young adult rat SMCs (20% and 18%, respectively) and the percentage of clones obtained after expansion up to the fifth passage (6% and 7%, respectively) were similar (Table 2). In contrast, these values were 34% and 12%, respectively, in old rat SMC clones.

Immunoblotting with anti-a-SM-1 (2 μg total protein loading) was performed on 7 SMC clones per age group (Fig 4). In clones derived from newborn rat SMCs, the a-SM actin content represented 100% to 180% of the parental population value. In clones derived from newborn and young adult rats, SM myosin, the number of positive cells generally reaching 100%; half of the clones were positive for desmin, 10 the number of positive cells varying from 30% to 100% (Table 2). Clones derived from young adult rat SMCs were all positive for both a-SM actin and SM myosin, similar to clones of newborn rats, but only 20% of them were positive for desmin, the number of positive cells varying from 30% to 100%. Thus, clones obtained from newborn or young adult rat SMCs were always more differentiated than the corresponding whole-cell populations. In contrast, clones derived from old rat SMCs showed a heterogeneity of a-SM actin expression: 15% were practically negative (only rare positive cells were scattered within the negative population); 22% were slightly positive (less than 30% of positive cells); and 63% showed more than 30% of positive cells, some reaching 100% of positive cells. None of the clones was positive for SM myosin; the only clone positive for desmin was also strongly positive for a-SM actin. Thus, clones derived from old rat SMCs were less differentiated than clones derived from newborn and young adult rat SMCs; moreover, a high proportion of the clones showed dedifferentiated features similar to or even less pronounced than those of their own parental population.

The proportion of total actin per total protein, analyzed by means of densiometric scanning of SDS-PAGE, was 12.4±0.3% in clones derived from newborn rat SMCs; it was significantly (P<0.01) lower in clones derived from newborn rat SMCs by 9.9±0.5% and 9.2±0.3%, respectively).

Immunoblotting with anti-a-SM-1 (2 μg total protein loading) was performed on 7 SMC clones per age group (Fig 4). In clones derived from newborn rat SMCs, the a-SM actin content represented 100% to 180% of the parental population value. In clones derived from young adult rat SMCs, the a-SM actin content represented in general more than 500% of the parental population values. In the old rat SMC parental population and in 4
of the tested clones, α-SM actin was not detectable with a 2 μg total protein loading, whereas the other clones were positive. When 10 μg total protein was loaded, 2 clones as well as the parental population showed comparable signals, whereas 2 other clones remained negative. The α-SM actin content of strongly positive clones represented more than 500% of the parental population value.

α-SM actin synthesis was evaluated in 2 SMC clones per age group. Among the clones derived from old rat SMCs, 1 clone had a high content of α-SM actin—positive cells by means of immunofluorescence, and the other was negative. The results confirmed that clones from newborn rat SMCs synthesize more α-SM actin (20.1±1.3% of total actin) than clones from young adult and old rat SMCs (11.8±1.2% and 10.6±1.1%, respectively, of total actin); in the negative clone α-SM actin synthesis was not detectable by means of immunofluorescence. Thus, despite the fact that whole-cell populations from young adult and old rats synthesize very low amounts of α-SM actin, this activity is maintained in clones from young adult rats and in some clones from old rats.

Hybridization with the total actin probe showed that the α-SM actin mRNA, expressed as a percent of total actin mRNA, was predominant in the two clones derived from newborn and young adult rat SMCs, representing 81.2±1.2% and 70.5±4.6%, respectively; it was also predominant in the positive clone derived from old rat SMCs, representing 80.2±4.5%, whereas it was undetectable in the negative one. Hybridization with the specific oligonucleotide (Fig 5) showed that the α-SM actin mRNA was higher in clones derived from newborn rat SMCs (178±4%) compared with the value of the parental population; it was higher or slightly lower in clones derived from young adult rat SMCs (166±7% and 80±2%, respectively) compared with the value of the parental population. In the positive clone derived from old rat SMCs, the α-SM actin mRNA was higher (500%) compared with the value of the parental population. Moreover, the α-SM actin mRNA level was
lower in the two clones derived from young adult rat SMCs and in the positive clone derived from old rat SMCs compared with that of clones derived from newborn rat SMCs.

**Discussion**

Arteriosclerosis is a complex disease in which, regardless of the mechanisms, the early involvement of SMC replication, migration, and dedifferentiation is well established. These changes can be due to microenvironmental influences (eg, the action and/or the absence of cytokines or extracellular matrix components) and/or to intrinsic properties of SMCs (for review see References 3, 26, and 27). This last mechanism presupposes a heterogeneity of SMC phenotype. SMC heterogeneity has been illustrated by several laboratories, including ours, using different criteria.16,28,29 Our present results provide some new data concerning the different growth and differentiation potencies of SMC whole populations and clones cultured from rats of different ages. It is known that rat SMCs spontaneously change their phenotypic features in vitro; nevertheless, these data may help in the understanding of the biological behavior of SMCs when submitted to growth stimuli and selection processes.

It is now generally accepted that cytoskeletal markers are reliable tools with which to define the modulation of SMC phenotypic features.4-6,9,30,31 Our data showed that whole populations of aortic SMCs from rats at different ages show different replicative activities and different phenotypic features when cultured in the presence of the same concentrations of FCS. In particular, SMCs from old rats show high replicative activity compared with SMCs from newborn and young adult animals. Interestingly, this behavior is not shared by fibroblasts cultured from the same animals, suggesting that cells from different tissues of old animals do not necessarily show a stereotyped decrease of replicative potentiality in culture, as is generally accepted (for review see Reference 32). Our results confirmed and extended data from our30 and other laboratories33-35 including the observation that whole populations of SMCs from old rats grew independently of serum factors (ie, in PDS), probably through the endogenous production of platelet-derived growth factor.35 Taken together, these results suggested that SMCs of old animals can react more intensely to (or even produce) growth factors that are normally present in serum. They also agree with previous work showing that in vivo SMCs of old rats replicate more actively after injury than those of young adult animals,33-34 and accord with the general belief that age is a strong independent risk factor for atheromatosis.36 It is noteworthy that, despite their different behavior in culture, SMCs freshly isolated from the aorta of young adult and old rats show similar phenotypic profiles and are all essentially in the G0-G1 phase, suggesting that microenvironmental factors actively maintain the differentiated features of SMCs of old animals in vivo. The possibility that locally liberated proteoglycans maintain differentiated features, including quiescence, of SMCs has been suggested on the basis of different observations (for discussion see Reference 37).

Our results also showed that, within each SMC population studied, a certain proportion of cells was capable of undergoing clonal growth in vitro. Moreover, clones from aortic media SMCs had different cytoskeletal features according to the age of the donor animal. In particular, only clones from old rats expressed a very dedifferentiated phenotype. SMC clones from newborn and young adult rats, despite some morphological differences among them (which will be the object of further study), expressed relatively uniform phenotypic features within a given age group; these features were different from those of the whole populations derived from rats of the same ages. Remarkably, clones from newborn rat aorta maintained highly differentiated features in vitro, suggesting that they have the intrinsic potentiality of doing so in vivo. This was also true, albeit to a lesser extent, for clones from young adult aorta, contrasting with the behavior of whole SMC populations from rats of the same age, which showed highly dedifferentiated features. In particular, clones from young adult rat aorta maintained the expression of SM myosin heavy chains in 100% of the cases, whereas the expression of this protein was practically lost in the whole-cell population. Whether these SMCs, potentially capable of producing a differentiated clonal population, play a role in vivo remains to be studied.

The capacity to differentiate was lost to a great extent in clones derived from old rat aorta. SM myosin heavy chains were absent in all cases and α-SM actin was absent in 15% of the clones. Thus, some clones from old rat SMCs did not express any cytoskeletal SMC differentiation marker among those tested here. However, unlike the whole-cell populations derived from old rat aorta, these clones did not grow independently of serum factors (ie, they did not grow in PDS), confirming the different behavior of clonal cells compared with whole-cell populations. Further studies are needed to establish whether products of SMCs in whole-cell populations influence the phenotype of SMCs in clones and vice versa. Moreover, work examining the differential expression of proteins or mRNAs in different SMC clones and whole populations will be important for the definition of different phenotypes. In this respect, interesting results have been recently reported on the differential expression of genes in an SMC population derived from newborn animals compared with a population derived from adult animals.38 Moreover, SMCs from old rats may represent a useful model for the study of the capacity of SMCs to undergo accelerated growth. Finally, it is noteworthy that clones from animals of different ages showed heterogeneous phenotypic features despite the fact that to reach the fifth passage they had to accomplish a similar number of cell divisions.

Cell differentiation has been interpreted for a long time in terms of genetically irreversible steps coinciding with mitotic events.39 More recently, it has been suggested that microenvironmental factors, such as locally liberated cytokines or extracellular matrix components, can also modulate differentiation features.37,40-45 It is conceivable that the differentiating activity of SMCs depends mainly on intrinsic factors in newborn and young adult rats, whereas it depends mainly on microenvironmental factors in old animals. Our results showed that rat aortic SMCs placed in culture produced populations and/or clones with different proliferative and phenotypic features according to the age of the donor. Our observations do not necessarily imply that similar phenomena occur in species other than rat or that in this species they occur in vivo; however, they suggest a possibility that can be further explored to understand the adaptation of SMCs to different stimuli and selection pressures.
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