Carotid Repair Using Autologous Adipose-Derived Endothelial Cells

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Background and Purpose—Adipose tissue is an abundant source of endothelial cells as well as stem and progenitor cells which can develop an endothelial phenotype. It has been demonstrated that these cells have distinct angiogenic properties in vitro and in vivo. However, whether these cells have the capacity to directly improve large vessel form and function after vascular injury remains unknown. To define whether delivery of adipose-derived endothelial cells (ADECs) would improve healing of injured carotid arteries, a rabbit model of acute arterial injury was used.

Methods—Autologous rabbit ADECs were generated using defined culture conditions. To test the ability of ADECs to enhance carotid artery repair, cells were delivered intraarterially after acute balloon injury. Additional delivery studies were performed after functional selection of cells before delivery.

Results—After rabbit omental fat harvest and digestion, a proliferative, homogenous, and distinctly endothelial population of ADECs was identified. Direct delivery of autologous ADECs resulted in marked reendothelialization 48 hours after acute vascular injury as compared to saline controls (82.2 ± 26.9% versus 4.2 ± 3.0% P < 0.001). Delivery of ADECs that were selected for their ability to take up acetylated LDL significantly improved vasoreactivity and decreased intimal formation after vascular injury.

Conclusions—Taken together, these data suggest that ADECs represent an autologous source of proliferative endothelial cells, which demonstrate the capacity to rapidly improve reendothelialization, improve vascular reactivity, and decrease intimal formation in a carotid artery injury model. (Stroke. 2009;40:1886-1891.)

Key Words: adipose • stem cells • restenosis • endothelial

Carotid artery and large vessel endothelial injury and dysfunction are a hallmark of many cardiovascular diseases.1,2 As such, strategies to enhance endothelial function and reendothelialization have been shown to have important beneficial effects on vascular structure and function.3,4 These strategies have included the use of cytokines and growth factors to stimulate resident endothelial growth. Strategies to more directly enhance reendothelialization have included attraction of circulating cells with endothelial potential as well as the direct delivery of cells with a more defined endothelial phenotype.5-9

Adipose tissue contains 2 potent sources of endothelial cells, namely resident microvascular endothelial cells and adipose-derived stromal cells.10-14 In humans, the former may be defined as CD34+/CD45- cells that express CD31 and CD144. Adipose derived-stem cells (CD34+/CD45- / CD31-) share many properties with marrow stromal cells, are capable of endothelial differentiation, and are found in close proximity to endothelial cells (ECs) within adipose tissue. Adipose-derived stem cells have been shown to have distinct angiogenic properties, which include the ability to improve blood flow in a murine hind limb ischemia model.12 As adipose tissue has been shown to be a potent source of endothelial cells, we hypothesized that delivery of ECs derived from adipose tissue (ADECs) in addition to their angiogenic potential may have the capacity to improve carotid artery form and function after acute vascular injury.

Methods

Cell Culture

All animal procedures were approved by the Mayo Clinic and Foundation Institutional Animal Care and Use Committee. Omental fat was harvested from New Zealand White rabbits (3.5 to 4.5 kg; Myrtle’s Rabbitry, Inc, Thompson’s Station, Tenn) under deep anesthesia with Isoflurane. Through a 1-inch incision in the epigastric region right below the sternum along the linea alba, about 3 to 5 g of omentum was exposed with a hook, and feeder vessels were ligated before the fat was removed. After mechanical disaggregation, the omentum was washed with Ca2+- and Mg2+-free PBS (Cellgro). A 1% solution of HBSS with Ca2+ and Mg2+ (Cellgro) and collagenase type I (Worthington, 142,926/mg) was used to digest the...
tissue. After 1 hour at room temperature, the supernatant was removed and the solution gently centrifuged at 1600 rpm. Cells were washed once with HBSS and once with EGM-2 (Lonza) and afterward plated on fibronectin (1 μg/cm²; Becton Dickson) in EGM-2 with either 2% autologous serum (AS) or 2% fetal bovine serum (FBS). Cells were passaged before reaching confluence, and the medium was changed daily until harvest.

Immunotyping of ADECs
Laser confocal microscopy was used to image cells on day 7. Cells were fixed, permeabilized, and blocked with either 10% normal goat serum or 10% normal donkey serum followed by incubation with 5 μg/mL mouse anti-eNOS (BD Transduction Labs). 0.7 μg/mL mouse antihuman smooth muscle actin (Dako), or 19 μg/mL sheep anti-human von Willebrand Factor (vWF; The Binding Site). Concentrated matched mouse and sheep IgG served as negative controls. eNOS and smooth muscle actin were visualized using goat anti-mouse fluorescein (Molecular Probes). vWF was visualized using donkey anti-sheep biotin followed by Streptavidin AlexaFluor 488 (Invitrogen). Hoechst staining identified the nuclei. Dil-acetylated LDL was also used as a co-staining with vWF eNOS and SM-actin as previously described for coexpression studies. Also, staining with Dil (1.1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodide) acetylated LDL (Biomedical Technologies Inc) and DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) acetylated LDL (Biomedical Technologies Inc) was performed. Where indicated, quantification (% positive or negative staining) was determined by counting under low-power confocal microscopy (25 fields). Flow cytometry for determination of surface hematopoietic antigen expression was performed using a fluorochrome-linked primary antibody to CD45 (Serotec MCA808).

Functional Analysis of Rabbit ADECs
To determine the proliferative capacity of ADECs and the impact of sources of sera, ADECs from 12 different rabbits were studied. Six cultures were grown with EGM-2 with 2% FBS and 6 cultures with EGM-2 and 2% autologous serum. Cells were detached using 0.05% Trypsin and 0.53 mmol/L EDTA in HBSS (Cellgro, Mediatech Inc) every other day, and cells were counted with a hemocytometer. To determine the angiogenic potential of the ADECs, tube forming assays were performed using Matrigel (Geltrex Reduced Growth Factor Basement Membrane Matrix, GIBCO). Plates were coated with 150 μL of Matrigel, and cells were plated in a density of about 1000 cells per 96-well plate. As a control, rabbit carotid artery endothelial cells were used. Matrigel cultures were imaged at 24 hours and imported into Image Pro Plus 5.1 (Media Cybernetics). The total length of each tube or the long axis of single cells or groups of adjacent cells was measured. The assay was performed in triplicate and repeated 3 times.

FACS Enrichment of Ac-LDL Positive ADECs
At least 36 hours after the last trypsination, cells were washed 2 times with PBS. 10 μg/mL DiO-Ac-LDL was diluted to the standard medium (EGM-2) and added to live cells for 4 hours. After 4 hours, the media containing the remaining DiO-Ac-LDL was removed and the cells washed again using PBS and twice using probe-free media. Cells were then trypsinated and washed 2 times with EGM-2 and afterward once with PBS. The cell suspension was filtered using MACS single cell filter from Milteny Scientif. Then the single cell suspension in PBS without Mg²⁺ and Ca²⁺ (to prevent cell attachment) was transferred under sterile conditions to the facility to proceed with the cell sorting. Unstained cells were used as negative control.

Animal Model of Carotid Injury and Cell Delivery
To determine whether ADECs would improve the vascular response to injury, a rabbit model of arterial injury was used. New Zealand White rabbits weighing 3.5 to 4.5 kg were anesthetized with Isoflorane. Rabbits were treated with aspirin (81 mg) 1 day before injury and daily throughout 28 days. The left and the right common carotid artery were exposed from the suprasternal notch to just below the internal/external bifurcation. After looping with a 4-0 suture, vessel clamps were used to isolate the vessels. An 8-0 purse-string suture was placed, through which a small arteriotomy was created. A 2F Fogarty balloon catheter (Baxter) was introduced antegrade into the vessel lumen. The balloon was inflated to cause a visible distension and withdrawn 3 times to demude a 3-cm length of artery as previously described. After balloon injury, local delivery of Dil labeled ADECs in PBS or PBS was performed. Through the arteriotomy, a 24-gauge catheter was placed in the vessel lumen and in the right carotid artery 250 μL of PBS without Ca²⁺ and Mg²⁺ and in the left carotid artery 250 μL of a single cell suspension of ADECs (2 to 3×10⁶) in PBS without Ca²⁺ and Mg²⁺. Cells were allowed to dwell in the absence of blood flow for 20 minutes. Afterward, the arteriotomy was closed with a purse string suture, and the vessel clamps were removed to restore antegrade flow.

Evaluation of Vascular Function and Structure
To determine whether delivery of cells resulted in attachment and residence (early reendothelialization), a group of rabbits were euthanized at 48 hours (n=20). Twenty minutes before sacrifice, rabbits received an intravenous injection of 20 mL of 0.5% Evan’s Blue dye (Sigma), an albumin-bound dye that stains blue areas of disrupted endothelial integrity. Reendothelialization was quantified using planimetric analysis (Image ProPlus 5.1, Media Cybernetics) of the nonstained segment within the injured vessel.

Segments of reendothelialized vessels were analyzed to determine whether the delivered cells were present after delivery. En face imaging to detect Dil was performed, and in addition, cross sections were stained with sheep-anti vWF (1:500, The binding Site) and Griffonia simplicifolia lectin I solectin B4 (1:100, Vector laboratories).

To determine the effects of ADEC delivery on vascular reactivity and neointimal formation after vascular injury, cells were used after 7 days in culture. Two series of studies were performed. In the first, Dil-labeled cells were delivered without selection (n=6). In the second (n=12) to improve homogeneity of delivered cells, cells were labeled with DiO acetylated LDL and selected by FACS before delivery. This procedure allowed for selection of functional (ability to take up Ac-LDL) features before delivery. Notably, cell selection did not affect subsequent proliferation but did shift the curve slightly to the right (data not shown) suggesting a need for recovery from the sorting process. Rabbits were euthanized at 28 days after injury and arterial sections were analyzed using previously described methods. Briefly, arteries were excised, dissected free of perivascular tissues, cut transversely into rings, and suspended in oxygenated Krebs in an organ chamber. After graded radial stretching and determination of maximal contraction with potassium chloride, rings were contracted to ~80% maximum and then challenged with incremental doses of acetylcholine to quantify endothelial-dependent relaxatory response. To determine the effects of cell delivery on neointimal formation, transverse sections (5 μm) of formalin-fixed arteries were stained with hematoxylin and eosin and analyzed with Image ProPlus software to evaluate neointimal formation. To index for variations in media thickness or off plane sectioning, intima to media ratios were calculated.

Statistical Analysis
Data were analyzed with SPSS software version 11.5 (SPSS Inc) and PRISM GraphPad version 4.03 (GraphPad Software Inc). Comparison between groups was performed with Student t test, and morphometric data were compared with unpaired t-tests. Vasoreactivity data were compared using ANOVA. A probability value <0.05 was considered as statistically significant. Graphs were created with Prism GraphPad version 4.03 and Sigma Plot version 9.0 (Systat Software Inc). Data are presented as mean±SEM.

Results
Rabbit ADEC Generation and Characterization
Autologous ADECs were obtained from 3 to 5 g of rabbit omental fat. Colonies were visible after 6 hours (Figure 1A)
and were expanded rapidly demonstrating cobblestone morphology (Figure 1B) and achieving greater than $4 \times 10^7$ cells within 14 days (Figure 1C). There was no difference in cell growth whether FBS or autologous serum was used (Figure 1D).

The endothelial nature of the ADECs was confirmed by immunophenotyping. After 7 days in culture, confocal immunofluorescence microscopy revealed that ADECs expressed vWF (97±3%), eNOS (98±3%), VEGFR2 (95±6%), while 97±3% were SM-actin negative (Figure 2). ADECs also uniformly bound Griffonia Lectin and demonstrated uptake of DiI acetylated LDL (94.1±1.2%, Figure 2).

By flow cytometry, cells were uniformly negative for the pan-hematopoietic surface antigen CD45. In a Matrigel tube-forming assay, ADECs formed more capillary-like tubes after 24 hours than rabbit carotid artery endothelial cells (Figure 2G and 2H), suggestive of a microvascular phenotype. These findings demonstrate that cells obtained and derived from omental fat generate a homogeneous and highly proliferative population of endothelial cells. In contrast with previous studies of rabbit blood-derived endothelial cells, the rapidity of cell-colony generation in the current study is suggestive, albeit not conclusive of a nonprogenitor origin.

**Effects of ADEC Delivery on Reendothelialization After Balloon Injury**

To study the potential of ADEC delivery to enhance reendothelialization, a rabbit model of acute carotid artery balloon injury was used. ADECs (2 to $3 \times 10^6$) were labeled with DiI acetylated LDL (for subsequent identification) before intraluminal delivery. Forty-eight hours after delivery, assessment of intraluminal coverage was measured by exclusion of Evans-Blue staining. At this time point, Evans Blue exclusion was demonstrated in 82.2±26.9% of the treated vessel compared with 4.2±3.0% of untreated vessels ($P<0.001$; Figure 3A). In cross-sections of the injured and treated vessels, staining for vWF was performed and showed immu-
noreactivity along the whole vessel wall (Figure 3B). En face and cross sectional imaging for DiI fluorescence demonstrated perilumenal residence of delivered cells (Figure 3C through 3F). These data suggest that delivered ADECs rapidly endothelialize injured arterial segments.

**Effects of ADEC Delivery on Vascular Reactivity and Neointimal Formation**

After delivery of unselected ADECs (Figure 4), there was improvement in response of precontracted rings to acetylcholine (maximal relaxation $62.2 \pm 8.6\%$ in unselected ADEC treated arteries versus $20.1 \pm 4.8\%$ in control arteries; $P<0.01$). Although there was a trend toward less intimal formation with unselected cell delivery, the differences were not statistically significant.

Labeling and selection of ADECs for Ac-LDL allowed for delivery of cells, which were capable of Ac-LDL uptake as an attempt to reduce fibroblast or smooth muscle cell contamination. After delivery of selected ADECs (Figure 5), there was improvement in response of precontracted rings to acetylcholine (maximal relaxation $66.3 \pm 4.1\%$ in selected ADEC treated arteries versus $13.2 \pm 4.9\%$ in control arteries; $P<0.01$). Delivery of selected cells resulted in a 40% decrease in intimal formation as assessed by the intima/media ratio ($0.30 \pm 0.03$ versus $0.49 \pm 0.05$; $P<0.002$). Notably, phenotypic analysis indicated that 99% of selected cells to be acetylated LDL positive (versus 94% in unselected) and $\approx 0.5\%$ to express smooth-muscle actin (versus $\approx 3\%$ in unselected). Taken together, these data suggest that the local delivery of selected ADECs modifies the vascular remodeling response to acute balloon injury. Additionally, these data suggest that increased purity of delivered cells resulted in a more potent vascular effect with improved structure and function.

**Discussion**

Endothelial disruption and dysfunction is central to the initiation and propagation of acute and chronic vascular disease. Enhancing endothelial healing has been an important
goal for many strategies to improve vascular remodeling. With the increased understanding of the dynamic interface between endothelium, blood, and tissue, the opportunities to regulate the endothelial healing process have increased. Several groups have used direct delivery of vascular and circulation-derived endothelial cells or cells capable of assuming an endothelial phenotype in animal models of vascular injury.5,6,8 These approaches have used autologous cells to avoid immunologic responses, but translation of these studies has been limited by practical and theoretical issues concerning cell phenotype, potency, and availability. Harvesting vascular tissue to isolate endothelial cells has the advantage of obtaining large vessel endothelium (albeit perhaps without great proliferative capacity) but has practical limitations. The use of circulation-derived cells mitigates the need for harvesting but is limited by the rare nature of rigorously defined “endothelial progenitor cells.” Also, the number and phenotype of circulation-derived cells are negatively affected by the presence of atherosclerosis risk factors and the presence of vascular disease.16,17 Thus, the need for an autologous and highly proliferative source of endothelial cells led to the isolation of adipose tissue-derived endothelial cells (ADECs).

ADECs are highly proliferative and fully endothelial in nature. Functionally, ADECs form tubes in vitro and take up acetylated LDL. A recent study has defined a close physical interaction between adipose stromal cells and microvascular endothelium (both CD34+/CD45−) in adipose tissue, and indeed, both cell types may be initially required for culture expansion of ADECs.14 As adipose stromal cells and microvascular endothelial cells share phenotypic markers and separation techniques remain limited, further clarification of the responsible cell type for ADECs generation is warranted. Unfortunately, reagents specific for the rabbit are unavailable to define the source of ADECs used here. The rapid growth of colonies might favor an abundant resident microvascular endothelial cell rather than a progenitor cell, but the question remains open.

ADECs have potent vasculoprotective capacity. In vivo, when delivered to injured arterial segments, ADECs functionally exclude Evan’s blue dye while lining the injured vessel. The rapid reendothelialization has important structural and functional consequences. Additionally, the ability of ADECs to take up acetylated LDL allowed for selection of cells by labeling with DiO-acetylated LDL and selection before delivery. This selection process diminished the potential for contaminating fibroblasts or smooth muscle cells that may have limited the functional effects of nonselected cells. However, one cannot exclude an alternative effect of selection on ADEC potency including removal of nonviable cells.

Adipose tissue is a rich source of mesenchymal stem cells and microvascular endothelial cells.10–12 As such, translationally relevant isolation procedures are being developed to facilitate cell separation and culture for use clinically.11 These approaches may include closed culture systems from harvest to delivery. In addition, access to autologous fat, although requiring a minimal incision, is generally feasible in the majority of patients. It is clinically apparent that adipose maintains its ability to grow (requiring blood supply)
throughout adult life. Whether the phenotype of ADECs is affected by diabetes, hyperlipidemia, or other cardiac risk factors remains to be determined.

In distinction to circulation-derived cells, the proliferative capacity of endothelial cells may allow for use in a clinically reasonable time frame. Although circulating monocyctic cells capable of assuming an endothelial phenotype might be available in reasonable numbers from blood, the poorly defined circulating endothelial progenitor that is capable of generating endothelial cells is quite rare in peripheral blood and generally requires 2 to 4 weeks in culture.18–20 Thus, ADECs represent a potent source of autologous endothelium for delivery.

Indeed, endothelial cell delivery may provide a unique opportunity to affect the care of patients with acute and chronic vascular disease. Autologous endothelium might be used to line prosthetic devices and grafts as well as for therapeutic angiogenesis. Delivery of cells and the intravascular retention of cells, although somewhat problematic, have been addressed by the use of biophysical and biochemical forces for delivery on stents and grafts. Magnetic properties have been used to maintain cells at target vessels and grafts,21,22 Taken together, these approaches may provide new opportunities for the translation of endothelial biology toward clinical therapies.

Conclusion
These data suggest that ADECs represent an autologous source of proliferative endothelial cells using endothelial culture conditions. Furthermore, ADECs demonstrate the capacity to rapidly improve reendothelialization and vascular reactivity and decrease intimal formation after vascular injury. Thus, ADECs may provide a potent autologous cell source with distinct vasculoprotective potential.

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Disclosures
None.

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