Adventitial Pericyte Progenitor/Mesenchymal Stem Cells Participate in the Restenotic Response to Arterial Injury

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Key Words

Adventitia • Coronary angioplasty • Mesenchymal stem cells • Pericyte-like cells • Pericytes • Restenosis

Abstract

Restenosis is a major complication of coronary angioplasty, at least partly due to the fact that the origin and identity of contributing cell types are not well understood. In this study, we have investigated whether pericyte-like cells or mesenchymal stem cells (MSCs) from the adventitia contribute to restenosis. We demonstrate that while cells expressing the pericyte markers NG2, platelet-derived growth factor receptor β , and CD146 are rare in the adventitia of uninjured mouse femoral arteries, following injury their numbers strongly increase. Some of these adventitial pericyte-like cells acquire a more MSC-like phenotype (CD90+ and CD29+ are up-regulated) and also appear in the restenotic neointima. Via bone marrow transplantation and ex vivo artery culture approaches, we demonstrate that the pericyte-like MSCs of the injured femoral artery are not derived from the bone marrow, but originate in the adventitia itself mainly via the proliferation of resident pericyte-like cells. In summary, we have identified a population of resident adventitial pericyte-like cells or MSCs that contribute to restenosis following

arterial injury. These cells are different from myofibroblasts, smooth muscle cells, and other progenitor populations that have been shown to participate in the restenotic process.

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Introduction

Restenosis has been called the Achilles' heel of coronary angioplasty, since constriction of the vascular lumen in surgically opened arteries is a major factor in compromising the success of coronary artery interventions. Although surgical improvements, including stent technology, have somewhat improved the situation, restenosis remains a pressing clinical challenge [1]. A better understanding of the molecular and cellular mechanisms underlying restenosis will be required to allow further progress in alleviating the problem.

One issue of significance is the identity and origin of cells that contribute to the formation and growth of restenotic lesions. Smooth muscle cells are among the most prominent cellular components of these lesions. Traditional views have maintained that these cells arise exclusively from the media of affected arteries [2]. However, research during the past decade demonstrates that the

origins of restenotic cells are more varied and complex than previously suspected [3]. For example, bone marrow progenitor cells are reported to differentiate into smooth muscle cells in atherosclerotic lesions and in the neointima of restenotic arteries [4], with bone marrow contribution to neointima formation varying in proportion to the severity of the lesion [5].

Another potential source of progenitor cells during restenosis is the adventitia. Previously thought to be relatively inert, this outermost layer of the arterial wall has now been shown to be a source of cells that can migrate into the lumen of injured arteries and contribute to neointima formation [6–8]. The identity of adventitial progenitor cells that contribute to neointima formation remains uncertain. While earlier reports suggested that adventitial fibroblasts might differentiate into myofibroblasts and migrate into the lumen of injured arteries [9], more recent studies have focused on adventitial cells expressing progenitor markers such as Sca1, c-kit, CD133, CD34, and Flk1. For example, adventitial cells expressing Sca1 have the capability to differentiate into smooth muscle cells both *in vitro* and *in vivo* [10].

Further confirmation of the cellular complexity of the restenotic response suggested by these disparate viewpoints is provided by our current work demonstrating the presence of numerous pericyte-like cells (henceforth called pericytes for simplicity) in the restenotic femoral artery. By definition, pericytes are the microvascular counterparts of smooth muscle cells in larger vessels, partnering with vascular endothelial cells during vessel development, maturation, and maintenance. However, in addition to their role as perivascular support cells, a subgroup(s) of pericytes is increasingly regarded as mesenchymal stem cells (MSCs) [11]. Pericytes exhibit tremendous plasticity not only in their developmental origins, but also in their differentiation potential. Developmentally, pericytes can arise not only from stem cell sources such as the bone marrow, but also from cells that reside in mature tissues such as adipose deposits, and both skeletal and smooth muscles [12–18]. The stem cell nature of pericytes is highlighted by several recent papers demonstrating the ability of pericytes to give rise to a variety of mesenchymal cell types in different organs, including skeletal muscle, smooth muscle, bone, cartilage, and adipose tissue [19–24]. Moreover, adventitial pericytes have been implicated in pathological processes such as wound healing and arterial calcification [25–27]. Pericytes therefore possess several of the properties associated with stem cells or adventitial progenitors that may participate in restenosis. In this communication, our

goals have been to characterize some of the properties of adventitial pericytes and to provide additional evidence for the contribution of these cells to neointima formation during restenosis.

Materials and Methods

Antibodies

Rabbit polyclonal antibodies against NG2 and platelet-derived growth factor receptor (PDGFR) β and guinea pig polyclonal antibody against NG2 have been described previously [28, 29]. Rat monoclonal F4/80 antibody was purchased from BioSource International (Camarillo, Calif., USA), and rabbit polyclonal CD146 antibody from Abcam (Cambridge, Mass., USA). Rat monoclonal antibodies against Sca1, CD11b, CD29, CD31, CD44, CD45, CD71, CD73, and CD90 were from BD Biosciences (La Jolla, Calif., USA). Cy3-coupled α -smooth muscle actin (SMA) antibody was purchased from Sigma (St. Louis, Mo., USA). 5-Bromo-2'-deoxyuridine (BrdU) antibody was from Serotec. Cy5-conjugated secondary antibody was obtained from Jackson ImmunoResearch (West Grove, Pa., USA). Alexa 488- and Alexa 568-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, Oreg., USA).

Animals

Adult, male, 8- to 12-week-old C57Bl/6 mice and C57Bl/6 mice expressing enhanced green fluorescent protein (EGFP) under control of the β -actin promoter (β -actin/EGFP; Jackson Laboratories) were used. Mice were maintained in the Sanford-Burnham Vivarium (fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). All animal procedures were performed in accordance with Office of Laboratory Animal Welfare regulations and were approved by Sanford-Burnham Animal Care and Use Committee review prior to execution.

Femoral Artery Injury Model

Femoral artery injuries were performed as previously described [4, 30]. Briefly, the left femoral artery was exposed by blunt dissection. After careful separation of the femoral nerve, both the femoral artery and vein were looped together proximally and distally with silk sutures for temporary vascular control. A small branch between the rectus femoris and the vastus medialis muscle was separated, proximally looped, and distally ligated with 4-0 sutures. The artery was then further separated from surrounding veins and connective tissue. After dilating the exposed artery with one drop of 1% lidocaine hydrochloride, a small incision was made into the muscular branch artery with Vannas style iris spring scissors and a spring wire (0.38 mm in diameter, No. C-SF-15-15; Cook, Bloomington, Ind., USA) was carefully inserted to a depth of approximately 8 mm toward the iliac artery. The wire was left in place for 1 min, to denude and dilate the artery, and then removed. The suture looped at the proximal side of the muscular branch artery was secured, blood flow was restored, and the skin incision was closed with sutures. In some cases, BrdU (80 mg/kg) was injected 3 and 4 days after femoral artery injury. At specified time points, mice were euthanized by CO₂ asphyxiation. Both the left and the right femoral artery were excised (the right artery as a control), fixed in 4% paraformaldehyde, cryoprotected

overnight in 20% (wt/vol) sucrose/phosphate-buffered saline (PBS) and frozen in OCT embedding compound (Tissue-Tek). Sections (25 μ m) were prepared using a Reichert cryostat microtome.

Bone Marrow Transplantation

Bone marrow transplantations were performed as previously described [17]. One β -actin/EGFP donor mouse per transplantation was euthanized by CO₂ asphyxiation. Femurs and tibiae were dissected, and the bone marrow was flushed out with 1 ml of PBS containing 2% fetal calf serum (FCS) and 5 mM EDTA (PBS/FCS/EDTA). The bone marrow cells were washed once with PBS/FCS/EDTA, and subsequently red blood cells were lysed on ice for 5 min with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4). After the lysis step, cells were washed twice with PBS/FCS/EDTA. Between 5 and 7 $\times$ 10⁷ cells were typically collected from a single donor. Immediately before injection into recipient mice, cells were passed through a 62- μ m nylon filter (Small Parts, Miami Lakes, Fla., USA) and washed once with Ringer's solution. Recipient mice were γ -irradiated (two 5-Gy doses administered 3 h apart) using a ¹³⁷Cs Gammacell-40 Exactor irradiator. Animals were immediately reconstituted via retro-orbital injection of 6 $\times$ 10⁵ bone marrow cells in 100 μ l of Ringer's solution and were maintained on antibiotic water (neomycin sulfate, 1.1 g/l and polymyxin B sulfate, 455 mg/l) for 6 weeks to allow hematopoietic re-establishment. Retro-orbital blood samples were taken from each recipient, and the extent of EGFP engraftment was determined by flow-cytometric analysis. Animals exhibiting at least 75% engraftment were used for femoral artery injury experiments.

Immunostaining and Confocal Microscopy

Immunostaining and confocal microscopy were performed as previously described [28, 29]. An inverted Radiance 2100 multiphoton confocal microscope (BioRad, Hercules, Calif., USA) was used to obtain serial 1- to 1.5- μ m optical sections across the entire 25- μ m thickness of the histological specimens. This confocal system provides for analysis of four fluorochromes, allowing us to perform quadruple labeling with DAPI and 3 antibodies, or with DAPI, 3 antibodies, and the EGFP transgene. Overlaid serial optical sections (z-stacks) were analyzed using Volocity 4D Rendering software (version 3.7) for unambiguous determination of the spatial relationship between cells. Differences between marker expression in the adventitia or neointima are determined either by analysis and counting of individual cells or by measurement of signal intensity with Volocity software on sections labeled and scanned under standardized conditions.

Ex vivo Aortic Explants

Thoracic aortas were isolated from wild-type C57Bl/6 mice and 3-mm pieces were maintained free-floating for 6 days at 37°C and 5% CO₂ in a cell culture dish containing DMEM, DMEM with 10% FCS, DMEM with 50 ng/ml PDGF-BB, or MSC expansion medium (MSCEM; Gibco). When indicated, 10 μ M BrdU were added to the culture medium for the entire incubation time. After incubation, aortic specimens were fixed with 4% paraformaldehyde, cryoprotected overnight in 20% (wt/vol) sucrose/PBS, and frozen in OCT embedding compound (Tissue-Tek). Sections (25 μ m) were prepared using a Reichert cryostat microtome. Sections were used for immunostaining and confocal microscop-

py, and expression differences between groups were determined by analysis and counting of individual cells with Volocity software.

Statistical Analysis

Unpaired Student's t tests were used to determine if measured differences between data were significant. A value of $p < 0.05$ is considered statistically significant. All values represent experiments done at least in triplicate and are given as means $\pm$ SEM.

Results

A Heterogeneous Population of Pericyte-Like Cells Expands within the Adventitia of the Injured Femoral Artery

In order to study the distribution and fate of putative progenitor cells within the arterial wall during restenosis, we used a femoral artery injury model [4, 30] that consistently resulted in significant medial thickening and formation of a neointima 9–12 days after the injury. We used immunostaining for NG2 and PDGFR β to detect the presence of pericytes/MSCs during the process of arterial remodeling after injury. Within the adventitia of healthy, uninjured femoral arteries, cells expressing these pericyte markers are found infrequently (fig. 1a). However, 7 days after injury, the number of NG2-positive cells in the adventitia increases dramatically (fig. 1b), with NG2 expression levels rising more than 7-fold (fig. 1c). A similar increase is observed for PDGFR β -positive cells in the adventitia (fig. 1b). Confocal co-localization analysis shows that about 40% of adventitial NG2-positive cells also express PDGFR β (fig. 1b), demonstrat-

Fig. 1. Pericyte-like cells in the adventitia. Pericyte-like cells expressing both NG2 (green) and PDGFR β (red; arrowhead) are found only rarely in the uninjured femoral artery (a). Seven days after femoral artery injury, the pericyte markers PDGFR β (red) and NG2 (green) are present on many cells in the adventitia (a). At this time point, the media (m) has undergone apoptosis (b). The increase in NG2 expression 7 days after injury, as determined by quantification of NG2 pixels, is roughly 7-fold (c; $p = 0.001$). Relative to NG2 (green), Sca1 (red) expression is relatively high in the adventitia of uninjured femoral arteries (d) and decreases in response to arterial injury (e, f; $p = 0.04$). NG2-positive pericytes (magenta) in uninjured femoral arteries co-express CD146 (red; arrowhead; h, j) but do not express CD90 (green; arrowhead; i, j). Seven days after femoral artery injury, the expression pattern of adventitial cells changes considerably: NG2+/CD146+ cells are found in higher numbers (k–n) and populations of cells expressing NG2, CD146, and CD90 (k–n) or NG2, CD90, and CD29 (o–r) can be detected. Blue = DAPI. Scale bars: 40 μ m.

ing that pericytes of the femoral artery may be a heterogeneous population, as has been suggested for pericytes in general [31].

In some tissues, pericytes are thought to be progenitors of myofibroblasts [32], a cell type proposed to contribute to restenosis [6]. However, we failed to detect the myofibroblast marker α -SMA [33, 34] in the injured adventitia. Similarly, a restenotic contribution has been suggested for adventitial Sca1-positive cells [10]. However, the behavior of Sca1-positive cells in the femoral artery injury model is distinct from that of NG2-/PDGFR β -positive pericytes. Sca1-positive cells are already numerous in the adventitia of the uninjured femoral artery, and Sca1 levels decrease after injury (fig. 1d–f). Moreover, there is little or no overlap between Sca1 and NG2/PDGFR β expression. Indeed, these two cell populations occupy different zones of the adventitia. NG2-/PDGFR β -positive pericytes are found almost exclusively near the boundary between the adventitia and media, while Sca1-positive progenitors occupy a more superficial layer of the adventitia. It has been suggested that the adventitial layer adjacent to the media is a vasculogenic zone that harbors progenitor cells [35].

In light of numerous reports identifying pericytes as MSCs, we aimed to determine whether NG2-positive adventitial cells express other markers associated with MSCs, such as CD29, CD44, CD71, CD73, CD90, or CD146 [11, 20]. Although abundant, CD44-positive cells do not co-express NG2, and cells expressing CD71 or CD73 are not detected in either healthy or injured arteries (table 1). The rare NG2-positive cells in the adventitia of the uninjured femoral artery are positive for CD146

expression, consistent with published observations that CD146 is expressed by both pericytes and MSCs [36, 37]. None of the other MSC markers are expressed by NG2-positive cells in the healthy adventitia (fig. 1g–j, 2e; table 1). However, following injury, the phenotype of NG2-positive cells becomes more complex, with CD90, CD29, and CD146 all being co-expressed to varying extents by NG2-positive cells (fig. 1k–r). Figure 1e, p illustrates a phenomenon that is sometimes observed for NG2 and CD29 immunostaining in this injury model; namely, an abundance of labeling in the apoptotic media. This is also seen in figure 1o, r and figure 2a, f, h. While we have speculated that this might represent binding of NG2 and β_1 -integrins to extracellular matrix material remaining after smooth muscle cell apoptosis, this has not been confirmed. Since this medial labeling is inconsistent and not understood, we have not included it in our analyses.

In accord with the idea that pericytes do not express hematopoietic, myeloid, or endothelial markers, NG2 is not expressed on CD31-positive (endothelial) or CD11b-positive (myeloid) cells either in uninjured or injured femoral arteries (data not shown). Also, fewer than 1% of NG2-positive cells in the injured artery express the hematopoietic marker CD45 (fig. 2a). In contrast to pericytes,

Table 1. Expression of pericyte and MSC markers in femoral arteries (FA) before and after injury

	Uninjured adventitia FA	Injured adventi- tia FA	Co-expres- sion with NG2 (inj. adv. FA)	Injured neointima FA	Co-expres- sion with NG2 (inj. neo. FA)
CD29	–	+	+	+	+
CD44	–	+	–	–	–
CD71	–	–	–	–	–
CD73	–	–	–	–	–
CD90	+	(few cells)	+	+	+
CD146	+	(few cells)	+	+	+
NG2	+	(few cells)	+	+	+
PDGFR β	+	+	+	+	+
Sca1	+	+	–	+	(few cells)

Fig. 2. Pericyte-like cells in the neointima. CD29 is not expressed in the uninjured femoral adventitia (e) but is found co-expressed (red) with NG2 (green) in the adventitia of injured femoral arteries (7 days after injury; h). The hematopoietic marker CD45 (red) is rarely co-expressed on NG2+ cells (green; <1%) in a superficial layer of the injured adventitia (arrowhead; 7 days after injury; a). Fifteen days after femoral artery injury, formation of a neointima (ni) can be observed (b, c, i–t). Although abundant on cells in the adventitia (a), Sca1 is only occasionally found on cells on the luminal surface of the neointima (b, j, l). NG2 is expressed on many cells throughout the neointima (c). Double labeling for NG2 (green) and α -SMA (red; c, d) reveals all possible combinations of staining: NG2-positive, SMA-positive (arrowhead in c); NG2-positive, SMA-negative (arrows in c); NG2-negative, SMA-positive, and NG2-negative, SMA-negative staining. At 15 days after injury, the neointima also contains cells co-expressing NG2 (magenta), CD90 (green), and CD146 (red; arrowheads; m–p) or NG2 (magenta), CD90 (green), and CD29 (red; q–t). Bone marrow transplantation experiments using β -actin/EGFP donors reveal that 7 days after femoral artery injury, the adventitia contains almost no EGFP+ cells (green) co-expressing NG2 (magenta; f) or Sca1 (red; g). The same is true of the neointima 15 days after injury (i–l). These findings suggest that NG2- and Sca1-positive adventitial progenitor cells that contribute to restenosis are not derived from the bone marrow. Blue = DAPI; α SMA = α -SMA. Scale bars: 40 μ m.

these rare NG2-positive/CD45-positive cells are always found in more superficial regions of the adventitia rather than in the region directly adjacent to the media. We also used the pan-macrophage marker F4/80 to further evaluate possible myeloid expression of NG2 7 days after injury. Although NG2 is known to be transiently expressed by macrophages in some models [17, 38], and although F4/80-positive macrophages are present in the adventitia of injured arteries, we did not detect co-expression of NG2 and F4/80 (data not shown).

NG2+, CD90+, CD146+, CD29+ Cells Also Appear in the Neointima of Femoral Arteries after Injury

To investigate whether cells expressing pericyte markers contribute to neointima formation, we used immunostaining to examine injured femoral arteries at later time points (9–30 days after injury). At 15 days after injury, Sca1 is rarely expressed by neointimal cells (fig. 2b), and Sca1-positive cells are only occasionally seen at the luminal surface of the neointima (fig. 2j, l). In contrast, NG2 is expressed 15 days after injury by many cells throughout the neointima (fig. 2c), some of which are also positive for α -SMA (fig. 2c, d). This is consistent with the fact that NG2 is known to be expressed by developing vascular smooth muscle cells, although the proteoglycan is usually down-regulated with differentiation [28, 39]. Interestingly, we found that CD29, CD90, and CD146 are expressed by NG2-positive cells in the neointima (fig. 2m–t), echoing the situation encountered in the adventitia. Since CD29, CD90 and CD146 are not expressed by smooth muscle cells in the femoral artery (fig. 1g–j, 2e), these findings are in accord with the idea that pericytes from the adventitia might contribute to neointima formation after arterial injury. In addition to neointimal smooth muscle cells that co-express NG2 and α -SMA and more mature smooth muscle cells that are α -SMA positive and NG2-negative, we also identified a population of NG2-positive cells that do not express α -SMA (fig. 2c, d). These NG2-positive, α -SMA-negative cells could represent pericytes prior to their differentiation to smooth muscle cells.

Adventitial Progenitor Cells Are Not Derived from the Bone Marrow

In order to determine if adventitial pericytes are derived from the bone marrow following femoral artery injury, we irradiated C57Bl/6 wild-type mice and reconstituted them with bone marrow cells from β -actin/EGFP transgenic mice. Six weeks after transplantation, we used these mice in the femoral artery injury model. It is known

that the contribution of bone marrow cells during restenosis is high in cases of severe arterial damage, such as the femoral artery injury model, while mild arterial injury induces little or no contribution from the bone marrow [5]. Consistent with this trend, we observed a very large number of bone marrow-derived cells in the restenotic femoral artery. These EGFP-tagged cells are present initially in the adventitia (7 days after injury) and later in the neointima (after 15 days; fig. 2f, g, k, l). Many of these cells are positive for F4/80, consistent with their identity as myeloid cells. Surprisingly, however, NG2- and Sca1-positive cells in the adventitia rarely express EGFP, showing that neither of these adventitial progenitor populations has a significant degree of origin from the bone marrow (fig. 2f, g). Comparable findings were made in the neointima. Careful analysis of confocal images revealed almost no co-expression of NG2 or Sca1 with the EGFP tracer (fig. 2i–l). Although co-expression of EGFP with NG2 or Sca1 could not be ruled out in a small number of cases (about 3% of the EGFP-positive cells), overall our data reveal that NG2-positive and Sca1-positive progenitor cells in the adventitia and neointima of femoral arteries are mostly derived from non-bone marrow sources.

Increased Adventitial NG2 Expression due to Both Up-Regulation of NG2 Expression and Expansion of NG2-Positive Progenitor Cells

Since the bone marrow transplantation experiments show that few NG2-positive cells are recruited to the injured artery from the bone marrow, NG2-positive cells must arise locally. These cells might arise either by up-regulation of NG2 expression in cells that were previously NG2 negative or else by proliferation of rare NG2-positive pericytes in the adventitia. Shi et al. [6] previously used a porcine coronary artery model to show that adventitial cells exhibit high levels of mitotic activity following injury. By administering BrdU 3 and 4 days following femoral artery injury, we also found that many adventitial cells are mitotic, including some cells expressing NG2 (data not shown). This suggests that at least part of the increase in NG2-positive adventitial cells after injury is due to cell proliferation. In order to more directly address the possibility of NG2 up-regulation in a way that excludes invading cells from other tissues, we turned to a cell culture model that allowed us to examine both cell proliferation and NG2 expression.

Our attempts to utilize the femoral artery for these cell culture experiments were frustrated by extreme variability in the viability of explants. In contrast, we

Fig. 3. Up-regulation of NG2 expression. When segments of the thoracic aorta are incubated for 6 days in DMEM supplemented with 10% FCS (**b, d**), DMEM supplemented with 50 ng/ml PDGF-BB (**c, d**), or MSCEM (**d**), many NG2-expressing cells (green) appear in the adventitia (**a**). Aortic explants incubated in DMEM for 6 days without any supplements contain few, if any, NG2-expressing adventitial cells (**a, d**). p values (**d**) represent statistically significant differences between untreated and treated aortic segments, and between MSC-treated and PDGF-BB-treated segments. Unlike smooth muscle cells of the femoral artery, which are mostly NG2-negative, α -SMA-positive smooth muscle cells (red) of the aortic media express high levels of NG2 (**a–c**). However, NG2-positive cells in the adventitia never express α -SMA (**a–c**), suggesting that adventitial NG2-positive cells in this experiment are not derived from the aortic media (**m**). When aortic explants are incubated with BrdU for 6 days in DMEM supplemented with PDGF-BB, BrdU (red) is incorporated into virtually all aortic NG2-positive cells (green; arrowheads; **e**). However, incubation of explants with BrdU for 6 days in MSCEM leads to a mixed population of mitotic (BrdU-positive, arrowheads) and non-mitotic (BrdU-negative, arrows) NG2-positive cells (**f**), providing evidence that NG2 expression by adventitial cells can increase not only via mitosis, but also via up-regulation. Blue = DAPI; α -SMA = α -SMA. Scale bar: 40 μ m.

were successful in reproducibly culturing dissected thoracic aortas from wild-type C57Bl/6 mice. We therefore used this model to obtain additional information concerning pericyte proliferation versus NG2 up-regulation. As in the adventitia of femoral arteries in situ, NG2 is only rarely found on aortic adventitial cells at the time of dissection or after 6 days in non-supplemented DMEM (fig. 3a, d). However, a large number of NG2-positive cells are present in the adventitia of the FCS-stimulated thoracic aortas (fig. 3b, d). A similar increase in adventitial NG2-positive cells is seen following stimulation of the explants with 50 ng/ml PDGF-BB (fig. 3c, d), an important factor for pericyte recruitment [40, 41]. In both of these latter cases, cell numbers in the outer

adventitial layer are increased by over 50% during the 6-day culture period, indicating that stimulation with either FCS or PDGF-BB might cause extensive cell proliferation or recruitment. Determination of BrdU incorporation into these cultures showed that, in the presence of either PDGF-BB or FCS, virtually all adventitial NG2-positive cells had incorporated BrdU and were therefore mitotic (fig. 3e). In contrast, when we used optimized MSCEM for the cultures, aortas contained higher numbers of NG2-positive adventitial cells than cultures grown with PDGF-BB or FCS (fig. 1d). In this case, we found that about 40% of NG2-positive cells in the adventitia had not incorporated BrdU (fig. 3f), consistent with the idea that both cell proliferation and NG2 up-regula-

tion can contribute to the increased abundance of NG2-positive cells. Rapid up-regulation of NG2 expression has also been reported in venules in response to vascular remodeling [42].

Discussion

The arterial wall has been proposed to harbor various types of progenitor and stem cell populations that contribute to both developmental and repair processes [43]. Based on reports that the adventitia provides a perivascular niche for progenitors that contribute to muscle regeneration, vasculogenesis, fibrosis, and atherosclerosis [6, 44–47], we have paid special attention to the adventitia in our analysis. Our hypothesis in this work has been that pericytes in the arterial wall might serve as a source of progenitors for vessel repair. Two of the most reliable and widely used markers for pericytes are PDGFR β and the NG2 proteoglycan [17, 28, 48]. While the adventitia of uninjured arteries contains very few cells expressing either NG2 or PDGFR β , there is a 7-fold increase in adventitial pericytes during the early stages of remodeling after the wire injury. These pericytes do not appear to be a homogeneous population, as evidenced by the fact that there is only a 40% overlap between NG2 and PDGFR β labeling.

Other markers indicative of an MSC phenotype (CD90, CD29, and CD146) also exhibit partial overlap with NG2, further underscoring the heterogeneity of adventitial pericyte/MSC populations. It remains to be demonstrated whether heterogeneity in marker expression is mirrored by heterogeneity in functional properties and/or developmental potential. Our experience in this regard echoes previous reports that single markers are not sufficient for the identification of pericytes/MSCs. Instead, a panel of markers is required for characterization of these cells. Double and triple labeling analyses of injured femoral arteries reveal that CD29 is almost always expressed on cells that also express NG2 and CD90 (fig. 1o–r). Thus, along with NG2 and CD146 in uninjured arteries, the NG2-CD90-CD29 combination has been the most useful set of markers in our hands for studying pericytes in the arterial injury model (fig. 1k–r). Interestingly, of the MSC markers tested, CD29 (β_1 -integrin) expression is most tightly linked with that of NG2. Since NG2 interacts physically with β_1 -integrins and enhances β_1 -integrin signaling to promote cell proliferation, motility, and apoptosis resistance [49–51], it is tempting to speculate that NG2/ β_1 -integrin-dependent

signaling could be important for some aspect of pericyte/MSC expansion and/or recruitment.

As arterial remodeling progresses, NG2-positive cells are also detected in the neointima. As in the adventitia, these NG2-positive cells also express various combinations of CD29, CD90, and CD146, consistent with the idea that pericytes from the adventitia may participate in the formation of the neointima. The expression of α -SMA by some of these NG2-positive cells suggests that adventitial pericytes/MSCs can serve as a source of neointimal smooth muscle cells.

Using a bone marrow transplantation approach, we found abundant bone marrow-derived macrophages in remodeling arteries, but were unable to detect any contribution of bone marrow cells to the generation of pericytes that are seen in the remodeling adventitia and neointima. This stands in vivid contrast to our previous work with neovascularization of FGF2-containing Matrigel plugs, in which we observed substantial bone marrow derivation of pericytes [17]. Other investigators using different types of models have also reported significant recruitment of pericytes from the bone marrow [13, 14]. It is important to note that bone marrow-derived pericytes/MSCs (resident MSCs) that occupy the adventitia prior to bone marrow transplantation and subsequent injury are not detected using our methodology.

The results of the bone marrow transplantation studies indicate that the adventitial pericyte population(s) mainly arises via expansion of rare cells in the arterial wall. Experiments with aortic explants support this possibility. NG2-positive cells are rare in the adventitia at the time of dissection, but become numerous 6 days after stimulation with fetal bovine serum, PDGF-BB, or MSCM. Quantification of BrdU incorporation reveals that cell proliferation is responsible for almost all of this increase in MSC numbers, except in the case when we used MSCM, which stimulates up-regulation of NG2 expression in addition to cell proliferation.

In summary, we have identified a population of pericyte-like or mesenchymal stem cells that contribute to restenosis following arterial injury. These cells are distinct from myofibroblasts, smooth muscle cells, and other progenitor populations that have been implicated in promoting the restenotic process, but might be related to other vessel wall progenitor cells that have been described as stem cell sources in various tissues [21, 52–54]. Pericyte-like cells are not derived from the bone marrow, but originate in the adventitia itself. The population expands in the adventitia via cell division rather than by recruitment from external sources. As restenosis progresses, the

pericyte-like cells begin to appear near the vessel lumen, where they give rise to α -SMA-positive cells that contribute to the assembly of the neointima. Manipulation of this population may offer one means of controlling restenosis.

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