Stress-induced senescence exaggerates postinjury neointimal formation in the old vasculature

Sheik J. Khan,² Si Pham,¹ Yunteo Wei,¹ Dania Mateo,¹ Melissa St-Pierre,¹ Terance M. Fletcher,² and Roberto I. Vazquez-Padron¹

¹Department of Surgery and Vascular Biology Institute and ²Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, Miami, Florida

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Khan SJ, Pham S, Wei Y, Mateo D, St-Pierre M, Fletcher TM, Vazquez-Padron RI. Stress-induced senescence exaggerates postinjury neointimal formation in the old vasculature. Am J Physiol Heart Circ Physiol 298: H66–H74, 2010. First published October 23, 2009; doi:10.1152/ajpheart.00501.2009.—This study aims to demonstrate the role of stress-induced senescence in aged-neointimal formation. We demonstrated that aging increases senescence-associated β-galactosidase activity (SA-β-Gal) after vascular injury and the subsequent neointimal formation (neointima-to-media ratio: 0.8 ± 0.2 vs. 0.54 ± 0.15) in rats. We found that senescent cells (SA-β-Gal+ p21+) were scattered throughout the media and adventitia of the vascular wall at day 7 after injury and reached their maximum number at day 14. However, senescent cells only persisted in the injured arteries of aged animals until day 30. No senescent cells were observed in the noninjured, contralateral artery. Interestingly, vascular senescent cells accumulated genomic 8-oxo-7,8-dihydrodideoxyguanine, indicating that these cells were under intense oxidative stress. To demonstrate whether senescence worsens intimal hyperplasia after injury, we seeded matrigel-embedded senescent and nonsenescent vascular smooth muscle cells around injured vessels. The neointima was thicker in arteries treated with senescent cells with respect to those that received normal cells (neointima-to-media ratio: 0.41 ± 0.105 vs. 0.26 ± 0.04). In conclusion, these results demonstrate that vascular senescence is not only a consequence of postinjury oxidative stress but is also a worsening factor for neointimal development in the aging vasculature.

vacular injury; aging; neointima; vascular smooth muscle cells

THE MECHANISM BY WHICH AGING exaggerates neointima formation after vascular injury has, so far, remained elusive (40, 42). It is known that aging alters vascular physiology (3) and arterial response to oxidative stress and injury (6, 35). One of the physiological changes that aging promotes, senescence, has recently been found to be involved in the development of cardiovascular diseases (14, 29–31). During senescence, cells undergo changes in morphology, physiology, and gene expression and permanently cease to proliferate but remain metabolically active for a long period of time (17, 19, 22, 41). A variety of physiological stimuli can provoke a cell to enter senescence. A few examples include telomere shortening (8, 39), oxidative stress (9, 12, 13), and activation of oncogenes (2, 32, 33).

Experimental evidences suggest the contribution of senescent cells to the development of vascular proliferative diseases. Human atherosclerotic plaques showed abundance of SA-β-Gal+ endothelial cells (EC) and vascular smooth muscle cells (VSMC) that exhibit morphological features of senescence (29, 32). On the other hand, Fenton et al. (15) have documented the presence of senescent cells in rabbit neointimas. More recently several authors have found that senescent VSMC (32), EC (26), and fibroblasts (7) produce de novo growth factors that may promote nearby cell growth and inflammation. Despite of all of these evidences, the role of senescence in neointimal formation has remained poorly understood.

Herein, we investigate the role of senescence in postinjury neointimal formation using the rat model of arterial balloon injury. We have found that aging exacerbates oxidative stress, vascular senescence, and neointimal thickness after injury. We have demonstrated that application of exogenous senescent VSMC around an injured artery increases neointimal thickness. These data are the first to suggest that oxidative stress-induced senescence of VSMC contributes to the age-related neointimal development in response to vascular injury.

MATERIALS AND METHODS

Animals. All animal procedures were previously approved by the Institutional Committee for Use and Care of Laboratory Animals at the University of Miami. Only male rats were included in the study. Aged Fischer 344 (>22 mo-old) rats were purchased from the National Institute of Aging (Bethesda, MD) from a colony at Harlan Laboratories (Indianapolis, IN). The young Fischer rats (2 mo-old) were directly obtained from Harlan Laboratories. The relative weight of young and aged Fisher rats was of 243 ± 5.2 g and 410 ± 7.9 g, respectively.

Transgenic inbred Lewis rats that express green fluorescent protein (GFP) under the control of ubiquitin-C promoter (24) were obtained from the Rat Resource and Research Center (Columbia, MO) and bred in our laboratories.

Rat balloon-injury model. All operative procedures were under isoflurane anesthesia (Baxter, IL). Balloon injury in the right iliac artery was inflicted with a 2F Fogarty catheter (Baxter, Irvine, CA) adapted to a custom angiographic kit (Boston Scientific, Scimed) (16). The balloon catheter was always inflated to yield a constant pressure between 1.5–1.6 atmospheres. Arterial specimens were collected 7 to 30 days after injury and fixed in 4% formalin-PBS (Sigma-Aldrich, St. Louis, MO) for 5 min. Specimens were rinsed with cold PBS before SA-β-Gal staining.

Senescence-associated β-galactosidase staining. Arteries were submerged in senescence-associated β-galactosidase (SA-β-Gal) staining solution for 24 h at 37°C (10). This solution was made of 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma-Aldrich), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl2, 2 mM MgCl2, and 40 mM trisodium citrate. The staining solution was titrated with 1 M NaH2PO4 to pH 6.0. After staining, vessels were rinsed with ice-cold PBS and immersed in 4% formalin-PBS for 24 h. Whole artery pictures were taken with a Leica M50 stereomicroscope (Bannockburn, IL). The activity of the senescence marker was scored as the percentage of dark blue areas. Stained
arteries were cut transversely into three equal portions, and paraffin was embedded on the same block. Tissue embedding and sectioning were performed by American Histolabs (Bethesda, MD).

Histological localization of senescent cells (SA-β-Gal+) was performed either under brightfield or fluorescence confocal microscope. Sections were deparaffinized and rehydrated by serially immersing them in xylene, alcohol, and water. For brightfield microscopy, nuclei were stained with Meyer’s hematoxylin (Sigma-Aldrich), and sections were mounted in Entellan mounting medium (Gibbstown, NJ). For fluorescence microscopy, sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The fluorescence microscope was a Leica SP5 confocal DMi6000 inverted microscope with a 633-nm laser (Leica Microsystems, Banockburn, IL).

**Morphometric analysis and neointimal cell number determination.** The area of each vascular layer and lumen was measured on Elastic Van Gieson (EVG)-stained slides to calculate the neointima-to-media ratio \[N/M = N/(M+N)\]. The total number of cells in each vascular layer was counted on hematoxylin and eosin-stained slides. Morphometric measurements and cell counting were performed on digital images using Image Pro Plus (Media Cybernetics, Bethesda, MD). All measurements were performed by an experienced observer blinded to the experiment.

**Immunohistochemistry and immunofluorescence microscopy.** Cross sections were taken from different levels of the paraffin-embedded arteries. After tissue rehydration, endogenous peroxidase was blocked with 3% hydrogen peroxide. Epitope retrieval was performed by boiling slides in citrate buffer (10 mM sodium citrate, pH 6.0) for 25 min. Nonspecific binding was blocked with 0.5% blocking solution (DAKO, Carpinteria, CA). Primary antibodies were incubated for 1 h at room temperature. Biotinylated secondary antibodies (DAKO Universal Link) were applied for 30 min, followed by washing steps with PBS and 15-min incubation with horseradish peroxidase-streptavidin solution (DAKO) at room temperature. Color was developed with a DAB chromogen solution (DAKO). Nuclei were counterstained with Meyer’s hematoxylin and mounted as described above. Images were taken with an Olympus IX71 camera fitted to an Olympus BX-40 microscope (Olympus America, Center Valley, PA). Primary antibodies were directed against: β-hydroxyethylethanolamine (AB5830; Millipore, Billerica, MA); nitrotyrosine (06–284; Millipore); 53BP1 (sc-22760); p21 (sc-6246); p27 (sc-528), p16 (sc-1207), Ku70 (sc-5309), and p53 (sc-99) (Santa Cruz Biotechnology, Santa Cruz, CA); CD8 (MCA48G); and CD68 (MCA341R; AbD Serotec, Raleigh, NC).

For immunofluorescence, blocking of nonspecific binding sites was achieved with PBS-FBS 15% for 20 min at room temperature. Sections were incubated overnight at 4°C with anti-smooth muscle actin (M0851; Dako) and either anti-Ki67 (4203; Epitomics, Burlingame, CA) or anti-GFP (Abcam; Cambridge, MA) antibodies in PBS-BSA 10%. After being washed twice with PBS-1% Tween 20 for 3 min, tissue sections were incubated with Alexa Fluor 546 goat anti-mouse and Alexa Fluor 488 donkey, rabbit, or goat antibodies (Invitrogen, Carlsbad, CA) for 90 min at room temperature. Sections were mounted in Vectashield DAPI.

**VSMC culture and induction of senescence.** Arterial rat VSMC were isolated from aged and young Fisher rats, cultured, and characterized as previously described (42). VSMC were also isolated from GFP transgenic and wild-type Lewis rats (2–4 mo old). To induce senescence, cells were cultured in complete medium supplemented with 100 μM H2O2 (Sigma-Aldrich). Completed medium was DMEM-F12 (50:50:30; Sigma-Aldrich) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.1 mM glutamine, 10 mM sodium pyruvate, and 0.75% sodium bicarbonate. Cells were passaged every 3 days. Senescence was apparent after two passages.

**Cellular senescence, population doubling calculation, and cell cycle determination.** VSMC morphological changes were examined by microscopy. Cells were counted at each passage using a Beckman Z1 Coulter particle counter (Fullerton, CA). Cell numbers were used to calculate cumulative population doublings (1). Population doubling was calculated according to the following ratio: \[\log_2 (\text{cell number harvested}) - \log_2 (\text{cell number inoculated})\]. Cumulative population doubling was merely the sum of all previous population doubling.

Cellular senescence was further confirmed by SA-β-gal staining. Briefly, cells were washed in 1× PBS, fixed with 0.25% glutaraldehyde (Sigma-Aldrich) in 1× PBS for 20 min at 4°C and then incubated overnight at 37°C with the SA-β-Gal staining solution (10). Positive staining (blue) was microscopically evaluated. Cell cycle progression was determined in senescent or nosenescent VSMC by fluorescence-activated cell sorting analysis of propidium iodide-stained cells (36). The cells were analyzed in a LSR System I (BD Biosciences, San Jose, CA). Cell cycle distribution was analyzed using Flowjo 7.2 (Tree Star, Ashland, OR).

**RESULTS**

**Aging exaggerates postinjury senescence and neointimal formation.** Postinjury SA-β-Gal was evident in injured arteries of both aged and young rats at day 7 postinjury. Nonetheless, postinjury vascular senescence was more intense in arteries of aged rats at days 14 and 30 than in those of their younger counterparts (Fig. 1A). At day 30, 23.07 ± 6.98% of the total vascular area of the injured artery turned positive for SA-β-Gal staining solution (10). As expected, the number of senescent cells was higher in injured arteries of aging animals at days 14 and 30 than in those of their younger counterparts (Fig. 1A). At day 30, 23.07 ± 6.98% of the total vascular area of the injured artery turned positive for SA-β-Gal in aged rats vs. 3.62 ± 1.14% (P > 0.001) in the young rats (Fig. 1C). In both young and aged animals senescence was more intense and lasting in the middle part of the injured artery than in the ends. Senescence was not detected in the noninjury, control arteries.

As expected, the number of senescent cells was higher in injured arteries of aged rats that in those from young animals. At day 14 after injury, 22.66 ± 9.38% of cells in arteries of aged rats were senescent vs. 10.75 ± 4.3% in the young animals. This difference remained until the end of the experiment at day 30 when injured arteries of aging animals contained a 43.13 ± 15.11% of senescent cells vs. a 25.38 ± 7.29% in those from their younger counterparts (P = 0.03, Fig. 1C and Supplemental Data Fig. I, available with the online version of this article). Interestingly, at day 7 postinjury, senescent cells were spread throughout the vascular wall of both aged and young animals. Afterward, senescent cells were...
found largely close to the external elastic lamina, in the interphase between the media and the adventitia (Fig. 1B).

Neointimal formation was assessed in EVG-stained cross sections of injured arteries from aged and young rats. The neointima appeared in both groups of animals as early as 7 days after balloon injury (data not shown). It was markedly engrossed at day 14 and reached the maximal thickness at day 30 after injury. Only at this time was the neointima significantly thicker in aging arteries with respect to their younger counterparts (N/M of 0.921 ± 0.76 vs. 0.466 ± 0.174, respectively, *P = 0.024 as calculated by the Mann-Whitney U-test for nonparametric data) (Fig. 1D). No neointima was found in contralateral, uninjured arteries.

Characterization of postinjury vascular senescent cells. SA-β-Gal⁺ cells in injured vessels were characterized by immunohistochemistry. Senescent cells (SA-β-Gal⁺) showed no staining for the known proliferative marker, Ki67 (>99%, Supplemental Data Fig. II). Senescent cells expressed cell cycle inhibitors p21 and p27 (>90%); yet they were negative for p16INK4a (Fig. 2). The absence of the proliferation marker and the presence of cell cycle inhibitors confirmed that those cells were cell-cycle arrested and had senescent phenotype.

Fig. 1. Injury exaggerates senescent-associated β-galactosidase (SA-β-Gal⁺) activity in arteries of aged animals. A: whole iliac arteries of aged and young rats harvested at 0, 7, 14, and 30 days postinjury and stained for SA-β-Gal⁺ (blue). B: cross sections of aged and young injured arteries showed senescent cells in media and adventitia at day 14 postsurgery. C: kinetic of appearance of postinjury senescence cells (SA-β-Gal⁺) in arteries of aged and young rats. D: arteries of aged rats (n = 9) developed significantly more neointima in response to injury than those of young animals (n = 11). Representative photomicrographs from Elastica-Van Giessen-stained sections show the neointimal development in aged and young rat arteries subjected to balloon injury 30 days prior to harvest. *P = 0.024. Statistical difference was calculated by the Mann-Whitney U-test for nonparametric data.

Fig. 2. Postinjury vascular senescence cells expressed cycle inhibitors p21 and p27. Injured arteries of aged rats were harvested 14 days after surgery and stained for the senescent marker SA-β-Gal (blue). The expression of the cell cycle inhibitors p16, p27, and p21 were detected in cross sections of those arteries by immunohistochemistry using specific antibodies (brown). Representative senescent cells positive for p21 and p27 are labeled with arrows. Nuclei were hematoxylin counter stained (purple). The media (M) and adventitia (A) are noted.

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Next we sought to identify the lineage of postinjured senescent cells in both aged and young injured arteries using specific antibodies. In injured arteries, most of the SA-β-Gal+ areas had low levels of smooth muscle cell actin (SMA) (Fig. 3, A–C). Only a few senescent cells (SA-β-Gal+) close to the external elastic lamina were double positive for SA-β-Gal and SMA (Fig. 3, B and C). Interestingly, SA-β-Gal+ cells in the media, but not in the adventitia, stained positively for vimentin, suggesting that they were from a VSMC lineage (Fig. 3, D and E). In addition, SA-β-Gal+ cells showed no costaining with anti-CD68, an antibody that detects macrophages or anti-CD8 an antibody that stains T-lymphocytes (Supplemental Data, Fig. III). Immunostaining showed abundant expression of SMA throughout the media of the noninjured arteries.

Postinjury vascular oxidative stress and senescence. To elucidate the role of oxidative stress in the postinjury vascular senescence, stable oxidative stress markers, 8-oxo-7,8-dihydrodeoxyguanine (8-oxo-G) and nitrotyrosine were detected by immunohistochemistry. In both groups of rats, aged and young, 8-oxo-G accumulation increased quickly after injury and reached its peak at day 7. Aging significantly increased the accumulation of arterial 8-oxo-G in the vascular wall of the injured arteries (Fig. 4, A–C). In arteries from young rats, 8-oxo-G declined by day 14 (Fig. 4, D–F), but those from aged animals still showed a significant accumulation of 8-oxo-G even at 30 days after balloon injury. Accumulation was predominantly in the nucleus of SA-β-Gal+ cells in the adventitia and, to a lesser extent, in the media. A significant amount of 8-oxo-G was also evident in nonsenescent neointima cells (Fig. 5).

SA-β-Gal+ cells also accumulated nitrotyrosine in an age-dependent manner (Fig. 5). In addition to the oxidative stress markers, the nuclei of senescent cells expressed high levels of DNA double-stranded break sensors, such as 53BP1, and DNA damage-signaling proteins Ku70 and p53 (Fig. 5).

Oxidative stress induces cell-cycle arrest and senescence in cultured VSMC. The presence of oxidative markers in SA-β-Gal+ cells following injury suggests that injury-induced oxidative stress stimulated DNA damage signaling resulting in senescence. To directly test the effect of oxidative stress and DNA damage on VSMC, we cultured rat VSMC from aged and young donors with sublethal doses of H2O2. This treatment induced distinct morphological changes like a vacuolated cytoplasm and elevated cytoplasmatic activity of SA-β-Gal (>90% of the cells, Fig. 6, A–C). These treatments also caused extensive physiological perturbation, culminating in a growth arrest of cells regardless of the donor’s age (data not shown). This growth arrest was assessed by calculation of the cumulative population doubling. For instance, the cumulative population doubling after four passages in the H2O2-treated cells was 3.50 ± 0.43 vs. 9.67 ± 0.45 recorded in the no-treated VSMC (Fig. 6D). Cell-cycle arrest in the H2O2-treated cells was further confirmed with propidium iodide staining followed by flow cytometry analysis. The 83.23 ± 5.68% of H2O2-treated cells were arrested in G0/G1 and only 14.91 ± 1.36% (P < 0.05) of those cells displayed signs of DNA replication (G2/M fraction) in response to serum stimulation (Fig. 6E). Untreated VSMC were fully able to proliferate in media for > 50 population doublings without any sign of reaching its Hayflick limit, which is the number of times a cell divides before telomeres reach a critical length and, therefore, stops division (data not shown) (18).

Implantation of exogenous senescent VSMC increases neointima formation after balloon injury. To directly test the role of senescent VSMC in the postinjury neointima formation, nonsenescent VSMC and VSMC that were induced to senescence in culture were embedded in Matrigel and seeded perivascularly around balloon-injured arteries of young rats. At day 30 postsurgery, injured and noninjured arteries were harvested.

**Fig. 3.** Smooth muscle actin (SMA) is downregulated in postinjury senescent cells. A: cross sections of injured arteries from aged rats harvested at 7, 14, and 30 days postinjury. Control arteries were contralateral, no-injured (NI) arteries. Senescent cells expressed the SA-β-Gal (light blue). The vascular smooth muscle cells (VSMC) marker, SMA (red), was detected by immunohistochemistry (IHC). B: SA-β-Gal positive (light blue) cells in the media and adventitia of an injured artery harvested 14 days postsurgery. C: detection of SMA (yellow) in SA-β-Gal positive cells by immunofluorescence microscopy. Images were taken using a confocal scanning microscopy. Nuclei were DAPI counter stained (dark blue). Representative SMA+ SA-β-Gal+ cells are indicated with arrows. Neointima (N), media (M), and adventitia (A) layers are noted. D: SA-β-Gal (red) positive cells in the media of injured arteries that later were stained with an anti-Vimentin antibody. Arrows, representative cells. E: detection of medial VSMC staining positively for SA-β-Gal (red) and Vimentin (green; arrows).
stained for SA-β-Gal, and neointimal formation was assessed. Injured arteries seeding with senescent VSMC developed two-fold thicker neointimas than those treated with nonsenescent VSMC or matrigel (N/M ratio of senescent vs. nonsenescent: 0.41 ± 0.10 vs. 0.26 ± 0.04, P = 0.014) (Fig. 7). The total number of cells in neointimas of arteries treated with senescent cells was 472 ± 181 cells per cross section vs. 374 ± 127 and 216 ± 153 (P < 0.05) cells in the neointima of arteries treated with nonsenescent and Matrigel, respectively. This suggests that seeding young rat arteries with senescent VSMC stimulates injury-induced neointima formation to a level observed in older rats. Interestingly, the postinjury senescence reaction at

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Fig. 4. Aging increases oxidative stress and the accumulation of 8-oxo-7,8-dihydrodeoxyguanine (8-oxo-G), a marker for DNA damage, after balloon angioplasty. A–F: injured arteries from aged (B–C) and young (E–F) animals harvested at 7 (B and E) and 14 (C and F) days postinjury and stained for SA-β-Gal (light blue)- and with 8-oxo-G (brown)-specific antibodies. Noninjured arteries (A and C) were used as controls.

Fig. 5. Accumulation of DNA damage-signaling proteins in senescent cells 14 days after balloon injury. Tissues were stained for SA-β-Gal (light blue) before fixing and paraffin embedding for histopathological analysis. Sections were double stained with SA-β-gal and indicated antibody by IHC (magnification, ×200). Representative positive SA-β-Gal⁺ cells are indicated with arrows. The control section was treated with an irrelevant antibody. Cell nuclei were counterstained with hematoxylin (dark blue).
arteries (Fig. 7, neointima and perivascular area of senescent VSMC). Interestingly, GFP was present in the neointima at day 30 postsurgery, but only a few senescent and nonsenescent VSMC were visible in the neointima by fluorescence microscopy. How- ever, only a few senescent and nonsenescent VSMC were present in the neointima at day 30 postsurgery (>3%, Fig. 8). Interestingly, GFP+ VSMC that migrated across the neointima were found close to the endothelium, at the luminal side of the neointima (Fig. 8). This result suggests that the mechanism by which senescent cells contribute to the neointimal formation might be more through the secretion of growth factors to stimulate nearby cell growth rather than by direct incorporation of those into the postinjury neointima.

**DISCUSSION**

The mechanisms by which aging contributes to the exaggerated neointima formation in response to vascular injury have remained elusive so far. Herein, we have demonstrated that aging exacerbates postinjury oxidative stress and cellular senescence in the vascular wall. We have also found evidence indicating that postinjury cellular senescence in the aging vasculature is not a mere consequence of the vascular remodeling, but it is, in fact, a worsening factor for neointimal formation.

Vascular senescence was established in our model of vascular injury based on three criteria: 1) SA-β-Gal activity, 2) absence of the Ki67 proliferation marker, and 3) accumulation of p21 and/or p27 cell cycle inhibitors. More than 90% of arterial SA-β-Gal+ cells stained negatively for Ki67 and positively for p21 or p27, which confirmed their senescent phenotype. We demonstrated, therefore, that postinjured senescent cells appeared in both young and aged animals at day 7 after surgery but that only in aged animals these cells remained perivascular until day 30 postsurgery. Senescence coincided with an exaggerated neointimal formation in the injured arteries of aged rats with respect to those from their younger counterparts suggesting a plausible association between neointima formation and cellular senescence in the aging vasculature.

We also sought to identify the causes of the exaggerated senescence reaction observed in the vasculature of aged rats in response to injury. Vascular cells undergo senescence either by shortening of telomeres (29) or by stress-mediated activation of the biochemical pathways that lead to senescence (32). Oxidative stress also induces senescence in VSMC (29) and EC (23). Our results indicated that postinjury senescent cells accumulated stable oxidative by-products, like 8-oxo-G, and that this accumulation is more intense in vessels of aged rats than in those of their younger counterparts. It is well described that aging exacerbates oxidative stress by chronic stimulation of NAD(P)H oxidase activity and by exhausting the intracellular antioxidant defenses (11, 34). One mechanism linking oxidative stress and cellular senescence is DNA-damage pathways. Severe damage of the DNA activates p53 signaling leading to cell-cycle arrest and senescence in vascular cells (25). In agreement with our finding, Zhang et al. (44) demonstrated the role of DNA-damage pathway on the postinjured neointimal formation after balloon injury. Martinet et al. (27, 28), on the other hand, documented the accumulation of 8-oxo-G and...
DNA damage-sensor proteins in rabbit atherosclerotic plaques (27, 28). Our study extends previous studies to demonstrate that oxidative stress and DNA damage is exacerbated with aging and that it may cause profound changes in cell physiology leading to senescence. Interestingly, immunohistochemical staining of tissue sections from injured arteries showed upregulation of Ku70 and 53BP1 proteins along with the DNA damage-marker 8-oxo-G in senescent cells as in nonsenescent neointimal cells. It could be postulated that oxidative stress plays a dual role in neointimal formation. Oxidative stress could either promote or suppress VSMC growth and neointimal thickness. Stimulation of cultured VSMC with xanthin/xanthin oxidase increases proliferation, whereas stimulation with hydrogen peroxide causes growth arrest and senescence (45). Therefore, it is plausible to suggest that senescent and neointimal cells in the aged vasculature originate from vascular cells that have unlike recovering from oxidative stress and DNA damage.

Identifying the cells that turned senescent in the vasculature in response to injury was also pursued as part of this study. We found senescent cells either scattered throughout the vascular wall of injured arteries at days 7 and 14 or perivascularly at day 30 postsurgery. These cells were more concentrated in the middle portion of the injured artery. However, Fenton et al. found mainly senescent cells in the neointimas of rabbit arteries at 6-wk postvascular intervention (15). Differences between species and type and degree of injury could account for such a discrepancy between studies. Interestingly, senescent VSMC (SA-β-Gal<sup>+</sup> cells in the media) stained positively for vimentin but mostly negative for SMA. Smooth muscle markers, which are mostly contractile proteins, are downregulated in VSMC during “contractile to synthetic” phenotypic switching (37, 43). Another group of senescence cells were found in the adventitia of injured arteries after day 14 following vascular injury. These cells have a fibroblastic appearance and stained negatively for the VSMC markers SMA and vimentin and for inflammatory cell markers, CD8 and CD68. Although we could not identify the lineage of the SA-β-Gal<sup>+</sup> cells, we propose, based on histological location and cell morphology, that those cells could be senescent adventitial fibroblasts involved in the vascular remodeling process. Senescent fibroblasts may secrete growth factors to favor tumor growth and vascularization (7).

Finally, to challenge the hypothesis that senescent cells were an active component in the neointima formation and not a mere passive consequence of a cell division and oxidative stress, we seeded exogenous senescent VSMC around injured arteries and...
looked for neointimal formation 4 wk later. Matrigel scaffolds provided physical properties ideal for perivascular implantation of VSMC. We found that senescent cells increased neointimal thickness in injured arteries with respect to nonsenescent VSMC as though seeding young rat arteries with senescent VSMC mimicked remodeling in aging rats.

These observations raise a pertinent question. How does a senescent cell contribute to neointimal hyperplasia? One explanatory theory would be that senescent VSMC produce growth factors to stimulate nearby cell proliferation, migration, and survival. It is supported by the fact that only a few senescent cells migrate into the postinjury neointima after implantation. Senescent human VSMC and EC have been found able to produce de novo IL-1β in culture (26, 32). Senescent fibroblasts secrete VEGF to stimulate tumor vasculogenesis (7). A second model would be that senescent cells may enhance extracellular matrix component production by local cells and therefore neointimal thickness (4, 21). Further studies are warranted to determine the mechanism by which senescent cells exacerbate aging-related postinjury neointimal formation.

This study also raises the apparent contradiction between an increased number of VSMC in injured arteries of aged animals and an exaggerated presence of nondividing senescent cells. This is not such a contradiction if one considers the following: 1) few senescent cells were found in the neointima, 2) neointimal cells may come from nonsenescent VSMC as not all VSMC become senescent after injury, 3) VSMC cells within neointima may have a clonal origin (38), and 4) it is likely that some of neointimal VSMC in postangioplastic restenosis models may have an stem cell origin (20). Based on our results, we favor the hypothesis that not all VSMC become senescent after injury, and those that do serve as signaling cells to guide the vascular remodeling process. Furthermore, perturbations in this signaling caused by cellular aging could lead to an exaggerated neointimal formation that will ultimately result in luminal narrowing.

In conclusion, our study demonstrates that senescent cells are a worsening factor for the development of vascular proliferative diseases in the aged vasculature and they are indeed an active player in the vascular remodeling process after acute injury.

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