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Modulation of the vascular response to injury by autologous blood-derived outgrowth endothelial cells

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Gulati, Rajiv, Dragana Jevremovic, Tyra A. Witt, Laurel S. Kleppe, Richard G. Vile, Amir Lerman, and Robert D. Simari. Modulation of the vascular response to injury by autologous blood-derived outgrowth endothelial cells. Am J Physiol Heart Circ Physiol 287: H512–H517, 2004; 10.1152/ajpheart.00063.2004.—Delivery of a heterogeneous population of cells with endothelial phenotype derived from peripheral blood has been shown to improve vascular responses after balloon arterial injury in an endothelium-dependent manner. Refinement of culture techniques has enabled the generation of outgrowth endothelial cells (OECs), a homogeneous population of distinctly endothelial cells expanded from circulating progenitor cells. The present study tested the hypothesis that OEC delivery would confer vascular protection after balloon arterial injury in a rabbit model. Rabbit peripheral blood mononuclear cells (PBMCs) were cultured in endothelial growth medium for 4–5 wk, yielding proliferative OECs with distinct endothelial phenotype (morphology, incorporation of acetylated LDL, and expression of endothelial nitric oxide synthase and caveolin-1 but not CD14). Animals underwent balloon carotid injury immediately followed by local delivery of autologous OECs for 20 min. Fluorescent-labeled OECs were detected in all layers at 4 wk, with immunostaining revealing maintenance of endothelial phenotype (von Willebrand factor-positive and RAM-11-negative) by luminal and nonluminal cells. To evaluate functional effects, additional animals received autologous OECs, saline, or freshly harvested PBMCs as noncultured cell controls by local dwell after balloon injury. Local OEC delivery improved endothelium-dependent vasoreactivity (P < 0.05 vs. saline and PBMC) and similarly reduced neointimal formation (P < 0.05 vs. saline and PBMC). These data suggest that OECs can be detected in injured arterial segments at 4 wk. Moreover, delivery of OECs conferred greater vascular protection than PBMCs or saline controls and may thus offer a novel, autologous strategy to limit the response to mechanical injury.

progenitor cells; balloon; vasoactivity; restenosis

ACCUMULATING EVIDENCE from animal and human studies suggests that circulating precursor cells may differentiate into new endothelial and smooth muscle cells at sites affected by atherosclerosis, restenosis, and transplant vasculopathy (2, 10, 23–25). Moreover, recent rodent studies have shown that incorporation of bone marrow-derived endothelial cells after balloon arterial denudation can be accelerated by pharmacological therapy (13, 26, 28). In these studies, the increased precursor cell contribution to reendothelialization was shown to be associated with an overall reduction in neointimal formation, and, although a direct causative link was not established, the results were consistent with the hypothesis that circulating endothelial precursor cells may inhibit the vascular response to mechanical injury.

A number of studies have identified cells with endothelial phenotype in adult peripheral blood. In a landmark study, Lin et al. (19) obtained outgrowth of endothelial cells from prolonged culture of the peripheral blood of gender-mismatched bone marrow transplant recipients. These outgrowth endothelial cells (OECs) were of donor karyotype and exhibited a distinctly endothelial phenotype, together with a remarkable proliferative capacity, suggestive of an origin from bone marrow-derived progenitors. Other groups have generated spindle-morphology cells with endothelial features after only short-term culture, reportedly endothelial progenitor cells (3, 15, 20). These cells are capable of tubulization in vitro (9) and can promote angiogenesis in vivo (15, 17). However, recent work suggests that these cells may in fact have a myelomonocytic origin without significant proliferative potential (22) and are therefore likely to be of distinct lineage to the proliferative OECs described by others (5, 19, 21), indicating heterogeneity in the population of peripheral blood mononuclear cells (PBMCs) capable of exhibiting endothelial features (7).

Nonetheless, the ability to generate endothelial-phenotype cells from peripheral blood has provided novel therapeutic opportunities. Transplantation of blood-derived cells appears to promote angiogenesis in ischemic tissue (17, 18), with recent human studies showing promising initial results (1). Blood-derived endothelial cells have also been used to generate nonthrombotic luminal surfaces for vascular prostheses (6, 16). Regardless of origin, endothelial-phenotype cells may afford vascular protection after acute arterial injury. For example, intravenously transfused cells promoted reendothelialization and inhibited neointimal formation in splenectomized mice (27). Moreover, in a rabbit model of balloon carotid injury, we demonstrated that local delivery of a heterogeneous population (which included monocyte-derived cells and true endothelial precursors) improved arterial vasoreactivity in addition to accelerating reendothelialization and reducing neointimal formation. In a similar model, others demonstrated that delivery of outgrowth endothelial cells, expanded from precursors and

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labeled with a retroviral marker, was also associated with accelerated endothelialization and reduced neointimal formation (6). Interestingly, in this study, genetically labeled cells were detectable on the luminal surface 2 wk after delivery, whereas at 4 wk none were apparent in the same location. These findings might suggest that the dominant functional benefit (endothelialization and neointima reduction) gained by cell therapy was provided in the early stages after delivery, although it is also possible that a proportion of delivered cells were resident elsewhere (deeper) in the vessel wall, thereby influencing postinjury proliferative responses through nonbarrier, paracrine mechanisms.

The present study used a rabbit model to evaluate the vascular distribution of a homogeneous population of autologous OECs after administration by local dwell at the time of balloon carotid injury. Furthermore, studies were performed to determine the effects of OEC delivery on endothelium-mediated vasorelaxation and neointimal formation at 4 wk. Finally, given that cultured cells of monocytic and precursor origin have previously been shown to confer vascular protection compared with saline controls (4, 6, 8, 27), we additionally elected to study the effect of noncultured PBMCs as an important control for cultured-cell therapy.

MATERIALS AND METHODS

Cell culture and labeling. All animal procedures were approved by the Mayo Clinic and Foundation Institutional Animal Care and Use Committee. New Zealand White rabbits weighing 3–3.5 kg were briefly sedated with 2 mg of subcutaneous acepromazine to facilitate a 30-ml blood draw via a 20-gauge cannula inserted into a central ear artery. An equal volume of dextrose-saline was administered subcutaneously as replacement. PBMCs were then isolated by density gradient centrifugation of heparinized blood with Histopaque-1083 (Sigma) at 1,600 rpm for 25 min (Beckman), yielding ~10^6 PBMCs per milliliter of blood. All cells were resuspended in 3 ml of EGM-2 (Clonetics) and plated in one well of a fibronectin-coated six-well plate (1 µg/cm^2; Sigma). Supplementary EGM-2 (1–2 ml) was added to wells at 24 h, and nonadherent cells were removed on the following day. The medium was then changed daily for 7 days and on alternate days thereafter. OEC colonies were passaged with 0.025% trypsin (GIBCO) to fresh fibronectin-coated wells or chamber slides when at ~80% confluence, typically around days 12–16 for the first passage, and were finally harvested for in vivo administration at 4–5 wk.

To track the fate of delivered cells, in some experiments, OECs were labeled with CM-1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (CM-Dil, 1 µg/ml), a fluorescent membrane dye (Molecular Probes), as previously described (8), and resuspended in 200 µl of saline for subsequent administration.

Characterization of OECs. Day 20 OECs were probed for expression of CD14 (a monocytic antigen) and the ability to incorporate acetylated LDL (an endothelial feature). Day 4 attached PBMCs served as positive controls for CD14. Cells were first incubated in medium with Dil-labeled acetylated LDL (Biomedical Technologies) at 37°C for 2 h in the dark. Cells were then fixed with methanol, blocked with 10% normal goat serum, and incubated with a murine primary antibody to CD14 (1:150; Dako) and then with FITC-conjugated goat anti-mouse secondary antibody (1:100; Molecular Probes). For detection of the functional endothelial proteins caveolin-1 and endothelial nitric oxide synthase (eNOS), OECs were permeabilized with 0.1% Triton X-100, fixed, blocked with 10% normal goat serum, and incubated with primary rabbit anti-caveolin-1 (1:200; Santa Cruz) and mouse anti-eNOS (1:50; BD Transduction Laboratories) as previously described (8). Cells were then incubated with goat anti-rabbit Texas red (1:500) and goat anti-mouse FITC secondary antibodies (1:1,000; both from Molecular Probes). In all cases, isotype-identical IgGs (Pharmingen) served as negative control antibodies. Hoechst blue (Sigma) was added to the penultimate wash to label nuclei.

Rabbit carotid injury. New Zealand White rabbits were anesthetized with an intramuscular cocktail of ketamine (50 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg). The right common carotid artery was exposed to just below the internal-external bifurcation. After clamp isolation, an 8-0 purse-string suture was placed anteriorly, through which a small arteriotomy was created. A 3F Fogarty balloon catheter (Baxter) was introduced retrogradely into the lumen, inflated to cause just visible distension, and withdrawn three times to denude a 3-cm-long section of artery. An adventitial suture was placed to mark the distal point of injury. Immediately after catheter withdrawal, residual material was removed, and 200 µl of saline with OECs, freshly harvested PBMCs, or saline alone were administered locally for 20 min by instillation through a 24-gauge catheter placed in the lumen. The arteriotomy was closed with the purse-string suture, and clamps were removed to restore antegrade flow.

Cell tracking and immunohistochemistry. Animals were euthanized after 4 wk with an overdose of pentobarbital sodium. Both carotids were excised, embedded in OCT (Tissue-Tek), and immersed in 2-methylbutane cooled by liquid nitrogen. Mounted 5-µm sections were examined under fluorescence microscopy for detection of CM-Dil-labeled cells. Appropriate sections were then fixed in acetone. For detection of von Willebrand factor (vWF) antigen, fixed sections were blocked with 10% normal donkey serum and incubated with mouse anti-rabbit vWF (1:1,000; Binding Site), an antibody previously shown to have rabbit endothelial cross-reactivity (29). Serial sections were blocked with 10% normal goat serum and incubated with mouse anti-rabbit RAM-11 or donkey anti-rabbit antibodies (Vector Laboratories; both: 1:200). Sections were then incubated with goat anti-rabbit Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 568 secondary antibodies (1:200; Molecular Probes). In all cases, isotype-identical Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 568 served as negative controls for CD14.

Arterial vasoreactivity. At 4 wk after balloon injury and local OEC, PBMC, or saline delivery, animals were euthanized (n = 16), and carotid arteries (injured right carotid artery and uninjured left carotid artery as internal control) were immediately immersed in cold Krebs solution. Arterial rings ~4 mm long (3 per artery) were dissected, connected to isometric force displacement transducers, and suspended in organ chambers filled with 25 ml of Krebs solution (94% O_2-6% CO_2). Rings were equilibrated for 1 h at 37°C and then incrementally stretched to 3 g. Viability and maximum contraction were determined with 60 mM KC_1. After three washes with Krebs solution and further equilibration, arteries were precontracted with phenylephrine in a titrated fashion to achieve ~80% stable maximal contraction. To study endothelium-dependent relaxation, acetylcholine (10^{-10}–10^{-5} M) was added to the organ bath in a cumulative manner. After three further washes and equilibration, the arteries were reconstructed, and viability was confirmed by assessment of endothelium-independent responses to sodium nitroprusside, an exogenous nitric oxide donor.

Morphometric analysis. Carotid arteries from vasoreactivity studies were cut into three equal-length segments, fixed with 100% methanol, and embedded in paraffin. Sections (5 µm) were generated at 400-µm intervals (6/segment), and paired slides were stained with Lawson’s elastic van Gieson or hematoxylin and eosin for morphometric analysis. The first two slides (400 µm apart) were analyzed to define the effects on neointimal formation. Endoluminal, internal elastic lamina, and external elastic laminar borders were manually traced using software (Image ProPlus) to calculate intimal and medial areas. Because native media thickness is variable (reflecting the diameter of the artery), it was used to index the area of neointima resulting from balloon injury. Accordingly, neointimal thickness was assessed in terms of intima-to-media area ratios.
RESULTS

Characterization of cultured OECs. OECs were obtained from 9 of 12 rabbit PBMC cultures. Typically about six proliferating cobblestone colonies (range 0–12) emerged at around day 10 in culture (Fig. 1A), equating approximately one colony per $5 \times 10^6$ plated PBMCs. OECs exhibited contact inhibition and proliferated rapidly, requiring multiple passaging. Double-staining experiments demonstrated that OECs incorporated DiI-labeled acetylated LDL, an endothelial feature, but lacked expression of CD14, a monocytic antigen (Fig. 1B). In contrast, PBMCs cultured for 4 days produced an attached population that was dominantly CD14 positive, indicating a different population of monocyte-derived cells (Fig. 1C). Moreover, confocal immunofluorescence revealed that OECs express the characteristic endothelial proteins eNOS and caveolin-1 (Fig. 1D) and form capillaries in Matrigel (not shown). Collectively, these data demonstrate that prolonged, defined culture of rabbit buffy coat PBMCs generates a homogeneous population of OECs that proliferate and exhibit functional endothelial features in vitro but do not express markers of monocytic lineage.

Delivered OECs are detectable 4 wk after balloon injury. A rabbit model was used to determine whether OECs incorporated into vascular segments after mechanical injury. Animals received $1.5 \times 10^6$ CM-Dil-labeled OECs by local dwell after balloon carotid injury and were killed at 4 wk. Specific red fluorescence identified the presence of labeled cells within arterial cross-sectional segments in a gravitational distribution. Labeled cells were seen dominantly in the media (Fig. 2, A and B) but were also observed in the neointima and adventitia. Some fluorescently labeled cells were detected on the luminal border expressing the endothelial antigen vWF (Fig. 2, C–E) but not RAM-11, a macrophage marker (Fig. 2F), or smooth muscle actin (not shown). However, only a minority of luminal endothelial cells at 4 wk were fluorescent, consistent with recent reports that used genetic labeling to identify delivered cells (6). Additionally, some delivered cells appeared to maintain an endothelial phenotype, despite being located in deeper vessel layers, which is not typical for endothelium in this model (Fig. 2, H–L). No staining for smooth muscle actin was seen. However, there were areas of fluorescence, particularly in the media, that did not appear to colocalize with conventional endothelial, smooth muscle, or macrophage markers, raising the possibility that these areas were nonviable cell fragments.

OEC delivery improves endothelium-dependent vasoreactivity at 4 wk. To investigate the effects of cell delivery on vascular function, endothelium-dependent vasoreactivity of carotid rings was evaluated 4 wk after balloon injury and delivery of $1.5 \times 10^6$ OECs suspended in saline ($n = 6$), the same number of PBMCs ($n = 4$), or saline alone ($n = 6$). After preconstriction in an organ chamber, ring relaxation in response to incremental doses of acetylcholine was assessed (Fig. 3). Maximal relaxation of vessel rings from OEC-treated animals was significantly enhanced compared with their PBMC- and saline-treated counterparts (76.4 ± 12.3, 50.1 ± 10.1, and 37.1 ± 13% relaxation for OECs, PBMCs, and saline, respectively, $P < 0.05$ for OEC vs. PBMC or saline). The concentration (−log M) of acetylcholine required to achieve 25% of maximal relaxation was similarly significantly lower for OEC-treated rings than for PBMC- or saline-treated rings (7.29 compared with 6.26 and 5.80, $P < 0.05$ for OEC vs. PBMC or saline). PBMC-treatment ring responses showed a trend toward enhanced relaxation compared with saline, although this was not statistically significant. Additionally, although these data demonstrate that OEC delivery at the time of balloon injury markedly improves subsequent vasoreactivity, the relaxation response to acetylcholine did not achieve that of normal, uninjured vessels ($P < 0.05$).

OEC delivery attenuates neointimal formation. Morphometric analysis of excised carotids demonstrated that OEC delivery was associated with a significant reduction in neointimal formation 4 wk after balloon injury. Neointimal formation was greatest in vessels that had been incubated with saline alone, although this was not significantly more than in PBMC-treated vessels (Fig. 4). Intima-to-media ratios were 0.44 ± 0.06, 0.94 ± 0.12, and 0.87 ± 0.18 for OEC-, saline-, and PBMC-treated vessels, respectively ($P < 0.05$ for OEC vs. each of saline and PBMC). This suggests that, in addition to improving...
endothelium-dependent relaxation, local delivery of OECs also attenuated neointimal formation after mechanical injury.

**DISCUSSION**

The present study demonstrates that locally delivered autologous OECs are detectable in rabbit carotid segments 4 wk after balloon injury. Moreover, OEC delivery appears to be associated with improved endothelium-dependent vasoreactivity and reduced neointimal formation compared with delivery of saline or PBMCs. The use of autologous cells as agents for vascular protection after arterial injury is an emerging strategy that has shown promising results in animal models (6, 8, 27). Our previous study showed that a heterogeneous population of input cells with endothelial phenotype would in vivo favor an endothelial

**Fig. 2.** Detection of OECs in arterial segments 4 wk after balloon injury. OECs were prelabeled with CM-Dil, a membrane dye that characteristically fluoresces red, for subsequent identification. A: low-power image of a single arterial section revealing scattered, discrete fluorescent cellular signals (red). B: section in A viewed with an FITC filter demonstrates autofluorescence of the elastic laminae (green), revealing the dominant fluorescent cellular localization to be in the media. Arrows in A and B indicate internal elastic lamina. Original magnification ×10. C: 5-μm transverse section showing red fluorescent cells on the luminal arterial border and deeper in the intima. D: immunostaining of an adjacent section shows that only luminal cells express von Willebrand factor (vWF), an endothelial marker protein. E: overlay of C and D. F: immunostaining for RAM-11, a macrophage marker, is negative. G: hematoxylin and eosin staining. Arrows in D, F, and G indicate luminal border. H: red fluorescent OECs located in the neointima, but not on the luminal surface. I: adjacent section demonstrating that some of these nonluminal cells maintained an endothelial phenotype as indicated by vWF expression in blue (arrowheads). J: overlay of H and I. K: adjacent section negative for RAM-11. L: hematoxylin and eosin staining. Arrowheads in H, I, and L denote colocalized red fluorescence and vWF immunoreactivity. Original magnification ×40.

**Fig. 3.** OEC delivery improves endothelium-dependent vasoreactivity after arterial injury. Carotid rings from OEC-treated rabbits show markedly enhanced vasoreactivity to acetylcholine 4 wk after injury. Values are means ± SE. *P < 0.05, OEC vs. saline and PBMC. However, uninjured left carotid arteries retained the largest relaxation responses. †P < 0.05, vs. OEC-treated rings.

**Fig. 4.** Local OEC delivery reduces neointimal formation after balloon injury. Significant attenuation of intima-to-media ratio in OEC-treated vessels compared with saline- and PBMC-treated control groups 4 wk after injury. *P < 0.05 vs. both.
phenotype on the luminal border but a macrophage phenotype elsewhere. The present study, delivering a homogeneous population of OECs, likewise demonstrated that at least some cells would retain endothelium-specific antigen expression on the luminal border. However, additional cells found deeper within the neointima also maintained endothelial marker expression, despite the atypical microenvironmental influences of this location. The tracking studies also demonstrated considerable fluorescent marking of the media in a cellular pattern, but immunostaining did not reveal any colocalization of endothelial, macrophage, or smooth muscle antigens. Although this may have been due to technical limitations in cell identification (limited reagents available for the rabbit), it is perhaps more likely that the media fluorescence represents nonviable cells or cell fragments. Ex vivo factors (induction of neoantigens during cell culture) and a nonpermissive in vivo microenvironment (media) might reduce viability of delivered OECs over time.

Local delivery of OECs after balloon injury appeared to be associated with a marked improvement in endothelium-dependent vasoreactivity and a reduction in neointimal formation at 4 wk. Retrospective comparison of these data with our previous work reveals strikingly similar results, even though the present study delivered ~10-fold greater cell numbers and used distinctly endothelial cells (as opposed to the previously used heterogeneous cell population that comprised mostly modified monocytes in addition to rare endothelial precursors) (8). Reasons that OECs did not demonstrate clear superiority might include reduced viability of OECs in vivo, related to prolonged periods of culture required ex vivo. Additionally, the mechanisms of benefit conferred by both populations may have differed, with modified monocytes delivered in the previous study perhaps providing a more robust source of growth factors and cytokines that would indirectly influence vascular behavior (11). Despite similarities in resultant effects of both cell populations, any translational considerations should take into account that in our and other studies examined short-term (4 wk) effects only; 2) unlike the earlier cultured monocyte-dominant populations, OECs are available in near limitless numbers from small blood volumes (7, 12, 19, 22); and 3) OECs may be markedly more susceptible to modification with therapeutic transgenes (14). Acknowledging limitations inherent to retrospective comparisons, the present study additionally evaluated the effect of delivering freshly harvested (noncultured) PBMCs as an important control for cultured-cell therapy. The data demonstrated that the effects of PBMC delivery on vasoreactivity and neointimal formation were not significantly different from the effects of saline alone.

The present study, together with the study reported by Griese et al. (6), raises important questions as to the timing and mechanisms of benefit conferred by OEC delivery. Griese et al. demonstrated that, after balloon endothelial denudation, delivery of retrovirally labeled OECs produced a progressive increase in reendothelialization and reduced neointimal formation over 4 wk, whereas the presence of (detectable) delivered cells on the luminal surface declined over the same period. Similarly, in the present study, only a minority of delivered cells were found on the lumen at 4 wk. Although it remains possible that technical factors may have accounted for some loss of label in both studies (e.g., decline in expression of genetic marker or progressive loss of fluorescent membrane dye with cell division), an alternative explanation might be that the dominant benefit gained by cell therapy occurs at an early time point. As an analogy, one might consider this a “cellular patch,” with OECs perhaps providing an early protective influence during the most vulnerable period immediately after acute injury, akin to the effect of a bandage on wound healing. Additionally, it is conceivable that nonluminal endothelial cells, as were also detected in this study, continued to exert a vascular protective effect through paracrine mechanisms, including the secretion of cytokines and nitric oxide.

Peripheral blood therefore appears to provide a robust source of proliferating OECs that can be harvested and delivered to attenuate vascular responses after balloon injury. Future efforts will include elucidation of some of the protective mechanisms involved and evaluation of the effects of cell delivery on chronic vascular injury processes such as atherosclerosis.

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