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Autologous Culture-Modified Mononuclear Cells Confer Vascular Protection After Arterial Injury

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***Background*—**Bone marrow–derived cells have been shown to contribute to endothelial replacement after vascular injury. In vitro culture of peripheral blood mononuclear cells produces cells with phenotypic characteristics of endothelium. To test the hypothesis that delivery of autologous culture-modified mononuclear cells (CMMCs) to injured arteries could attenuate the vascular response to injury, a rabbit model was studied.

***Methods and Results*—**Rabbit peripheral blood mononuclear cells were cultured in endothelial growth media for 7 to 12 days, yielding highly proliferative cells with distinct endothelial phenotype (expressing CD31 and endothelial nitric oxide synthase and capable of acetylated LDL uptake). A rabbit model of balloon carotid injury was used to evaluate the effect of day 7 CMMC delivery on vascular responses. Animals underwent balloon injury and immediate delivery of autologous CMMCs or buffered saline by 20 minutes of local dwelling. Fluorescence-labeled CMMCs were detected in all vessel layers 4 weeks after delivery. Colonies of cells that localized to the lumen and stained for endothelial markers were also identified. Local CMMC administration at the time of balloon injury accelerated reendothelialization at 4 weeks compared with saline ($P<0.05$). Moreover, CMMC delivery markedly improved endothelium-dependent vasoreactivity at 4 weeks compared with saline ($P<0.005$). Finally, CMMC treatment reduced neointimal formation by 55% at 4 weeks ($P<0.05$).

***Conclusions*—**These data demonstrate that delivery of CMMCs to balloon-injured arteries is associated with accelerated reendothelialization, enhanced endothelium-dependent vasoreactivity, and reduced neointimal formation. Thus, delivery of autologous CMMCs represents a novel vasculoprotective approach to attenuate the response to acute vascular injury. **(*Circulation*. 2003;108:1520-1526.)**

Key Words: atherosclerosis ■ endothelium ■ cells ■ restenosis

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Recent experimental observations suggest that bone marrow–derived cells may contribute to both endothelial replacement and neointimal smooth muscle in multiple models of vascular injury.[1–3] Furthermore, the identification of circulating vascular progenitors in adult blood and the ability to generate enriched populations in vitro[4–7] have provided new opportunities to directly modify the response to vascular injury. It was recently shown that statin therapy accelerated the incorporation of bone marrow–derived endothelial cells in a rat model of balloon arterial injury and reduced neointimal formation.[8,9] Whether the effect of statins on neointimal formation was a direct result of incorporation of bone marrow–derived cells was not established. Taken together, however, these studies have led to the hypothesis that circulating endothelial progenitor cells or their progeny would incorporate into injured arteries and attenuate the vascular response to injury.

The population of circulating cells with endothelial potential may be heterogeneous, including CD14+ monocytes and an infrequent population of cells expressing CD133.[5,10–14] Whether or not these cell populations share similar ontogeny remains to be clarified. In addition, it may be that mixed cell populations engraft to a higher degree,[12,15] suggesting an importance of intercellular cooperation. Therefore, rather than isolating defined cells, culture conditions were optimized to generate a heterogeneous population of culture-modified mononuclear cells (CMMCs) derived from rabbit peripheral blood mononuclear cells intended to be inclusive of these cell types. This study was designed to deliver autologous CMMCs to injured arterial segments and to assess the effects of CMMC delivery on structural and functional parameters in a rabbit model of balloon injury.

Methods

Cell Culture and Labeling

All animal procedures were approved by the Mayo Clinic and Foundation Institutional Animal Care and Use Committee. Periph-

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eral blood mononuclear cells were isolated from 25 mL of rabbit peripheral blood by density-gradient centrifugation with Histopaque-1083 (Sigma) at 1800 rpm for 25 minutes. Cells (5×10^6/mL) were resuspended in EGM-2 (Clonetics) and plated in wells of a fibronectin-coated 6-well plate (1 μg/cm^2; Becton Dickinson). Adherent cells were passaged on day 3 to fresh fibronectin-coated wells or chamber slides, and the medium was thereafter changed daily until harvest.

To track the fate of delivered cells, in some experiments, day 7 CMMCs were labeled with CM-DiI (1 μg/mL), a fluorescent membrane dye (Molecular Probes), as described previously[12] and resuspended in 100 μL saline for administration.

Characterization of Cultured Cells

Immunofluorescence imaging was performed under laser confocal microscopy. Fixed day 7 and day 12 cells were blocked (normal goat serum) and incubated with primary antibodies to CD31, CD14, RAM-11 (all Dako), caveolin-1, and endothelial nitric oxide synthase (eNOS) (both BD Transduction) alone or in combination. FITC- or Texas Red–conjugated IgGs (Vector) served as secondary antibodies where appropriate and isotype-identical IgGs (Pharmingen) as controls. Hoechst blue (Sigma) was added before the final wash to stain nuclei. For coexpression studies, cells were incubated with acetylated LDL as previously described[6] before immunostaining. Fluorescence-activated cell sorting (FACS) detection of CD31 was performed as previously described.[6]

Rabbit Carotid Injury

New Zealand White rabbits weighing 3 to 3.5 kg were anesthetized with ketamine/xylazine. 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 3 times to denude a 3-cm length of artery. An adventitial suture was placed to mark the distal point of injury. Immediately after catheter withdrawal, residual material was removed, and 100 μL of saline with CMMCs or saline alone (control) was administered locally for 20 minutes 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 weeks with an overdose of pentobarbital. 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-DiI–labeled cells. Appropriate sections were fixed in acetone, blocked with 10% normal goat serum, and incubated with antibodies to CD31 (1:40), RAM-11 (macrophage marker, 1:100), or control IgG (matched concentrations). Sections were then incubated with either goat anti-mouse biotinylated antibody (Amersham) and alkaline phosphatase–linked streptavidin or with FITC-conjugated secondary IgG. Vector blue (Vector) was used to identify RAM-11 staining and FITC fluorescence microscopy to detect CD31. Staining for the endothelial marker *Griffonia simplicifolia* lectin I isolectin B4 (BS-1 Lectin, Vector) was performed as for RAM-11, omitting the blocking step.

Reendothelialization

Animals underwent balloon injury and local delivery of 10^5 CMMCs (n=5) or saline (n=6); euthanization was at 4 weeks. Thirty minutes before they were killed, rabbits received an intravenous injection of 5.0 mL of 0.5% Evans blue dye (Sigma) to allow macroscopic examination of the remaining denuded area. Arteries were fixed and incised longitudinally to expose the luminal surface. Planimetric analysis was performed (Image ProPlus) to calculate the reendothelialized area, defined as that not stained with Evans blue.

Arterial Vasoreactivity

Four weeks after balloon injury and local CMMC (or saline) delivery, animals were euthanized (n=12), and carotids (injured right carotid artery; uninjured left carotid artery as internal control) were immediately immersed in cold Krebs solution. Arterial rings ≈4 mm in length (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 hour at 37°C and then incrementally stretched to 3 g. Viability and maximum contraction were determined with 60 mmol/L KCl. After 3 washes with Krebs solution and further equilibration, arteries were precontracted with phenylephrine in a titrated manner to achieve ≈80% stable maximal contraction. To study endothelium-dependent relaxation, acetylcholine (10^{-9} to 10^{-5} mol/L) was added to the organ bath in a cumulative manner. After 3 further washes and equilibration, the arteries were recontracted, and viability was confirmed by assessment of endothelium-independent responses with incremental doses of sodium nitroprusside (10^{-9} to 10^{-5} mol/L), an exogenous NO donor.

Morphometric Analysis

Carotids from vasoreactivity studies (n=12 animals) were cut into 3 segments of equal length, fixed with 100% methanol, and embedded in paraffin. Sections (5 μm) were generated at 400-μm intervals (6/segment), mounted on slides, and stained with hematoxylin and eosin for morphometric analysis. The first 2 slides (400 μm apart) were analyzed to define the effects on neointimal formation. Endoluminal, internal elastic laminar, and external elastic laminar borders were manually traced with software (Image ProPlus) used 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.

Statistical Analysis

All data were analyzed with the SAS software. Normal distribution was tested with the Shapiro-Wilks statistic. Vasoreactivity data were analyzed with ANOVA for repeated measures; direct pairwise comparisons between groups were made with Scheffé's *t* test. Morphometric data (endothelialization and intima-to-media area ratios) were compared with unpaired *t* tests. A value of $P<0.05$ was considered to be statistically significant. Data are presented as mean±SEM.

Results

Characterization of Cultured Cells

Day 7 adherent cells were morphologically heterogeneous and gave rise to highly proliferative cobblestone colonies of cells at approximately day 9 to 12 (Figure 1A). These cells maintained a monolayer, demonstrated contact inhibition, and proliferated through multiple passages. Cells at both day 7 and day 12 readily formed capillaries in Matrigel and stained with BS-1 lectin[6] (not shown).

CMMCs were probed for additional endothelial features as well as for coexpression of monocytic markers. Confocal microscopy demonstrated ≈80% of day 7 CMMCs to both express CD14 (a monocytic antigen) and incorporate acetylated LDL, a commonly used identifier of endothelial function (Figure 1B). A similar proportion of cells stained for RAM-11 (a macrophage marker). Interestingly, day 12 CMMCs exhibited more pronounced expression of endothelial marker CD31 but were negative for monocyte/macrophage markers (Figure 1, B–D). Cells from both time points demonstrated distinct intracellular staining for eNOS (Figure

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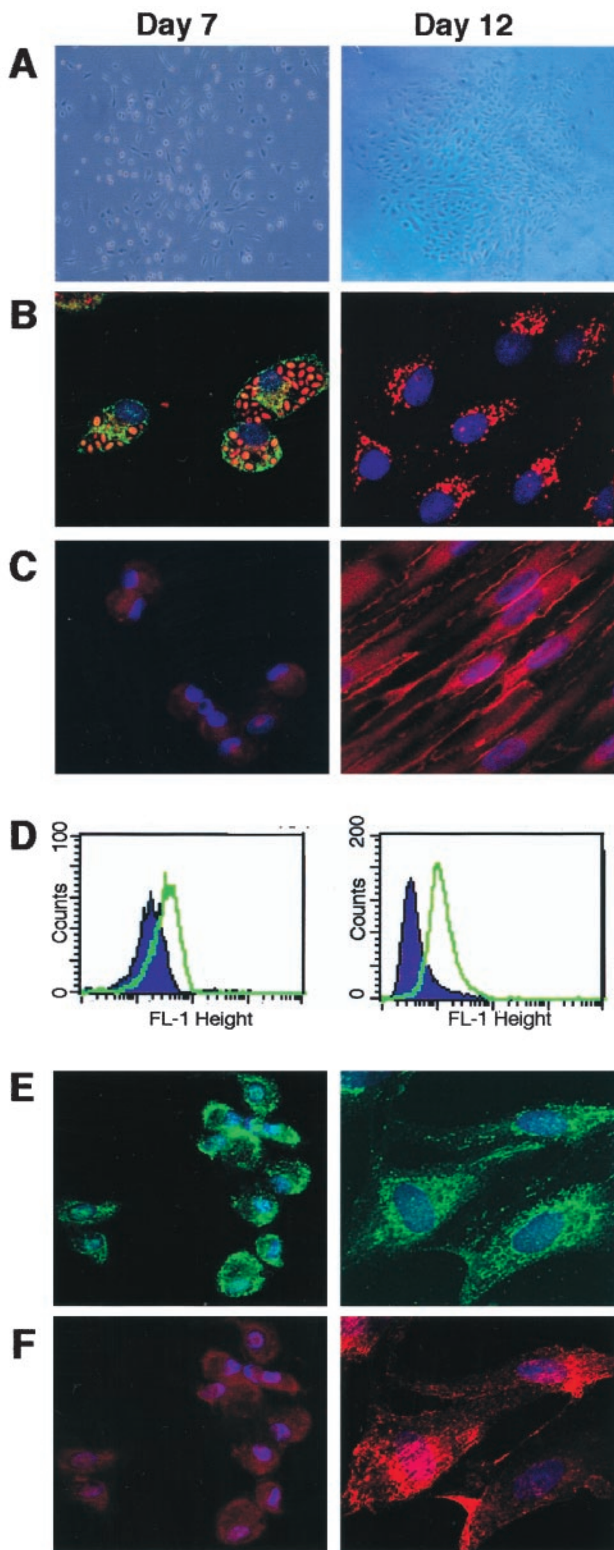

Figure 1. Cultured rabbit peripheral blood mononuclear cells develop endothelial characteristics in vitro. A, Light microscopic images of CMMCs in culture (magnification ×20). B, Fluorescent images demonstrating CD14 expression (green) in day 7 but not day 12 CMMCs. Uptake of DiI-acetylated LDL (red) is seen at both time points (magnification ×100). C and D, More pronounced membrane staining for CD31 in day 12 CMMCs as shown by immunofluorescence (red) and quantitative FACS. Open histograms represent CD31 antibody, and filled histograms represent isotype-matched control IgG. E and F, Confocal immunofluorescence of CMMCs with primary antibodies to eNOS (green) and caveolin-1 (red). Intracellular punctate staining for eNOS is seen at both time points, whereas caveolin-1 is seen only in day 12 CMMCs (magnification ×100).

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1E). However, in contrast to day 7 CMMCs, only cells at day 12 (in colonies) expressed caveolin-1, a protein known to associate with eNOS in mature endothelium (Figure 1F). All cells were negative for smooth muscle actin, and isotype-identical IgG control antibody staining showed negative/background fluorescent staining only (not shown). Collectively, these data demonstrate day 7 CMMCs to be composed predominantly of cells that share functional, antigenic, and cord-forming features in common with endothelial cells while maintaining monocytic features. Furthermore, the day 7 population was capable of producing highly proliferative colonies typical of endothelium in vitro.

Delivered CMMCs Are Detectable 4 Weeks After Balloon Injury

To define the therapeutic potential of CMMCs in modulating the vascular response to injury, a rabbit model was used to first determine whether labeled CMMCs incorporated into the injured vessel wall. Animals received 10^5 autologous CM-DiI–labeled CMMCs (day 7) by local dwelling after balloon carotid injury and were killed at 4 weeks. Specific red fluorescence identified the presence of labeled cells within the neointima, media, and adventitia of injured segments. No labeled cells were identified in uninjured control arteries. Labeled cells were seen lining the lumen that costained for endothelial markers CD31 and BS-1 lectin but not the macrophage marker RAM-11 (Figure 2, A–E). Labeled cells were also identified in other layers, some of which demonstrated macrophage phenotype (Figure 2, F–I). No labeled cells were seen that costained for smooth muscle actin. These data suggest that CMMCs can be delivered locally to injured arterial segments. Furthermore, delivered cells demonstrate heterogeneity in spatial localization and lineage markers 4 weeks after injury.

CMMC Delivery Enhances Reendothelialization at 4 Weeks

Because CMMC-derived cells were detectable at 4 weeks, the effect of local CMMC delivery on arterial reendothelialization after balloon injury was determined. Evans blue dye was administered premortem to stain nonendothelialized areas. The area of original balloon denudation (area between sutures) did not differ between the 2 groups. The reendothelialized area was 91±7% in CMMC-treated vessels versus 67±6% in controls ($P<0.05$) (Figure 3A). In additional studies, CMMCs were labeled with CM-DiI before administration. Luminal staining for Evans blue was absent, indicating complete reendothelialization of the injured artery. En face fluorescence microscopy demonstrated multiple colonies of fluorescence-labeled cells on the luminal surface. However, fluorescent cells composed only ≈5% of the total surface. This suggests direct but incomplete participation of delivered CMMCs in arterial reendothelialization (Figure 3B).

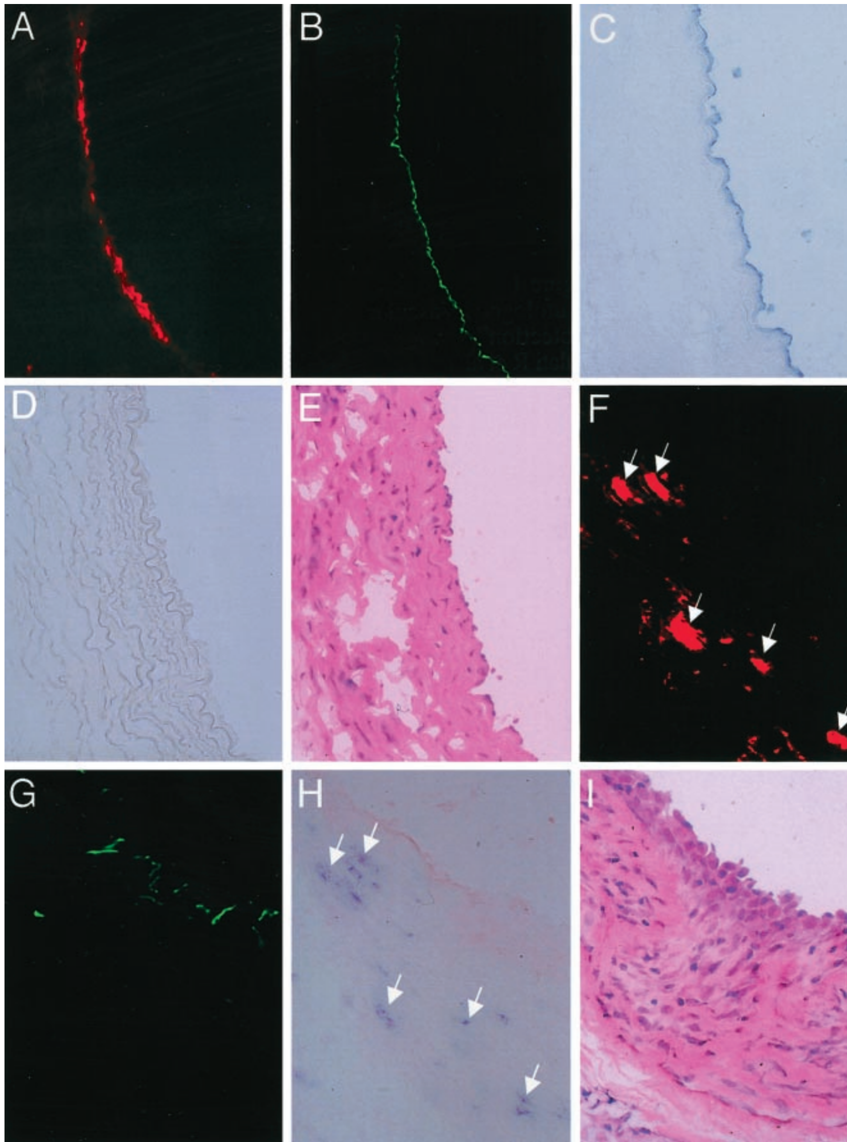

Figure 2. Administered CMMCs incorporate into vessel wall after balloon carotid injury. A, Carotid section demonstrating labeled CMMCs on luminal border 4 weeks after local delivery. Colocalization staining of endothelial antigens CD31 (B) and BS-1 lectin (C) but (D) negative staining for macrophage marker RAM-11. E, Hematoxylin and eosin staining of adjacent arterial section. F, Labeled cells also detected in neointima that (G) do not costain for CD31 but (H) do stain for RAM-11, consistent with macrophage lineage. I, Hematoxylin and eosin staining of adjacent arterial section. Red indicates CM-DiI fluorescence; green, CD31; arrows, colocalization of neointimal CMMCs with RAM-11 (blue). Magnification ×40.

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CMMC Delivery Enhances Endothelium-Dependent Vasorelaxation 4 Weeks After Balloon Injury

To investigate the effect of CMMC delivery on vascular function, endothelium-dependent vasorelaxation of carotid rings was examined at 4 weeks. After precontraction with phenylephrine in an organ chamber, ring relaxation in response to incremental doses of acetylcholine was assessed (Figure 4). Maximal relaxation of vessel rings from CMMC-treated animals was significantly enhanced compared with saline-treated counterparts (77.6±4.6% versus 28.2±10.3%, $P<0.005$). The concentration (−log mol/L) of acetylcholine required to achieve 25% of maximal relaxation (EC_{25}) was 7.19±0.04 in CMMC-treated rabbits compared with 5.38±0.06 in saline-treated animals ($P<0.005$). Although the data clearly demonstrate that CMMC delivery markedly enhanced endothelium-dependent vasorelaxation, responses did not achieve those of uninjured vessels, which retained the largest responses to acetylcholine ($P<0.05$ for maximal relaxation and EC_{50} compared with CMMC treatment).

CMMC Delivery Attenuates Neointimal Formation

Because early reendothelialization has been shown to attenuate neointimal formation,[16] the effect of CMMC delivery on neointimal formation was determined. Morphometric analysis of excised carotids showed a significant reduction of neointimal thickness in vessels from CMMC-treated animals 4 weeks after balloon injury (Figure 5). Intima-to-media ratio was 0.39±0.08, versus 0.86±0.17 for saline treatment ($P<0.05$). This suggests that in addition to improving endothelium-dependent relaxation, local delivery of CMMCs also attenuated neointimal formation after mechanical injury.

Discussion

New paradigms regarding the inherent role of circulating cells, including precursor cells in postnatal neovascularization, have presented novel therapeutic opportunities. In this regard, the studied applications of endothelial-lineage cell therapy have demonstrated enhancement of new capillary formation in ischemic tissue (therapeutic vasculogen-

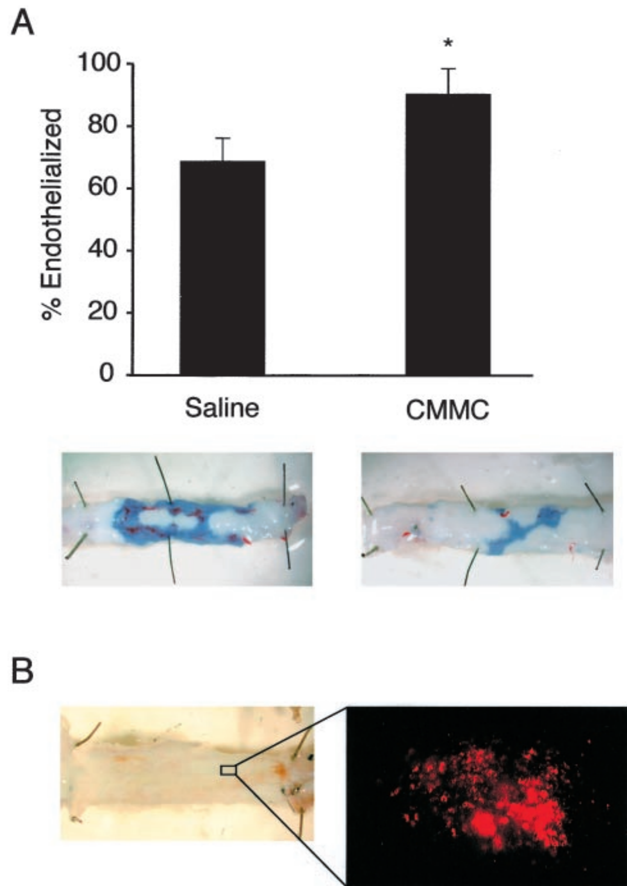

Figure 3. Local CMMC delivery accelerates reendothelialization after balloon injury. A, Reendothelialized area at 4 weeks is significantly greater in carotids from CMMC-treated animals than saline-treated counterparts, *$P<0.05$. Representative photographs of exposed carotid lumens 4 weeks after balloon injury and delivery of CMMCs or saline. Reendothelialized areas do not stain blue. B, En face lumen microscopy 4 weeks after injury and delivery of fluorescence-labeled CMMCs. Absence of Evans blue staining suggests complete reendothelialization. Multiple colonies of fluorescence-labeled cells were seen on luminal surface, suggesting direct participation in reendothelialization (representative example of 1 colony).

esis)[6,17–19] and generation of antithrombogenic luminal surfaces in prosthetic grafts.[20] The present study tested whether peripheral blood mononuclear cells, after culture modification toward an endothelial phenotype, may have an applied role in modulating the vascular response to balloon injury. It is reported here that incorporation of CMMCs into injured arterial segments is associated with accelerated reendothelialization and markedly enhanced endothelium-dependent vasoreactivity. Furthermore, CMMCs delivered at the time of injury effect an overall reduction in subsequent neointimal formation.

Even though the suggestion that blood can give rise to endothelium is not new,[21] the characterization of putative endothelial precursor populations is recent and ongoing.[4–6,13] Although outgrowth of highly proliferative typical endothelial cells from rare circulating blood precursors has been demonstrated,[5,10] it also appears that a much larger population of CD14+ monocytes is capable of adopting endothelial features and incorporating into neovasculature.[11,12] It is also notable that the majority of blood-derived endothelial progenitor cells defined by Kalka et al[6] and Rehman et al[13] express both monocytic and endothelial markers. The extent to which these cells act purely by forming structural channels as has been described with macrophages[22] or whether the cells adopt sustained and truly functional endothelial properties (antithrombogenic, vasoregulatory, etc) remains to be clarified. Given the difficulties in defining and isolating putative progenitors and given that cooperation between mixed cell phenotypes may be advantageous,[12] an inclusive approach was adopted for this study. In vitro culture of rabbit

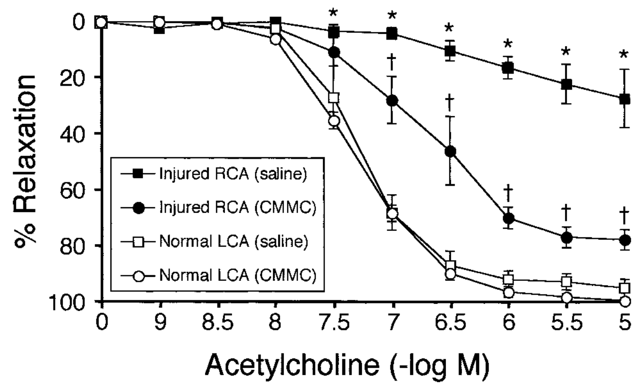

Figure 4. Enhanced endothelium-dependent vasoreactivity in carotid rings from CMMC-treated rabbits. Vessels from CMMC-treated rabbits show markedly enhanced vasoreactivity to acetylcholine 4 weeks after injury. Data expressed as mean % relaxation±SEM (*$P<0.05$ for CMMC- vs saline-treated injured arteries). Uninjured left carotid arteries retained largest responses (†$P<0.05$ for maximal relaxation and EC_{50} compared with CMMC rings). RCA indicates right carotid artery; LCA, left carotid artery.

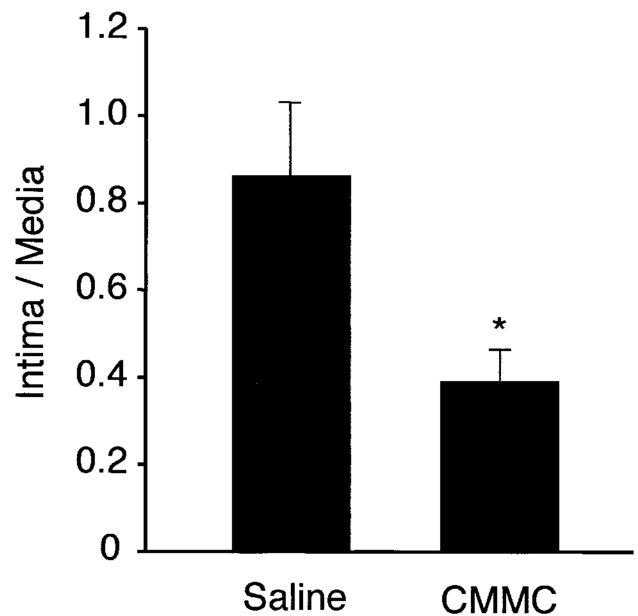

Figure 5. Local CMMC delivery reduces neointimal formation after balloon injury. Significant attenuation of intima-to-media ratio in arteries from CMMC-treated animals vs saline-treated control group (*$P<0.05$).

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peripheral blood under specific conditions for 7 days produced a cell population 80% of which coexpressed monocytic and endothelial features. Continued culture produced rapidly expanding, uniquely endothelial (CD14/RAM-11 negative) colonies thereafter. Similarly, cells with distinct endothelial (but not monocytic) phenotype were identified in vivo on the luminal border at 4 weeks. Given that ≈20% of the input CMMC population was negative for monocyte markers, it is not possible to determine whether the endothelial phenotype cells at day 12 in vitro or at 4 weeks in vivo arose from monocytic transdifferentiation (loss of markers) or from a nonmonocytic starting population.

The finding of delivered cells over a small proportion of the luminal surface suggests direct but incomplete participation in endothelial resurfacing. Although the proportion may have been underestimated because of loss of fluorescence with cell division, it remains somewhat difficult to reconcile such a small area of direct contribution with the overall increase in reendothelialization reported. It is thus speculated that indirect mechanisms may also be involved. CMMC incorporation may alter the kinetics of the denuded surface to induce proliferation of neighboring resident endothelium or recruit additional circulating precursors. In support of this possibility, it was shown that bone marrow–endothelial lineage cells express angiogenic ligands and cytokines[13,23] and induce proliferation of preexisting vasculature in the vicinity of myocardial infarcts.[15]

The margin by which CMMC delivery improved endothelium-dependent vasoreactivity is a striking feature of this study. The effect is likely to be mediated at least in part by accelerated reendothelialization. However, nonluminally located cells (as were also found in this study) could also influence vascular reactivity and smooth muscle proliferation through paracrine mechanisms, including the release of NO (via eNOS) into the surrounding milieu. Indeed, adenoviral gene transfer of eNOS to the adventitia has been shown to improve NO production and vasoreactivity even in arteries without endothelium.[24]

Circulating cells that may contribute to neointimal formation include endothelial and smooth muscle precursors,[2,3] in addition to monocytes and lymphocytes.[25] Cell delivery to injured arterial segments could thus also theoretically be deleterious. Indeed, fluorescent RAM-11–staining cells were seen after local delivery, implying macrophage phenotype or phagocytosis of delivered cells by resident macrophages. Although CMMC-derived smooth muscle cells were not detected, the presence of smooth muscle precursors in the delivered population cannot be excluded.[7] Furthermore, direct or induced generation of adventitial vasa vasorum increasing plaque vascularity[26] or induction of transdifferentiation processes[27] may serve to hasten intimal expansion. Yet the results reported here show that the effect of CMMC delivery is an overall reduction in cellular accumulation. This reduction may be mediated in part by accelerated restoration of an endothelial barrier. It is also conceivable that the provision of supplementary sources of NO and other secreted factors deeper in the vessel wall may suppress activation and recruitment of additional plaque-forming cells. Future studies will be designed to test these hypotheses and to address the important question of whether delivery of defined cell populations is superior to the inclusive, heterogeneous approach used here.

Overall, the findings reported in this study expand the potential applications of cell therapy to direct modulation of vascular responses to injury. Furthermore, they have implications for gene therapy and tissue engineering approaches to vascular disease.

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