AGING AND ARTERIOSCLEROSIS

The Increased Proliferation of Arterial Smooth Muscle Cells Isolated from Old Rats Is Associated with Increased Platelet-derived Growth Factor–like Activity

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Aging is a strong independent risk factor for the development of cardiovascular disease. The association of other risk factors, such as cigarette smoking and hyperlipidemia, with cardiovascular disease decreases with age (1). Investigations in both humans and other species suggest that the proliferation of vascular smooth muscle cells (SMCs)[1] is an early pathological feature of arteriosclerosis (2). In animal models of arteriosclerosis, aging accelerates injury-induced SMC proliferation. Stemerman et al. (3) observed more rapid, more pronounced, and more prolonged incorporation of tritiated thymidine by arterial cells in old compared with young rat aortas after balloon catheter–induced injury. By performing aortic transplants between old and young rats, Hariri et al. (4) demonstrated that exaggerated intimal plaque formation in aortas from old rats was a characteristic of the age of the artery, not the age of the animal. Combined, these studies suggested that the increased susceptibility of aged arteries to arteriosclerosis is related to an exaggerated proliferative response by arterial cells after injury.

We have examined the proliferative capacity of SMCs isolated from aortas of old and young rats to provide insight into the increased susceptibility to arteriosclerosis with aging. We now report evidence that aging is associated with dysregulation of the proliferative responses of SMCs from old aortas. This dysregulation is characterized by increased proliferation of old SMCs both in the presence and absence of serum, and is associated with enhanced growth-factor activity and decreased growth-inhibitory activity of the old SMCs.

Materials and Methods

Reagents. RPMI 1640 and Medium 199, both containing 25 mM Hepes, FCS, newborn calf serum (NCS), plasma-derived serum (PDS), trypsin–EDTA solution, and gentamycin

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[1] *Abbreviations used in this paper:* ANOV, analysis of variance; BASMC, bovine aortic smooth muscle cell; HBS, Hepes-buffered saline; NCS, newborn calf serum; PDGF, platelet-derived growth factor; PDS, plasma-derived serum; SMC, smooth muscle cell.

sulfate were purchased from Flow Laboratories, Inc. (McLean, VA). Hepes-buffered saline (HBS) consists of 0.15 M NaCl, 0.025 M Hepes, 0.040 M KCl, pH 7.4. Serum-free media (HL-1, Ventrex Labs, Portland, ME) contained no platelet-derived growth factor (PDGF), but contained progression factors including insulin, transferrin, and selenium. All sera used were tested for capacity to support growth of young adult SMCs. Media containing 2% PDS did not permit proliferation of young SMCs, but allowed maintenance of a stable cell number. PDGF was purchased from Bethesda Research Laboratories (Gaithersburg, MD) or from Collaborative Research (Lexington, MA); both products were reported as >97% pure by SDS-PAGE and NH_2-terminus sequencing. Antibody to PDGF (rabbit anti-human PDGF) was kindly provided by Drs. G. Pierce and T. Deuel (Washington University, St. Louis, MO). 120 U/mg Sodium heparin (Gibco, Grand Island, NY) was prepared as a stock solution of 1 mg/ml in media and sterilized by passage through a 0.22-μm filter (Millex GS, Millipore Co., Bedford, MA). [^{3}H]TdR (2 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

Isolation of Rat SMCs. Old (>19 mo) and young (<3 mo) male Fischer 344 rats were acquired from the NIA Harlan-inbred line. Rat aortic SMCs were isolated from aortas by modifications of published methods (5). Immediately after death, the aorta was removed from the aortic arch to the iliac bifurcation using aseptic technique. Gross adventitial attachments were removed and the aorta was opened longitudinally with iris scissors. The adventitia was peeled from the ablumenal surface with watchmakers forceps, and both the lumenal and ablumenal surfaces were scraped with a scalpel blade to remove endothelial cells and remaining fibroblasts, respectively. Comparison of histological sections from gross vs. cleaned aorta indicated that both old and young aortas were reduced to aortic media as clearly defined by the presence of internal and external elastic laminae. The vessels were then washed with HBS, minced, and the fragments placed into 10 ml of Medium 199 plus 0.2% collagenase (CooperBiomedical, Inc., Malvern, PA) and 0.1% elastase (type III; Sigma Chemical Co., St. Louis, MO). The aortic tissue was digested at 37°C with continuous agitation, and the enzyme mixture was changed at hourly intervals until the tissue was completely dispersed. The enzymes were inactivated in the digestion medium immediately after removal from incubation by adding equal parts of fresh medium containing 20% FCS. All enzyme-containing digests were pooled and filtered through a 30-μm nylon mesh; cells were pelleted by centrifugation; and ~50,000 cells were plated in growth medium per 25-cm^2 tissue culture flask. SMCs from old and young rat aortas, harvested on the same day and cultured identically, were used as matched cultures in all experiments. The cells exhibited a classic SMC phenotype and were studied during the third to sixth passage.

Cell Culture Conditions. SMC were routinely maintained in medium consisting of RPMI 1640 and Medium 199 (1:1) containing 0.025 M Hepes, 20 mM L-glutamine, 50 μg/ml gentamycin, and 10% FCS. Cells were grown to confluence and subpassaged by treatment with trypsin-EDTA. Cultures were maintained in 75-cm^2 plastic culture flasks (Corning Glass Works, Corning, NY) in a water-saturated 5% CO_2–95% air atmosphere.

Proliferation Assay. To determine the time course of serum-induced proliferation, old and young SMCs were plated at equal density (20,000/well) on microtiter plates and growth arrested for 5 d in 2% PDS. Before stimulation by serum, five wells per age group were used for cell counts to confirm equal plating. After the replacement of the medium with fresh 2% PDS, FCS was added to groups of wells at a final concentration of 10% for 0–48 h before harvest. 1 h before harvest, [^{3}H]TdR was added to a final concentration of 1 μCi/ml and incubated at 37°C for 1 h. The cell layers were then washed, cells were detached with trypsin, and the DNA was recovered onto fiberglass filter paper using an automated cell harvester (Skatron Inc., Sterling, VA). The filter discs were placed in borosilicate glass vials containing 6 ml of liquid scintillation fluid, Aquasol (DuPont Co., Wilmington, DE). Radioactivity of the filter discs was measured by liquid scintillation counting. Replicates (n = 5) were averaged and expressed as a percent change above the lowest [^{3}H]TdR incorporation in each experiment.

Continuous Label. To determine the total number of cells entering the S-phase of the cell cycle during the observation period, old and young SMCs were plated in 8-well slide

chambers (Labtek; Miles Laboratories Inc., Naperville, IL), growth arrested, and stimulated at the appropriate times by addition of 10% FCS plus 0.1 μCi/ml [^{3}H]TdR. At harvest, the SMCs were washed, fixed with 4% paraformaldehyde, washed twice with cold 10% TCA, and autoradiographically exposed. Cells were scored manually at a magnification of 1,250. Data were expressed as the percent of labeled cells.

PDGF Dose–Response Protocol. Matched cultures of old and young SMCs were plated at 20,000 cells/well side by side on microtiter plates in medium containing 2% PDS and growth arrested for 5 d. The cells were then changed to fresh medium containing 2% PDS and increasing doses of PDGF. 18 h later, [^{3}H]TdR was added as 20 μl/well (final concentration 1 μCi/ml) for 4 h after which the DNA was harvested as described above. The data from three assays were pooled after being normalized by expressing the dpm's as a percent of the maximal incorporation (100%) recorded in each assay.

Serum-free Proliferation of SMC. Cultures of old and young SMCs were harvested with trypsin, counted, and plated onto 96-well microtiter plates in serum-free media (Hl-1) supplemented only with L-glutamine and antibiotics. After 24 h (day 0), 5 wells of old and young SMCs were harvested to confirm the similarity of plating densities. Cells were refed every 4 d and on days 1, 7, and 14 were harvested with trypsin and counted in an electronic cell counter (model ZBI; Coulter Electronics Inc., Hialeah, FL). Data were expressed as the mean ± SEM (n = 5).

Mitogenic/Inhibitory Activity of SMC Lysates. Old and young SMCs plated at equal cell densities between 0.5 and 2.0 × 10^6 cells/75 cm^2 were cultured in serum-free medium and then were harvested by scraping in HBS containing 0.02% EDTA. The cells were collected by centrifugation, washed, and resuspended in 1 ml HBS per flask, and lysed by rapid freeze-thawing three times. Cellular debris was removed by centrifugation at 10,000 g for 20 min and the cleared lysates were adjusted to equal protein concentrations (6) for old and young SMC lysates prepared at each cell density. Bovine aortic smooth muscle cells (BASMCs) were used as target cells to assay mitogenic activity of cell lysates. BASMCs at 20,000 cells/microtiter well were partially growth arrested in medium containing 2% PDS for 3 d. The test lysate (typically 20 μg protein in 20 μl) was diluted with medium to 100 μl and then mixed with an equal volume of medium containing 4% PDS, 100 μg/ml gentamycin, and [^{3}H]TdR (2 μCi/ml). BASMCs (n = 6 wells per group) were incubated in this mixture for 24 h, washed, and the cells harvested. DNA-associated radioactivity was determined as described above.

The negative control consisted of SMCs maintained in medium with 2% PDS only and the positive control consisted of SMCs maintained in medium with 2% PDS and 4 U/ml PDGF. To minimize interassay variation, the incorporation of [^{3}H]TdR by the negative controls (0%) was subtracted from all values and the remainder was expressed as a percent of the [^{3}H]TdR incorporation by SMCs stimulated with PDGF (100%). The presence of PDGF-like molecules in the lysates was examined by the ability of PDGF antibodies to reduce the mitogenic activity of the lysate. The antibody (1:200 dilution of 3 mg/ml rabbit IgG) was preincubated with the test agent (lysate or PDGF) 15 min before application to the BASMCs. This particular antibody (anti-PDGF$_{II}$) is known to be PDGF specific while demonstrating wide cross-species reactivity, including rat cellular PDGF.

Inhibition of SMC Proliferation by Heparin. The antiproliferative effects of sodium heparin were assessed by two methods. First, serum-stimulated (5% NCS) SMC proliferation was measured after nine d of growth with refeeding at 3-d intervals. Heparin was added to the medium from stock solutions to achieve final concentrations of 1, 10, or 100 μg/ml. At the end of the 9 d in medium with or without heparin, the cells were washed, detached with trypsin, and counted in an electronic cell counter as described. Second, [^{3}H]TdR incorporation was measured in a protocol identical to the PDGF dose–response protocol except that concurrent with addition of PDGF (4 U/ml) or control buffer, sodium heparin (10 μg/ml) was added to the wells. Incorporation of [^{3}H]TdR into DNA by old and young SMC incorporation was adjusted to dpm per cell.

Statistical Methods. Data were compared by analysis of variance (ANOV) for old vs. young with repeated measures on the time factor, dose factor, or on cell density. For continuous-label autoradiography, data were examined by the chi-square test. For serum-

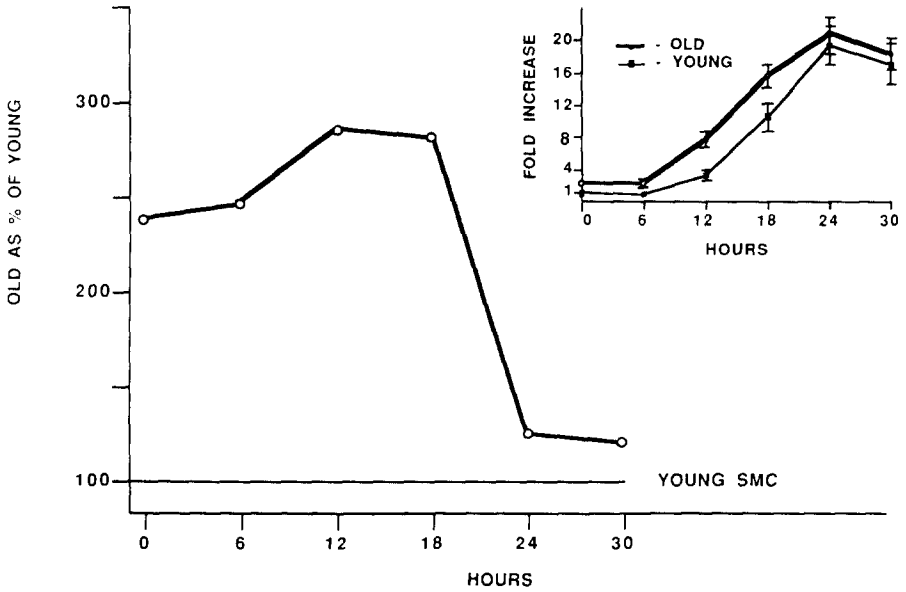

FIGURE 1. Influence of age on the time course of incorporation of [^{3}H]TdR incorporation into DNA of confluent, cultured aortic SMCs stimulated by exposure to 10% FCS. Values for old expressed as a percent of those for young cells at times specified following stimulation of quiescent cultures by serum. (*Inset*) (○) SMC from old (>19 mo) rats; (■) SMC from young (<3 mo) rats. Data expressed as increase above baseline activity, mean ± SEM. Composite data from eight experiments with $n = 6$ per group within each experiment.

free proliferation and heparin-inhibition studies, a paired t test or ANOV with repeated measures on dose of heparin was used. Differences were considered statistically significant if $p < 0.05$.

Results

Effect of Donor Age on the Time Course of Serum-stimulated Thymidine Incorporation in Arterial SMC Cultures. Equal numbers of SMCs from old (mean ± SEM/well: 23,345 ± 5,906) and young (23,874 ± 5,062) rat aortas were placed into culture. The [^{3}H]TdR incorporation into DNA during a 1-h pulse was measured at 0, 6, 12, 18, 24, and 30 h after initiating stimulation with 10% FCS. Both old and young SMCs increased [^{3}H]TdR incorporation during the first 24 h after addition of serum (Fig. 1, *inset*). However, over the first 18 h after serum stimulation, old SMCs incorporated [^{3}H]TdR at a significantly higher average rate (638.9 dpm/well per h) than comparable numbers of young SMCs (309.5 dpm/well per h; $p = 0.005$, ANOV). SMCs from old aortas incorporated twice as much [^{3}H]-TdR as SMCs from young aortas at baseline and this difference increased to nearly threefold at 12 h (Fig. 1). However, by 24 h the [^{3}H]TdR incorporation rates for the two groups were statistically comparable ($F = 0.06$, $p > 0.05$, ANOV). This increase in DNA synthesis was followed by cell proliferation, with cell numbers increasing for both old (2.8-fold) and young (2.5-fold) SMCs by 48 h of serum stimulation.

To rule out possible artefacts such as differences in nucleotide pool sizes that might affect [^{3}H]TdR incorporation, the cumulative numbers of cells in the total

TABLE I
Entry into Cell Cycle S-phase by Cultured SMC Isolated from Young and Old Rats

Culture period (h)	Percent labeled cells	
	Old	Young
With serum		
6	32.8*	13.8
12	51.1	44.2
18	88.2*	71.9
24	93.8	89.9
30	96.8	96.3
No serum (control)		
30	65.0*	46.0

SMCs from young and old rats were cultured in slide chambers in the absence or presence of 10% FCS for the times specified. [^{3}H]TDR was added to the cultures concurrently with the FCS. At the times specified, the cells were fixed with paraformaldehyde and prepared for autoradiography as described in Materials and Methods. Cells with >20 grains/nucleus were considered to have entered the S-phase of the cell cycle. The data presented are the means of two comparable experiments assessing >125 cells/point in duplicate.
* Significantly different from young: $p < 0.05$.

old or young SMC population that entered the S-phase of the cell cycle after serum stimulation was determined autoradiographically by continuous [^{3}H]TdR labeling over the same time period (Table I). During the first 18 h after serum stimulation, a greater percentage of old SMCs enter S-phase of the cell cycle than young SMCs similarly treated. By 30 h in the presence of serum, as had been seen in the pulse-label studies, there was no significant difference between old and young SMCs, as nearly all cells in both populations had entered the S-phase of the cell cycle. In contrast, after 30 h in the absence of serum, significantly more old than young cells had entered the proliferative cycle. Thus, the critical age–related differences are most prominent in the absence of serum or in the initial responses to proliferative stimuli.

The Effect of Donor Age on PDGF-stimulated [^{3}H]TdR Incorporation. Because the predominant serum mitogen for SMCs is PDGF, we investigated whether old SMCs were more responsive to PDGF than young SMCs. Consistent with the previous studies, quiescent, confluent old SMCs maintained in medium plus 2% PDS incorporated twofold more [^{3}H]TdR than young SMCs. When increasing doses of PDGF were added to cultures maintained in 2% PDS, both old and young SMCs increased [^{3}H]TdR incorporation in a dose dependent manner ($F = 35.5$, $p < 0.001$) (Fig. 2). The maximal response of old SMCs was greater than that of young SMCs and occurred at a lower dose of PDGF (2 U/ml). Old SMCs incorporated more [^{3}H]TdR than young SMCs at all doses of PDGF tested ($F = 33.1$, $p < 0.001$). However, because young SMC had lower baseline [^{3}H]TdR uptake, they showed a greater relative increase in the presence of PDGF. The high baseline activity observed with the old SMCs in the absence of added PDGF is a consistent characteristic of the aged SMCs that was examined in the following experiment.

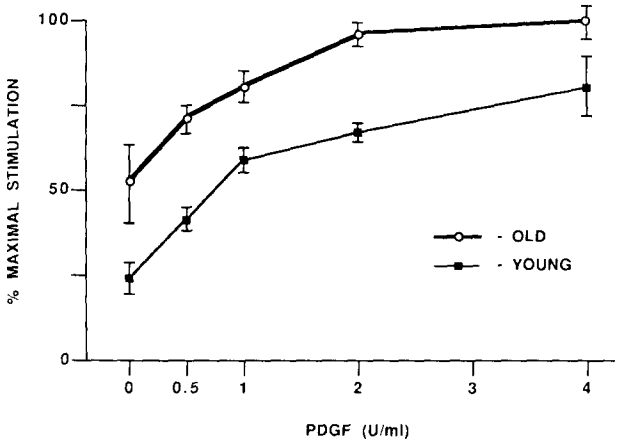

FIGURE 2. The proliferative response of old and young SMCs to increasing concentrations of PDGF. Symbols as in Fig. 1. [^{3}H]-TdR incorporation 22 h after addition of human PDGF and 4 h after addition of isotope to growth-arrested cultures of SMC in medium containing 2% PDS. Results expressed as mean ± SEM percent of maximal dpm's observed in each of three experiments.

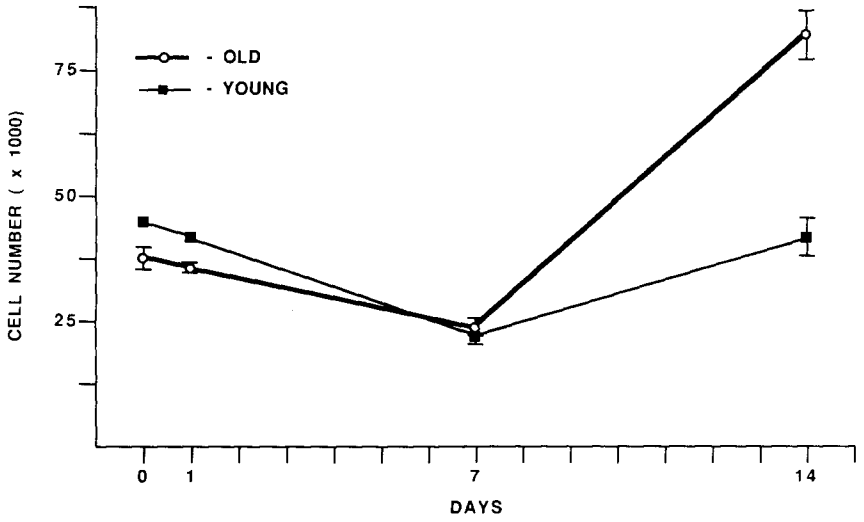

FIGURE 3. The effects of age on SMC proliferation in serum-free medium. Old and young SMCs were plated in serum-free HL-1 medium on day 1. Cell counts were made at days 0, 1, 7, and 14. Symbols as in Fig. 1. Representative experiment with $n = 5$ wells per group.

Serum-free Proliferation of Rat Aortic SMCs. Increased mitotic activity of old SMCs in the absence of serum and under low-serum conditions, combined with their pattern of responses to stimulation by serum and PDGF, suggested the possibility that old SMCs may be capable of autocrine production of growth factors. We examined the ability of SMCs from old and young rats to proliferate in serum-free medium (HL-1) that contained cell-cycle progression factors (i.e., insulin, transferrin), but no detectable competence factors. Fig. 3 demonstrates that the number of old SMCs after 14 d of culture in serum-free medium was significantly greater than the number of young SMCs ($t(8) = 6.5$, $p < 0.001$) even though the initial plating density of the young SMCs was slightly higher.

Mitogenic Effects of Cell Lysates from Old and Young Rat SMC. The ability of the old SMCs to proliferate in the absence of exogenous growth factors suggested that they may be capable of producing and using their own growth factors,

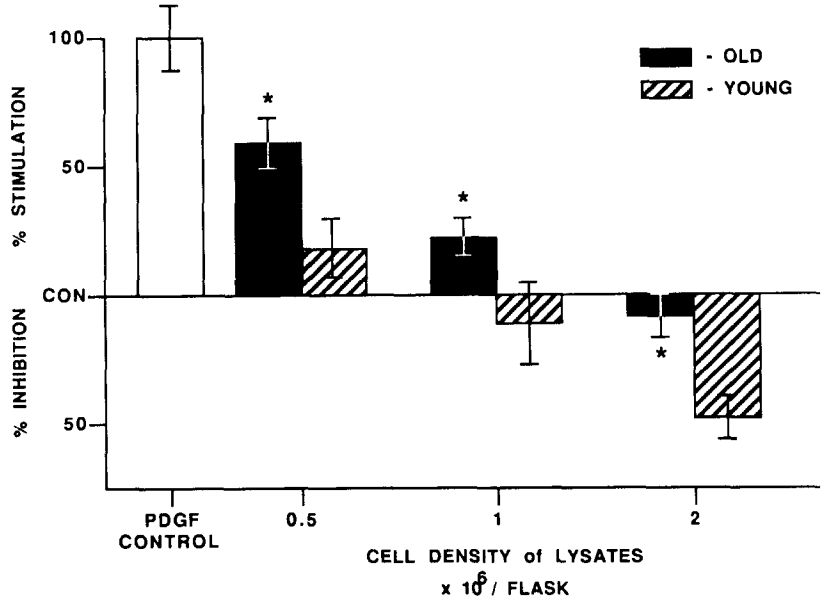

FIGURE 4. Influence of lysates of young and old aortic SMCs on proliferative responses of target cells (BASMCs). Effects of age and of cell density of cultures used to prepare lysate. (*Ordinate*) Percent stimulation indicates lysate-stimulated thymidine incorporation compared to 2% PDS control (Con, 0%) and to 4 U/ml PDGF (100%). Data as mean ± SEM, duplicate experiments on separate cell lines, $n = 6$ per experiment. (*Open bar*) PDGF control; (*solid bars*) old SMCs; (*slashed bars*) young SMCs; * indicates $p < 0.05$.

particularly PDGF. Thus, we examined the capacity of cell-associated materials prepared from old and young SMCs to influence the proliferation of quiescent target BASMCs. Freeze–thaw lysates were prepared from old and young SMCs cultured in serum-free media. Lysates containing equal amounts of protein stimulated or inhibited [^{3}H]TdR incorporation into DNA of BASMCs depending upon two factors: the cell density of the rat SMC culture used to prepare the lysates, and the age of the rats from which the SMC were isolated (Fig. 4). The amount of lysate loaded was chosen from pilot dose–response studies to provide a subsaturating stimulation of [^{3}H]TdR incorporation. In general, lysates prepared from preconfluent cultures (0.5×10^6 cells/75 cm^2) stimulated proliferation, and lysates prepared from confluent cultures (2×10^6 cells/75 cm^2) were inhibitory when tested in this assay (ANOV, $F = 38$, $p < 0.001$). Lysates from old SMCs grown at 0.5 and 1.0×10^6 cells/75 cm^2 flask were significantly more stimulatory than lysates from young SMCs ($F = 23.6$, $p < 0.001$). Likewise, old SMCs from confluent cultures (2.0×10^6 cells/flask) were less inhibitory than were similar lysates from young SMC cultures containing the same protein concentration. When preconditioned media from similarly treated cultures were examined for growth factor activity, we observed that, though more variable with respect to overall stimulatory activity, the preconditioned media rendered results qualitatively similar to those presented in Fig. 4. Thus, old SMCs contained either greater amounts of growth stimulators or lower amounts of growth inhibitors than young SMCs.

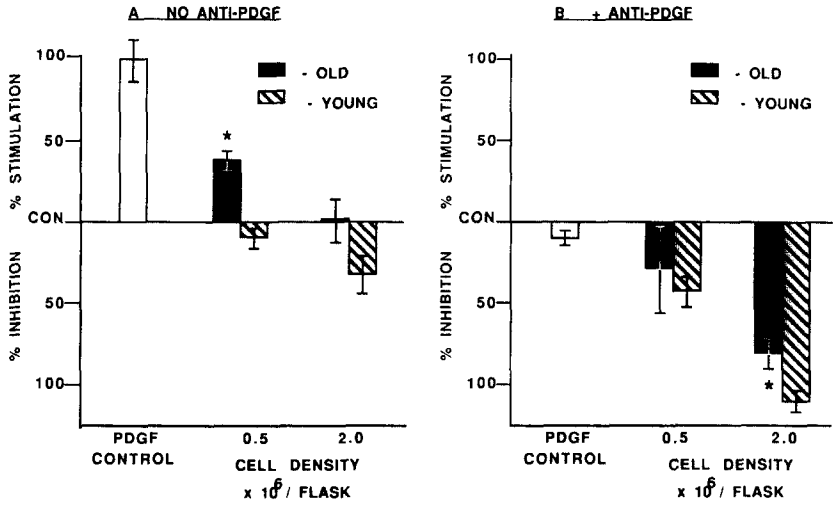

FIGURE 5. Effects of antibody to PDGF on proliferative responses of target BASMCs to lysates of aortic SMCs from old or young rats. Effects of lysates from sparse (0.5×10^6 cells/flask) or confluent (2.0×10^6 cells/flask) rat aortic SMC cultures are compared with PDGF. (*A*) Proliferative effects of lysates in the absence of anti-PDGF. (*B*) Proliferative effects of lysates after their treatment with anti-PDGF. Ordinate and symbols as in Fig. 4.

The contribution of PDGF-like molecules to the mitogenic activity observed was evaluated by incubating the lysates with antibody to PDGF before adding them to target cells. The anti-PDGF antibody completely blocked the stimulatory effect of purified PDGF in the BASMC assay (Fig. 5). Nonimmune rabbit IgG at a similar dilution (1:200) had no effect on the activity of purified PDGF: PDGF-stimulated activity = 1,840 ± 184 dpm (mean ± SEM), plus nonimmune IgG = 1,790 ± 156 dpm ($p > 0.05$). Addition of anti-PDGF to the lysates eliminated their stimulatory effect on [^{3}H]TdR incorporation ($F = 64.8$, $p < 0.001$) and revealed different degrees of inhibition by lysates from old and young SMC. Antibody-treated lysates from old SMCs had less inhibitory activity at high cell density than lysates from young SMCs ($t(10) = 2.3$, $p < 0.05$). This suggests that PDGF-like activity accounts for some of the growth-stimulating activity in the lysates, but raises the possibility that old and young SMCs may differ in the levels of growth-inhibiting agents.

Effects of Growth Inhibitors on Proliferation of Old and Young SMCs. The importance of differences in growth inhibitors on the proliferative capacity of SMCs from old and young rat aortas is suggested by studies with heparin, a major inhibitor of SMC growth. In the absence of heparin, serum-stimulated old SMCs proliferated significantly better than young SMCs (Fig. 6, $t(8) = 4.4$, $p = 0.002$) as we have shown earlier. However, addition of heparin to the cultures not only reduced SMC proliferation in a dose-dependent fashion ($F = 23.6$, $p < 0.001$) in all SMC cultures but also abolished the proliferative advantage of the old SMCs ($F = 0.089$, $p > 0.1$).

The effect of heparin on proliferation under nonstimulated or PDGF-stimulated conditions, two other areas in which old and young SMCs differ, corroborated the difference in sensitivity of old and young cells to growth inhibition.

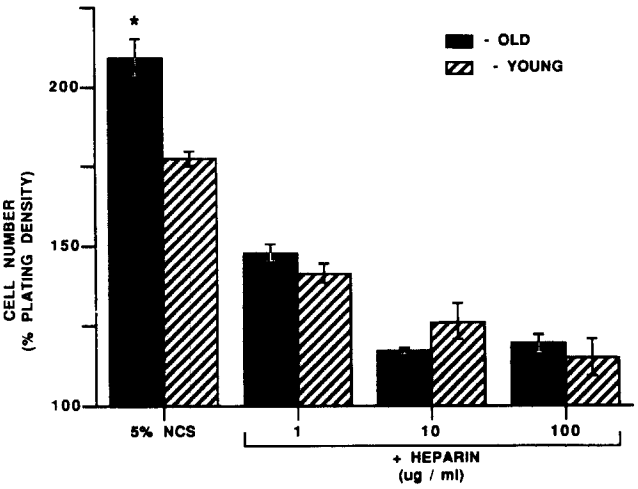

FIGURE 6. Effects of heparin on the serum-stimulated proliferation of old and young SMCs. Cell number over a 9-d period with refeeding every 3 d. 100% indicates no change from initial value. Data as mean ± SEM; representative experiment, $n = 5$; * indicates $p < 0.05$.

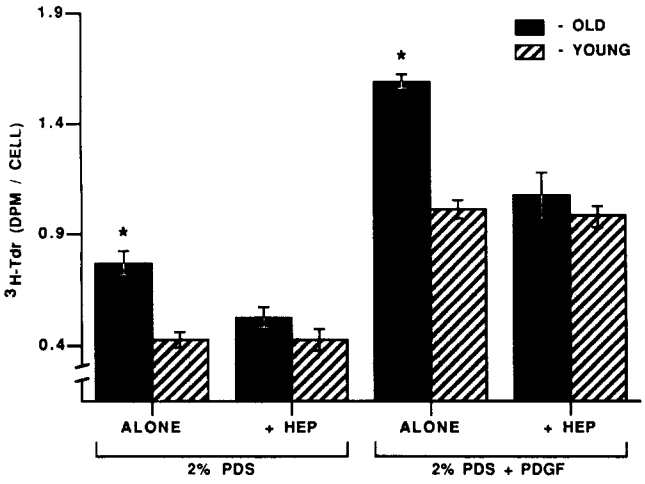

FIGURE 7. Effects of heparin on PDGF-stimulated and unstimulated incorporation of [^{3}H]TdR. Activity determined (dpm/cell) during a 4-h incubation, 18 h after exposure to heparin (10 μg/ml). Data as mean ± SEM; representative experiment, $n = 5$; * indicates $p < 0.05$.

Using a short-term [^{3}H]TdR incorporation assay, we observed that heparin (10 μg/ml) abolished both the significantly elevated baseline ($t = 4.9$, $p = 0.001$) and PDGF-stimulated [^{3}H]TdR incorporation by old SMCs ($t = 10.5$, $p < 0.001$) (Fig. 7). Thus, in the presence of heparin, proliferative responses of old and young SMCs become comparable.

Discussion

Invasion of the intima by medial smooth muscle cells and their proliferation within the arterial wall represent key pathological steps in arteriosclerosis. Aging appears to augment the development of sustained arteriosclerotic lesions in animal models and may be related to this process in humans. We have previously reported that the increased susceptibility of the aorta of old animals to arteriosclerosis is an intrinsic property of the vessel and not of the host (4). The studies reported here demonstrate the increased proliferation in tissue culture of aortic SMCs isolated from old rats as compared to those of young rats grown under

identical conditions. Thus, the exaggerated SMC proliferation observed after vascular injury of aged animals in vivo is paralleled by the in vitro growth pattern of SMCs derived from uninjured vessels. This finding is consistent with our hypothesis that the increasing risk of arteriosclerosis with age reflects a dysregulation of cell growth and differentiation during the aging process.

The augmented proliferative response of SMCs from old animals is characterized by three pertinent attributes. First, SMCs from old animals incorporate more [^{3}H]TdR into DNA under serum-free baseline conditions after growth arrest than do SMCs from young animals. Second, quiescent old SMCs respond more rapidly when exposed to progression factors and incorporate more [^{3}H]-TdR into DNA after exposure to serum than young SMCs, suggesting that some old SMCs "rest" in an early G_1 stage of the cell cycle, requiring only progression factors to enter mitosis. Third, old SMCs reach maximal levels of [^{3}H]TdR incorporation at lower concentrations of exogenous PDGF than young SMCs. Indeed, equivalent levels of incorporation could not be achieved in the young SMCs at testable concentrations of PDGF. These observations imply that more SMCs from old animals remain in cell cycle under conditions that inhibit proliferation of young SMCs, and therefore old SMCs respond more rapidly and more vigorously to introduction of progression factors. These conclusions are based not only on data from thymidine incorporation, which may be influenced by intracellular thymidine pool size or by polyploidy, but also on increased cell counts of cultures of old and young SMCs after exposure to serum or PDGF. Both flow cytometric analysis (Hariri, R. J., D. P. Hajjar, D. Coletti, D. R. Alonso, and E. Rabellino, submitted for publication) and autoradiography of continuously [^{3}H]TdR–labeled cells also reveal an increased number of old SMCs in S-phase before and soon after serum stimulation.

This evidence suggested to us that growth of aortic SMCs was altered in relation to age. We investigated whether more growth factor activity might be associated with old compared to young SMCs. Several studies of rat arterial SMCs have demonstrated the presence of PDGF-like proteins (7–9) or the expression of genes coding for the A-chain (10) and, in injured SMCs (11) the B-chain (c-sis) of a PDGF-like protein. We predicted that old SMCs may have higher levels of a cellular PDGF that rendered them partially independent of exogenous growth factors. This hypothesis was supported by the fact that lysates prepared from old SMCs stimulated the proliferation of target BASMCs more than did lysates from young SMCs and that the mitogenic activity was completely blocked by specific antibody to PDGF. Blockade of PDGF-like activity by specific antibody further exposed the inhibitory action of the cell-associated inhibitors and demonstrated that lysates of confluent old SMCs are less inhibitory than lysates of young SMCs.

Arterial SMCs grown in vitro are known to produce heparan sulfates that increase in growth-inhibiting activity as the cells reach confluence (12). Since these heparin-like molecules appear to be important in maintaining quiescence of confluent SMCs in vitro or in situ, we compared the effects of exogenous heparin on the growth of old and young SMCs. Proliferation of serum-stimulated old SMCs was particularly sensitive to inhibition by heparin. This inhibition of old SMC proliferation was also observed under serum-deprived conditions in

which old, but not young, SMCs grow. Together with our finding that old SMCs produce less growth-inhibiting activity (presumably heparins) than young SMCs and are more easily blocked from proliferation by exogenous heparin, these data suggest that a deficit in the amount and/or inhibitory efficacy of cellular heparins at confluence contributes to the enhanced proliferative activity of old aortic SMCs. Thus, it is possible that heparin might mitigate the excessive proliferative response of the aged vessel to injurious conditions in vivo. It is known that intimal hyperplasia in rats (12) and rabbits (13) after experimental vascular injury is inhibited by heparin. Characterization of the rate of production of growth-inhibiting heparin-like glycosaminoglycans by old and young vascular SMCs will help to determine if defects in production or in the processing of cellular heparins accounts for the age-related proliferative differences we have observed.

We conclude that age-related changes in aortic plaque formation after injury in vivo involve changes in the behavior of the vascular SMCs. The in vivo proliferative response parallels intrinsic, age-related differences in the proliferative behavior of isolated aortic SMCs in vitro. These studies emphasize that the mechanism underlying the differences in proliferation of old and young aortic SMCs involves at least two interacting processes: an increased cellular PDGF-related activity by old SMCs, and a concomitant decrease in cell-associated growth inhibitors.

Summary

In vivo studies have suggested that the aorta from an old animal responds to injury with an exaggerated proliferation of smooth muscle cells (SMCs) compared with the response of this aorta from a young animal. In this study we compared proliferation of SMCs derived from uninjured old (<19 mo) and young (3–4 mo) rat aortas. Old SMCs grew more rapidly than young SMCs in the presence of medium containing competence factors (10% FCS or platelet-derived growth factor [PDGF]) as well as in their absence (2% PDS or serum-free media) as determined both by a short-term thymidine incorporation assay and by cell counts. Lysates prepared from old SMCs that had been grown in the absence of serum or PDGF stimulated proliferation of target cells more than lysates prepared from young SMCs; the effect was inversely related to cell density of the SMCs. This stimulatory effect of lysates was completely blocked by antibody to PDGF. After the growth-promoting activity of lysates was eliminated by anti-PDGF, growth-inhibiting activity was revealed. Lysates prepared from old SMCs had significantly less capacity to inhibit target cell growth. In the presence of exogenous heparin both the serum- or PDGF-stimulated proliferation and serum-free proliferation of old SMCs were decreased to the level of proliferation of young SMCs. These results suggest that the balance between growth-promoting and growth-inhibiting factors is altered in SMCs from old rats. This may contribute to the increased proliferative capacity of these cells in culture and may facilitate the development of atherosclerosis with age.

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