Aging exacerbates negative remodeling and impairs endothelial regeneration after balloon injury

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Torella, Daniele, Dario Leosco, Ciro Indolfi, Antonio Curcio, Carmela Coppola, Georgina M. Ellison, Viviana G. Russo, Michele Torella, Giovanni Li Volti, Franco Rengo, and Massimo Chiariello. Aging exacerbates negative remodeling and impairs endothelial regeneration after balloon injury. Am J Physiol Heart Circ Physiol 287: H2850–H2860, 2004; doi:10.1152/ajpheart.01119.2003.—Many older patients, because of their high prevalence of coronary artery disease, are candidates for percutaneous coronary interventions (PCI), but the effects of vascular aging on restenosis after PCI are not yet well understood. Balloon injury to the right carotid artery was performed in adult and old rats. Vascular smooth muscle cell (VSMC) proliferation, apoptotic cell death, together with Akt induction, telomerase activity, p27kip1, and endothelial nitric oxide synthase (eNOS) expression was assessed in isolated arteries. Neointima hyperplasia and vascular remodeling along with endothelial cell regeneration were also measured after balloon injury. Arteries isolated from old rats exhibited a significant reduction in VSMC proliferation and an increase in apoptotic death after balloon injury when compared with adult rats. In the vascular wall of adult rats, balloon dilatation induced Akt phosphorylation, and this was barely present in old rats. In arteries from old rats, Akt-modulated cell cycle checkpoints like telomerase activity and p27kip1 expression were decreased and increased, respectively, compared with adults. After balloon injury, old rats showed a significant reduction of neointima formation and an increased vascular negative remodeling compared with adults. These results were coupled by a marked delay in endothelial regeneration in aged rats, partially mediated by a decreased eNOS expression and phosphorylation. Interestingly, chronic administration of L-arginine prevented negative remodeling and improved reendothelialization after balloon injury in aged animals. A decreased neointimal proliferation, an impaired endothelial regeneration, and an increase in vascular remodeling after balloon injury were observed in aged animals. The molecular mechanisms underlying these responses seem to be a reduced Akt and eNOS activity.

restenosis; Akt; cell cycle; nitric oxide

AGING IS THE MAJOR RISK FACTOR for the development of vascular diseases such as hypertension and atherosclerosis (25, 26). Because the prevalence of coronary artery disease is higher in older patients, many of these subjects are candidates for percutaneous coronary interventions (PCIs). To this regard, following acute myocardial infarction, PCI compared with thrombolytic therapy seems to be the revascularization strategy of choice in elderly patients (31). However, restenosis remains the major limitation of PCI (16, 20), although eluting stents have demonstrated striking results in its prevention (16, 20).

Earlier clinical studies have reported a low rate of procedure success and high rates of major complications in older patients undergoing balloon angioplasty (27). Risks to elderly patients undergoing PCI are two- to fourfold higher than those to younger patients, and these are strongly influenced by comorbidities (27). Data on the safety, efficacy, and clinical outcomes of coronary stenting in elderly are still limited, even if age-related risk for PCI has decreased as a result of the stent era (2, 6, 10).

There is considerable interest in understanding the mechanisms that underlie the vascular remodeling that occurs with advanced aging. Age-associated remodeling of the arterial walls in rodents includes mainly intima-media thickening of large vessels and endothelial dysfunction (25, 26). Previous studies specifically evaluating the effects of aging on VSMC proliferation after vascular injury have not always produced unequivocal and consistent data. On the basis of careful reports from several laboratories, it is generally considered that rat aortic vascular smooth muscle cells (VSMC) from aged animals have a greater proliferative capacity than those derived from younger animals (11, 25, 26, 34). However, it should be noted that the increased growth capacity of VSMC in older rats has not always been observed (29, 38). In contrast to data from rats, VSMC derived from humans clearly demonstrate an age-related decline in proliferative capacity, and the reduced ability of aged vascular cells to proliferate may also contribute to the pathogenesis of plaque vulnerability (32). On the other hand, endothelial dysfunction, including decreased endothelial cell (EC) proliferation and migration, has been repeatedly reported as a hallmark of vascular aging (1, 12, 25, 26).

Recent characterization of protein kinase B (PKB)-Akt has uncovered an essential role for this serine-threonine kinase in the control of VSMC and EC proliferation, migration, and survival (13, 33). Generally, cellular aging has been defined by a decline in growth rate and proliferative activity. It has also been associated with a reduced ability to activate Akt-dependent signaling, leading to an enhanced sensitivity and vulnerability of aged cells toward cell death (36).

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Therefore, the aims of the present study were to assess the effects of balloon injury on neointimal hyperplasia (regarded as in vivo VSMC proliferation) and vascular remodeling (involving cell apoptosis and endothelial regeneration) in aged rats. Subsequently, the role of Akt in the regulation of the different age-dependent responses to injury was also evaluated.

METHODS

Experimental balloon angioplasty. Adult Wistar rats (at 3 mo of age, 520 ± 50 g) and old Wistar rats (at 27 mo of age, 600 ± 30 g) were included in the present study and assigned to the following groups: 2 days (n = 5 adult and n = 5 old); 7 days (n = 5 adult and n = 5 old); and 14 days (n = 6 adult and n = 6 old) after balloon injury. Angioplasty of the common carotid artery was performed using the Clowes method as previously described and extensively validated in our laboratory (14–24). Finally, an additional group of rats (n = 5 adult and n = 5 old) was anesthetized and underwent only the surgical procedure without the balloon injury (sham adult and sham old).

Morphology. Fourteen days after the balloon injury, animals were anesthetized, the carotid arteries were fixed, and the anatomic measurements of vascular dimensions were carried out as previously described (14–24). To investigate the effect of balloon dilation on arterial remodeling, the ratio between the external elastic lamina described (14 – 24) was calculated and expressed as the arterial remodeling index (ARI) (19, 23, 24).

Immunohistochemistry. The amount of VSMC proliferation was quantified from carotid arteries excised at 2 days after injury, using an antibody against MIB1, specifically targeting the Ki-67 antigen (24). Furthermore, p27kip1 immunoreactivity was detected as previously described (21).

To measure the reendothelialized area 14 days after balloon-induced endothelial denudation, cross sections from the proximal, mid, and distal portions of the injured artery were stained with a polyclonal antibody against endothelial nitric oxide synthase (eNOS) or CD31 (Santa Cruz Biotechnology) (23).

The amount of reendothelialization was determined by measuring the volume percentage of positive eNOS (or CD31) staining at the inner surface of the entire luminal circumference in each section (from proximal, mid, and distal segments of the injured arteries), and the mean value of all fields for each animal was calculated (23).

Immuno blottings. Isolated carotid arteries from animals euthanized at 2 and 7 days after balloon injury were pulverized and proteins extracted as previously described (23). The following antibodies were used for Western blot analysis: mouse monoclonal Akt (Upstate Bio; Purchase, NY), rabbit polyclonal phospho-Akt (Ser473) (Cell Signaling Technology; Beverly, MA), rabbit polyclonal p27kip1, goat polyclonal CD31 (Santa Cruz Bio; Santa Cruz, CA), mouse monoclonal eNOS (BD Transduction Laboratories), and rabbit polyclonal phospho-eNOS (BD Ser1177) (Cell Signaling Technology). Specific proteins were detected as previously described (23). To compare specific protein expression with the level of a stable protein, we analyzed the expression of tubulin or actin, running a parallel gel with identical samples, and using a mouse monoclonal anti-tubulin antibody (Santa Cruz Bio) or an anti-actin antibody (Sigma, St. Louis, MO).

Telomeric repeat amplification protocol. The telomeric repeat amplification protocol assay (Intergen) for quantification of telomerase activity in carotid arteries isolated 2 days after balloon injury was performed as previously described (36, 37).

Statistical analysis. All data are shown as means ± SE. Statistical analysis between groups was performed by ANOVA using a SPSS 10.0 program. When a significant overall effect was detected, Tukey’s test was applied to compare single mean values. A P value < 0.05 was considered significant.

RESULTS

Aging decreases VSMC proliferation after balloon angioplasty. Proliferating cell nuclear antigen (PCNA), thymidine, and bromodeoxyuridine (BrdU) have been extensively used for the detection of cycling VSMC (7, 17, 18, 21–23). However, PCNA cannot discriminate between cells traversing the cell cycle or undergoing DNA repair (3–38). Thymidine and BrdU incorporation during the S phase cannot address whether DNA synthesis is associated with repair processes, ploidy formation, or cytokinesis (3–38). These factors could lead to a deceptive estimation of true dividing cells. Expression of Ki-67 is a requirement for cells to traverse the cell cycle and undergo cell division (3, 24, 37). It is a nuclear antigen expressed in all phases of the cell cycle except Go (3, 24, 37). Therefore, Ki-67 is preferable to thymidine, BrdU, and PCNA for labeling proliferating cells. We assessed VSMC proliferation by immunostaining for Ki-67 at 2 days after vascular injury (22). In the carotid arteries isolated from old rats, a strikingly lower ratio of Ki-67-positive nuclei to total cells (proliferation index, PI = 6 ± 2%) was observed compared with carotid vessels from adult animals (PI = 52 ± 10%; P < 0.001 vs. old rats) (Fig. 1, A and B). This is indicative of a decreased VSMC proliferation in arteries from old animals after balloon injury.

Fig. 1. A: representative photomicrographs of carotid arteries isolated from adult and old rats fixed 2 days after balloon injury and stained with an anti Ki-67 monoclonal antibody. B: bar graphs showing the cumulative percentage of Ki-67-positive vascular smooth muscle cells (VSMCs) in the injured carotid arteries. *P < 0.01 vs. adult. Results are means ± SD of 5 independent measurements.
Aging suppresses Akt activation after balloon injury. The serine-threonine kinase Akt, or PKB, lies at the cross roads of multiple cellular signaling pathways and is particularly important in mediating cell survival, opposing apoptotic cell death; particularly, it is a critical factor in the proliferation and migration of VSMCs and the formation of neointimal tissue after balloon injury (13, 33). Thus, to elucidate the role of Akt in the mechanisms of vascular remodeling, protein extracts of arteries isolated 2 days after balloon injury from both adult and old rats were analyzed by Western blot analysis for Akt protein expression and its phosphorylation status. Neither aging by itself nor vascular injury changed Akt total protein levels. This was evident through there being no difference in Akt protein expression between isolated left noninjured and right balloon-injured arteries from both adult and old rats (Fig. 2A). Nevertheless, balloon injury caused a significant activation of Akt through its serine-phosphorylation in the carotid arteries from adult animals (Fig. 2, A and B). On the contrary, in the arteries isolated from old animals, balloon injury failed to activate Akt as demonstrated by weak Akt-phosphorylation (Fig. 2, A and B).

Effects of aging on Akt-modulated cell cycle check points after balloon angioplasty. Upon stimulation by arterial injury, the cell cycle machinery controls VSMC proliferation and migration (5, 20, 30). Cell cycle progression is regulated by the activity of cyclin/cyclin-dependent kinase complexes and their endogenous inhibitors termed the CDK inhibitors (5, 20, 30, 35). Between them, p27<kappa>1 is an important regulator of the VSMC cycle (5, 20, 30, 35). It is interesting to note that Akt has been demonstrated to modulate p27<kappa>1 expression by phosphorylating it and increasing its cytoplasmatic retention and degradation, and, consequently, this facilitates VSMC progression into the cell cycle (39). Therefore, protein extracts from the arteries isolated 2 days after balloon injury from both adult and aged rats were analyzed by Western blot analysis for p27<kappa>1 expression. Left noninjured arteries from old rats contained a slightly increased amount of p27<kappa>1 compared with adults (Fig. 3, A and B). As expected, balloon injury produced a significant decrease in p27<kappa>1 protein levels in the vascular wall of adult animals, reflecting the high rate of VSMC proliferation and migration in these arteries (Fig. 3, A and B). Even if stimulated by balloon injury, carotid arteries from old rats failed to decrease their p27<kappa>1 protein expression, consistent with a failure to reenter the cell cycle (Fig. 3, A and B). Furthermore, the localization of p27<kappa>1 in the vascular wall was further analyzed by immunohistochemistry (Fig. 3). A significantly higher fraction of VSMCs (47 ± 5% positive cells) expressing p27<kappa>1 2 days after balloon injury was detected in the arteries isolated from old rats compared with adult rats (10 ± 7% positive cells; P < 0.001 vs. aged animals) (Fig. 3, C and D).

Furthermore, Akt is a key modulator of telomerase by phosphorylating a serine residue of telomerase and, through this, increasing its activity (36). Telomerase is a cellular reverse transcriptase that adds TTAGGG repeats onto preexisting telomeres, opposing telomere erosion and dysfunction during the accumulation of cell doublings, preventing cellular senescence (4). Conversely, telomerase-competent cells show telomerase activity only when they have entered the cell cycle (4).

Therefore, telomerase activity was measured and compared in isolated carotid arteries from adult and old rats. Formation of the typical 6-bp ladder was barely detectable in the noninjured arteries from adult and old animals, reflecting the presence of quiescent cells (Fig. 4, A and B). Compared with noninjured arteries, balloon injury to injured arteries in adult animals resulted in a 10-fold increase in telomerase activity (Fig. 4, A and B). Interestingly, injured arteries isolated from old rats after balloon injury showed a slight increase in telomerase activity compared with the uninjured, which proved to be significantly lower compared with injured arteries isolated from adult animals (Fig. 4, A and B).

Aging increases vascular apoptosis after balloon angioplasty. To investigate further the observed differences in Akt activation, we tested whether suppression of vascular Akt signaling in aging would produce different levels of apoptosis in the vascular walls of old compared with adult animals after balloon injury. Thus we assessed caspase 3 activation by detecting its active cleavage product in isolated arteries 2 days after balloon injury. Moreover, we determined the activity of caspase-3 by assessing cleavage of poly(ADP-ribose) polymerase (PARP) into its 89-kDa form, which is considered a

Fig. 2. A: representative Western blot analysis showing Akt phosphorylation and its total protein levels in not-injured and balloon-injured arteries isolated from adult and old rats 2 days after balloon angioplasty. B: arbitrary optical density (OD) units of Akt phosphorylation. *P < 0.01 vs. all. Not-injured, contralateral noninjured left carotid arteries isolated from adult and old animals. Balloon-injured, right balloon-injured carotid arteries isolated from adult and old animals. Results are means ± SD of 5 independent measurements. **P < 0.01 vs. adult.
hallmark of apoptosis (7). Immunoblot analysis of protein extracts from injured carotid arteries of adult rats showed a small cleavage of caspase-3 (Fig. 5, A and B). In contrast, balloon injury produced a remarkable caspase 3 cleavage in the carotid arteries of old animals (Fig. 5, A and B). Consistent with these findings, PARP cleavage was significantly increased in the injured arteries from old compared with adult rats (Fig. 5, A and C). Furthermore, a significant increase in DNA fragmentation was observed in the injured arteries from old rats, which was basically absent in arteries from adult rats 2 days after balloon injury. *P < 0.01 vs. adult. Results are means ± SD of 5 independent measurements.

Age-dependent effects on vascular structure after balloon angioplasty. The above experiments showed a decreased cell proliferation and increased cell death 2 days after balloon angioplasty in the walls of injured arteries from old compared with adult rats. Therefore, we evaluated whether this would result in a different response of the wall, in terms of neointimal hyperplasia and negative remodeling, following balloon injury. As expected, balloon injury produced a significant neointima formation in carotid arteries from adult rats (Table 1, Fig. 6, A and C). In contrast, old rats displayed a significantly reduced neointima after balloon injury compared with adult animals (Table 1, Fig. 6, A and C). Digital planimetry revealed vascular negative remodeling in the adult group compared with sham animals 14 days after balloon angioplasty (Table 1, Fig. 6, B and C). Interestingly, aging was associated with a significant worsening of negative remodeling (Table 1, Fig. 6, B and C). The final luminal area was not statistically different between adult and old rats (Table 1), reflecting a similar late lumen loss in the two groups.

**Aging delays reendothelialization after balloon injury.** Fourteen days after balloon angioplasty, eNOS labeling of the vascular wall showed that aging resulted in a decreased reendothelialization in all the analyzed injured arterial segments (proximal, middle, and distal) compared with the arteries isolated from adult animals (Fig. 7A). Indeed, in the old group, the new endothelium on the neointima layer circumference was severely reduced proximally and distally of the balloon-injured segment (respectively, 45.5 ± 3.4% and 40.5 ± 4.7%) compared with the same segments in the adult group (proximally, 79.6 ± 7.4; P < 0.01 vs. old group; distally, 85.5 ± 5.2; P < 0.01 vs. old group) (Fig. 7A). Furthermore, the middle portion of the injured segment in the adult animals, which is the last part to be recovered by new ECs after balloon-induced endothelium denudation, showed an incomplete reendothelialized circumference (26.5 ± 4.3%) that was almost absent in the same carotid injured segment of old rats (5.5 ± 5.1%; P < 0.01 vs. adult group) (Fig. 7A). Similar results were obtained with CD31 staining (data not shown).

**Fig. 3.** A: representative Western blot analysis showing p27kip1 protein levels in the not-injured and injured arteries isolated from adult and old rats 2 days after balloon injury. B: arbitrary OD units of p27kip1 protein expression. *P < 0.01 vs. adult; **P < 0.01 vs. all. Results are means ± SD of 5 independent measurements. C: representative immunohistochemical photomicrographs showing the levels of p27kip1 in the injured arteries from adult (left) and old (right) rats 2 days after balloon injury. D: bar graphs showing the cumulative percentage of p27kip1-positive VSMCs in the injured carotid arteries isolated from adult and old animals 2 days after balloon injury. *P < 0.01 vs. adult. Results are means ± SD of 5 independent measurements.
Aging leads to a reduction of eNOS protein and its phosphorylation. We next investigated the possible mechanisms by which aging leads to a decreased endothelial regeneration after balloon injury. Endothelial NO, produced by the activity of eNOS, promotes EC growth, and eNOS upregulation in the vascular wall speeds up reendothelialization after vascular injury (23). Akt modulates eNOS function, increasing its activity by its phosphorylation on Ser1177 and promoting EC proliferation and survival (8). Moreover, previous studies have demonstrated that eNOS expression and endothelial NO generation are reduced in ECs with increasing age (12, 25, 26). Therefore, we evaluated the expression and the phosphorylation of eNOS by Western blot analysis. It was found that eNOS protein levels were reduced in left noninjured carotid arteries isolated from old rats compared with adult rats (Fig. 7, B and C). To ensure that the reduction of eNOS was not due to the unspecific downregulation of proteins or to a reduced EC number, we determined the expression of a specific endothelial protein CD31 (Fig. 7, B and E). No difference was detected between CD31 expression in noninjured arteries from old and adult rats, confirming the finding of an actual decrease in eNOS amount in the vascular wall of old animals compared with adults (Fig. 7, B and E). Subsequently, phospho-eNOS was absent in the noninjured arteries from adult as well as old rats (Fig. 7, B and D).

We then evaluated the eNOS expression in arteries isolated from adult and old rats 7 days after balloon injury (Fig. 7, B and C). Compared with the contralateral noninjured arteries, balloon-injured arteries from adult animals showed a decreased eNOS protein expression, reflecting the endothelium-denuding effect of the balloon injury (Fig. 7, B and C). This was confirmed by a reduction of CD31 expression, parallel with eNOS expression in the balloon-injured arteries of adult animals (Fig. 7, B and E). Interestingly, a significant eNOS phosphorylation was observed in the injured vessels from adult rats (Fig. 7, B and D). On the contrary, in old animals 7 days after balloon angioplasty, total eNOS was basically absent, along with a detectable CD31 expression, which was still significantly lower compared with CD31 expression at this time point in the adult animals (Fig. 7, B, C, and E). Phospho-eNOS in injured arteries of old rats was not detectable (Fig. 7, B and D). This would suggest a decreased endothelial regeneration, secondary to a reduced eNOS production (also confirmed by the decreased eNOS-to-CD31 ratio in the old animals after balloon injury, Fig. 7F) and its phosphorylation in old animals.

L-Arg supplementation prevents negative remodeling after balloon injury in aging. The altered pathophysiology (i.e., increased vascular death and reduced reendothelialization) observed here in old rats after balloon injury could be the consequence of the decreased eNOS vascular expression with aging. Therefore, to test this hypothesis, L-arginine (L-Arg) was administered to a group of old animals (n = 7) undergoing balloon injury. Briefly, starting 7 days before balloon injury, the old animals were assigned to treatment with daily doses of L-Arg (500 mg·kg⁻¹·days⁻¹·ip) for 21 days (old + L-Arg group). In an additional set of experiments, 7 days of L-Arg treatment did not change the vascular dimensions of carotid arteries isolated from old animals, therefore excluding the possibility that a different vessel size at the moment of injury could bias the results at 14 days after the balloon angioplasty (data not shown). Interestingly, when compared with the old group, chronic L-Arg supplementation prevented negative remodeling (Table 1 and Fig. 8) and increased reendothelialization (proximally from 45.5 ± 3.4 to 88.3 ± 5.6%, P < 0.01; distally from 40.5 ± 4.7 to 82.5 ± 6.5%, P < 0.01; and in the midsegment from 5.5 ± 5.1 to 35.5 ± 6.1% P < 0.01) determining an increased lumen area 14 days after balloon angioplasty (Table 1 and Fig. 8). This result supports the concept that eNOS-NO modulation plays a pivotal role in the pathophysiology of vascular repair after vessel injury in the aged.

DISCUSSION

The major findings to emanate from the present study are that: 1) aging affects vascular structure after balloon angioplasty resulting in a reduced neointimal tissue growth but a worsening of negative remodeling compared with adults; 2) in aged animals, a reduced reendothelialization was consistently observed after vascular injury; and 3) Akt and eNOS activation and expression are reduced in aged rats after balloon injury, resulting in an increased apoptotic cell death, suggesting that aging is associated with a decreased capacity of vascular cells to survive, reenter the cell cycle, and proliferate in response to balloon injury.

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Restenosis has been traditionally considered the “Achilles’ heel” for balloon angioplasty (16, 20). The only accepted way to reduce restenosis so far has been stent deployment (20). However, VSMC proliferation after stent deployment is still a clinical challenge, even though recent data reported the promising effects of eluting stents in preventing the rate of restenosis (20).

Elderly patients have been considered for a long time a high-risk population for PCIs with a higher incidence of peri-procedural failure and complications compared with younger patients (27). Whether aging puts patients at a higher risk by increasing VSMC proliferation, exacerbating restenosis after balloon angioplasty, or particularly after stent deployment has never been established.

To this regard, it has recently been reported that elderly patients have greater absolute risk reductions associated with contemporary percutaneous revascularization than do younger patients, suggesting that the benefits of aggressive revascularization therapies may extend to a subset of patients in older age groups (2, 6, 10). However, few data are available on the possible role played by age in the mechanisms responsible for the restenosis phenomenon.

Previous in vitro and in vivo studies aimed at determining the effects of aging on VSMC proliferation have so far been a matter of debate (11, 25, 26, 29, 32, 34, 38).

In the past, many investigators (5, 14 –16, 30) have focused their attention on the intracellular molecules that regulate VSMC proliferation, migration, and survival after vascular injury.

These processes are regulated in turn by several intermediate signaling proteins like Akt/PKB (13, 33). Notably, reduced Akt signaling is actually apparent only in carotid arteries isolated 2 days after balloon injury from old rats. This pattern of DNA damage was basically undetectable in arteries isolated from adult animals, *P < 0.01 vs. adult. Results are means ± SD of 5 independent measurements.

### Table 1. Cumulative morphological results in the groups included in the study

<table>
<thead>
<tr>
<th></th>
<th>SO Adult</th>
<th>SO Old</th>
<th>Adult</th>
<th>Old</th>
<th>Old + L-Arg</th>
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<tr>
<td>Neointimal CSA, mm²</td>
<td>0.00 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>0.215 ± 0.021*</td>
<td>0.053 ± 0.020†</td>
<td>0.054 ± 0.020†</td>
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<td>N/M ratio</td>
<td>0.00 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>1.042 ± 0.122*</td>
<td>0.251 ± 0.048†</td>
<td>0.217 ± 0.086†</td>
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<tr>
<td>Lumen CSA, mm²</td>
<td>0.360 ± 0.028</td>
<td>0.370 ± 0.025</td>
<td>0.125 ± 0.012‡</td>
<td>0.139 ± 0.044‡</td>
<td>0.259 ± 0.028*</td>
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<td>EELc_left, mm</td>
<td>2.892 ± 0.052</td>
<td>2.890 ± 0.059</td>
<td>2.887 ± 0.058</td>
<td>2.890 ± 0.068</td>
<td>2.885 ± 0.049</td>
</tr>
<tr>
<td>EELc_right, mm</td>
<td>2.889 ± 0.050</td>
<td>2.885 ± 0.061</td>
<td>2.785 ± 0.053*</td>
<td>2.459 ± 0.104*</td>
<td>2.863 ± 0.058</td>
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<tr>
<td>ARI</td>
<td>0.999 ± 0.001</td>
<td>0.998 ± 0.010</td>
<td>0.965 ± 0.007*</td>
<td>0.851 ± 0.022*</td>
<td>0.992 ± 0.026</td>
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Values are means ± SE. SO Adult, sham-operated adult animals; SO Old, sham-operated old animals; Adult, adult animals subjected to carotid balloon injury; Old, old animals subjected to carotid balloon injury; Old + L-Arg, old animals subjected to balloon injury + l-arginine administration; Neointim. CSA, neointimal cross-sectional area; N/M ratio, neointima-to-media ratio; Lumen CSA, lumen cross-sectional area; EELc_right, external elastica lamina circumference of right injured carotid artery; EELc_left, external elastica lamina circumference of left controlateral not-injured artery; ARI, arterial remodeling index = EELc_right/EELc_left. *P < 0.01 vs. All; †P < 0.01 vs. SO Adult, SO Old, and adult; ‡P < 0.01 vs. SO Adult and SO old.
activation in VSMCs leads to diminished VSMC proliferation and a consequent reduced neointima formation after vascular injury (13). It should be noted that in the present study we observed an impaired Akt activation in old rats after balloon injury. This was associated with a decreased VSMC proliferation as demonstrated by the reduced Ki-67-positive cells in the injured vascular wall of old animals and also by the significant reduction of neointima formation. These results strongly suggest a decreased cell proliferative response to balloon injury with aging.

Recent studies have demonstrated that telomerase activity correlates with VSMC cell proliferation and survival (28). Indeed, inhibition of telomerase diminished growth of VSMCs, which suggests a crucial role for telomerase activation in the regulation of VSMC proliferation (28). Here, we have shown that telomerase activity was significantly lower in the arteries of old rats after balloon injury compared with the injured arteries isolated from adult animals. This finding further supports the concept of a decreased cell proliferative response to balloon angioplasty with aging.

To this regard, a recent study elegantly demonstrated that aortic VSMCs from aged mice exhibited an impaired proliferative capacity in vitro (29). This behavior of VSMCs from aged mice was related to the dysregulation of cell cycle-regulating protein p27Kip1. In fact, levels of the cyclin-dependent kinase inhibitor p27Kip1 failed to decrease in response to mitogenic stimuli in aged VSMCs (29). In the present in vivo study, we consistently observed a negligible neointimal tissue growth after balloon angioplasty in aged rats that was associated with an absence of p27Kip1 reduction, which was the common consequence of balloon injury in adult animals (21).

Akt has been demonstrated to modulate both the phosphorylation of p27Kip1, increasing its cytoplasmatic retention and degradation (39), and of telomerase, increasing its activity by a posttranslational modification of the telomerase catalytic subunit (4, 36). Even if in the present study we did not directly address these possibilities, it is likely that the absence of Akt activation observed in the vascular wall of old animals after balloon injury could have led to the increased p27Kip1 expression and to the reduced telomerase activity with aging.

Recently, human studies have shown that arterial circumference can change in response to arterial intervention (so called “negative” remodeling), and this can explain the late lumen loss after balloon angioplasty (40). It is worth noting that in the present study, late lumen loss after balloon angioplasty in aged rats is mainly due to neointimal hyperplasia and in part to negative remodeling whether in the aged rats late lumen loss is almost entirely due to inward remodeling. Sham Adu, sham-operated adult group; Sham Old, sham-operated old group; Adu, adult group; Old, old group.

Fig. 6. A: bars representing neointima area and neointima-to-media ratio of common carotid arteries 14 days after balloon angioplasty. *P < 0.01 vs. adults. B: bars representing arterial remodeling index of common carotid arteries after balloon angioplasty from each group of animals studied. ARI, ratio between external elastic lamina (EEL) circumference of right injured artery (EELC_{right}) and EEL of left noninjured artery (EELC_{left}) *P < 0.01 vs. all; **P < 0.01 vs. old. C: representative histological sections stained with van Gieson’s solution of common carotid arteries from adult and old rats before (top, left and right, respectively) and after balloon angioplasty (bottom, left and right, respectively). It is evident that late lumen loss after balloon angioplasty in adult rats is mainly due to neointimal hyperplasia and in part to negative remodeling whether in the aged rats late lumen loss is almost entirely due to inward remodeling. Sham Adu, sham-operated adult group; Sham Old, sham-operated old group; Adu, adult group; Old, old group.
Endothelial NO synthesis inhibits apoptosis, and aging is associated with a decreased NO synthesis and concomitantly with an increased sensitivity of apoptosis (1, 12, 25, 26). NO, synthesized by eNOS, seems to be involved in the regulation of vascular remodeling after balloon injury (23). Specifically, vascular remodeling after arterial injury is altered in eNOS-deficient mice (9). Previous data from other groups has clearly indicated that eNOS expression and activity decreases with aging (25, 26). Akt phosphorylates eNOS, increasing EC migration and proliferation in vitro and in vivo (8). In the present study, we observed a reduction of basal eNOS vascular expression in aged animals. Our data also indicate that aging nearly abolishes eNOS vascular expression and its phosphorylation after balloon injury. The latter could partially explain the reduced reendothelialization after balloon angioplasty observed in the aged rats compared with adult animals. On the other hand, the reduced vascular eNOS expression could have even contributed to the increased cell apoptosis after balloon angioplasty.
angioplasty in aged animals. Interestingly, L-Arg supplementation reversed the effects of aging on vascular remodeling after balloon injury. This outlook gives rise to a plausible speculation that the reduced Akt activation was responsible for the reduced eNOS activity, yielding reduced endothelial regeneration after balloon injury in aged animals. However, further studies are needed to verify this hypothesis.

**Limitations and clinical implication of the study.** In the present study, we reported a decrease in neointimal formation after balloon injury in aged rats compared with adult rats. This is in contrast with previous results demonstrating that VSMCs within balloon-injured aortas of aged rats exhibited higher proliferation rate than that of younger adult rat aortas (11, 34). In these studies, the authors reported that aging produces a change in the vascular VSMC that enhances proliferation (11, 34). In fact, they showed an increased [3H]thymidine incorporation in injured aortas isolated from old compared with young rats (34). However, [3H]thymidine incorporation has the limitation of not to distinguish between cell proliferation or DNA repair (3). Furthermore, VSMC proliferation was addressed as [3H]thymidine incorporation per milligram of DNA isolated from the total arterial tissue, which cannot distinguish among cell types as well as cell to cell (34). In fact, the increased [3H]thymidine incorporation could be the result of either increased DNA repair in the arteries from old animals or due to proliferation of cells not involved in the intima formation. Moreover, the morphological analysis of neointima formation involved the count of intimal cell nuclei per cross section, which by itself does not really give reliable information on the vascular dimensions, i.e., neointima area and lumen area. Furthermore, the method of aortic balloon injury used in the two studies is different from the carotid balloon injury method used in our study (14–24), as well as the different rat strain could have contributed in some extent to the different observations. It is interesting to note that in the study by Hariri and coworkers (11) the method of vascular injury did not include arterial dilation. Therefore, it is hardly comparable to the actual angioplasty procedure in humans. Additionally, the studies showing a growth advantage of VSMCs isolated from aged rats were performed using VSMCs from aortas in vitro, whereas in our study, we assessed VSMC proliferation in in vivo injured carotid arteries, establishing a completely different experimental setting. It is important to remark that our study has the major aim to evaluate the aging effect on restenosis, whereas

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Fig. 8. A: bars representing neointima-to-media ratio and lumen cross-sectional area (CSA) of common carotid arteries isolated 14 days after balloon angioplasty from old and old + L-arginine (L-Arg) (old animals treated with L-Arg) groups. *P < 0.01 vs. old. B: bars representing arterial remodeling index (ARI) of common carotid arteries after balloon angioplasty from sham-operated old animals, old and old + L-Arg groups. *P < 0.01 vs. all. C: bars representing the percentage of reendothelialized circumference of common carotid arteries 14 days after balloon angioplasty. *P < 0.01 vs. all except for old + L-Arg distal; #P < 0.01 vs. all; $P < 0.01 vs. all except for old + L-Arg proximal. D: representative histological sections stained with hematoxolin and eosin of common carotid arteries from old rats treated (old + L-Arg) and not-treated (old) with L-Arg 14 days after balloon angioplasty.


11. Hariri RJ, Alonso DR, Hajjar DP, Coletti D, and Weksler ME. Therefore, aggressive medical therapy for old patients after stent deployment aimed at reducing thrombotic events and increasing endothelial survival and proliferation more than dealing with VSMC proliferation should become a necessity. Nevertheless, it is really hard and highly speculative to merge experimental findings into a clinical scenario, and therefore, further appropriate clinical study should provide additional answers to this problem.


20. Indolfi C, Mongiardol A, Cucito A, and Torella D. Molecular mecha-


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the two mentioned studies were mainly focusing on atherosclerosis (11, 34). To this regard, the reduced ability of aged vascular cells to proliferate may also contribute to the pathogenesis of plaque vulnerability and atherosclerosis (32). These different aspects of the observations may have caused apparent contradictory results.

One of the current clinical problems for interventional cardiologists is the right decision on the treatment of elderly patients with coronary artery disease. For quite a long time, the elderly have been considered a procedural risk factor for PCI (27). However, the stent era seems to have improved the outcome of PCI in aged patients (2, 6, 10). This preliminary finding could correlate with a decreased VSMC proliferation after stent deployment (which is actually the main factor determining restenosis after stenting) in elderly patients. Our results are comparable with these findings because we have demonstrated a decreased VSMC proliferation in aged animals. This is of particular interest, considering the rapid spreading of the two mentioned studies were mainly focusing on atherosclerosis (11, 34). To this regard, the reduced ability of aged vascular cells to proliferate may also contribute to the pathogenesis of plaque vulnerability and atherosclerosis (32). These different aspects of the observations may have caused apparent contradictory results.

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