Endothelial Cells Overexpressing Interleukin-8 Receptors Reduce Inflammatory and Neointimal Responses to Arterial Injury

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Background—Interleukin-8 (IL8) receptors IL8RA and IL8RB on neutrophil membranes bind to IL8 and direct neutrophil recruitment to sites of inflammation, including acutely injured arteries. This study tested whether administration of IL8RA- and/or IL8RB-transduced rat aortic endothelial cells (ECs) accelerates adhesion of ECs to the injured surface, thus suppressing inflammation and neointima formation in balloon-injured rat carotid arteries. We tested the hypothesis that targeted delivery of ECs by overexpressing IL8RA and IL8RB receptors prevents inflammatory responses and promotes structural recovery of arteries after endoluminal injury.

Methods and Results—Young adult male rats received balloon injury of the right carotid artery and were transfused intravenously with ECs (total, $1.5 \times 10^6$ cells at 1, 3, and 5 hours after injury) transduced with adenoviral vectors carrying IL8RA, IL8RB, and IL8RA/IL8RB (dual transduction) genes, AdNull (empty vector), or vehicle (no EC transfection). ECs overexpressing IL8Rs inhibited proinflammatory mediators expression significantly (by 60% to 85%) and reduced infiltration of neutrophils and monocytes/macrophages into injured arteries at 1 day after injury, as well as stimulating a 2-fold increase in reendothelialization at 14 days after injury. IL8RA-EC, IL8RB-EC, and IL8RA/IL8RB-EC treatment reduced neointima formation dramatically (by 80%, 74%, and 95%) at 28 days after injury.

Conclusions—ECs with overexpression of IL8RA and/or IL8RB mimic the behavior of neutrophils that target and adhere to injured tissues, preventing inflammation and neointima formation. Targeted delivery of ECs to arteries with endoluminal injury provides a novel strategy for the prevention and treatment of cardiovascular disease. (Circulation. 2012;125:1533-1541.)

Key Words: restenosis | endothelial cells | inflammation | receptors, interleukin-8 | tissue therapy

The endothelium is a dynamic component of the cardiovascular system that plays an important role in health and disease. It is not only a barrier between the circulating blood and vascular smooth muscle cells but also the source of mediators that regulate vascular tone and growth, platelet function, and coagulation.1 Endothelial damage/dysfunction in vivo leads to loss of its antiaggregative, antithrombotic, anti-inflammatory, and antivasovasomotor smooth muscle cell activation/growth properties.2,3

Clinical Perspective on p 1541

Neutrophils form the early line of defense against tissue injury. Neutrophils migrate to injured tissue in response to the chemoattractant interleukin-8 (IL8), which is expressed and released in large amounts by injured or infected tissues. IL8 binds to the selective IL8 receptors IL8RA and IL8RB on the neutrophil surface.4-5 Activated IL8RA and/or IL8RB induce expression of chemotactic mediators that trigger local inflammation. Neutrophils are the main leukocyte subset that interacts with the damaged endothelium, triggers the initial proinflammatory response, and facilitates the influx of other classes of inflammatory cells, eg, monocytes/macrophages and T cells, in the setting of acute vascular injury.6,7

Using the rat carotid artery balloon injury model, we tested the hypothesis that targeted delivery of endothelial cells (ECs) by overexpressing IL8RA and IL8RB prevents inflammatory responses and promotes structural recovery of arteries after endoluminal injury. Rat aortic ECs were transduced with adenoviral vectors carrying neutrophil IL8RA and IL8RB and intravenously transfused into rats with balloon injury of the...
common carotid arteries. We demonstrated that administration of ECs equipped with this homing device mimicked the behavior of neutrophils at the site of vascular injury, thus attenuating the infiltration of neutrophils and suppressing inflammation. We further demonstrated that ECs overexpressing IL-8 receptors accelerated reendothelialization and inhibited neointima formation in injured arteries.

**Methods**

**Animals and Procedures**

Twelve-week-old male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories and maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (6 AM–6 PM) and fed a standard rat pellet diet ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Rats were weighed before and 2 or 4 weeks after carotid injury.

One day before carotid injury, rats were anesthetized with 80 mg/kg ketamine and 5 mg/kg xylazine and subjected to cannulation of a femoral vein. On the next day, rats were subjected to balloon injury of the right carotid artery. The contralateral unjured left carotid artery served as a control.

**Plasmid and Adenoviral Vector Generation**

ECs that overexpress IL8RA and/or IL8RB were generated by use of adenoviral vectors that contain human IL8RA and/or IL8RB cDNAs and the green fluorescent protein (GFP) gene with the AdEasy Adenoviral Vector System (Stratagene) as summarized in the text and in Figure 1 of the online-only Data Supplement.

**In Vitro Characterization of Rat Aortic ECs Transduced With IL8RA-GFP, IL8RB-GFP, or AdNull-GFP Adenoviral Vectors**

Rat aortic ECs and the EC growth medium MCDB-131C were purchased from VEC Technologies (catalog No. RAEC/T-75 and MCDB-131C). ECs were cultured to 80% confluence and transduced with the adenoviruses containing IL8RA-GFP, L8RB-GFP genes, or AdNull-GFP (empty adenoviral vector control). More than 80% of the ECs became green 48 hours after transduction. To examine the expression and distribution of IL8RA, IL8RB, or GFP in transduced ECs, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS and then stained with selective primary antibodies against IL8RA (catalog No. ab60254, Abcam) or IL8RB (catalog No. ab24963, Abcam) and a Texas Red–labeled goat anti-mouse IgG secondary antibody (catalog No. 115–075–003, Jackson ImmunoResearch Laboratory). After additional nuclear staining with DAPI, ECs were mounted for confocal fluorescent microscopic analysis with a computerized Zeiss-Axioskop system.

Separate groups of ECs transduced with both IL8RA and IL8RB (IL8RA/RB) were used to examine the effects of overexpression of IL8RA and IL8RB on cell proliferation, migration, and apoptosis in vitro. Untransduced ECs (vehicle-EC) and ECs transduced with AdNull vectors were used as controls. Proliferative activity was assessed with direct cell counts at 1, 4, 9, and 24 hours after cell seeding (cells reached 100% confluence at >30 hours of culture). Migratory activity was assessed with the Boyden chamber technique as described previously and in the online-only Data Supplement. Apoptotic activity was assessed with the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (Cell Technology).

To confirm that IL8RA-, IL8RB-, and IL8RA/RB-ECs can functionally mimic neutrophil binding to IL8, the ability of transduced ECs to attach to activated endothelial monolayers and to compete with neutrophils for binding to the monolayers was assessed. In the first experiment, the attachment of transduced ECs to endothelial monolayers was assessed. Endothelial monolayers in 24-well plates were treated with tumor necrosis factor-α (TNF-α; 10 ng/mL) for 16 hours before IL8RA, IL8RB, IL8RA/RB, or AdNull ECs (1.0×10⁶ cells) were added. At 15, 30, 45, and 60 minutes, ECs were washed 3 times with PBS, and GFP-labeled ECs adherent to the activated monolayers were counted. In the second experiment, activated neutrophils were prepared as described previously and as described in the online-only Data Supplement. Various concentrations (0.5, 1.0, and 1.5×10⁶ cells) of IL8RA/RB-ECs (GFP labeled) were applied to TNF-α (10 ng/mL)–pretreated endothelial monolayers. Neutrophils (1.0×10⁶ cells, prelabeled with CellTracker CM-Dil in red) were added 30 minutes after the application of IL8RA/RB-ECs and incubated for an additional 1 hour before cell (red neutrophils and green ECs) counting.

**In Vivo Transfusion Regimen**

Rat aortic ECs transduced with AdNull-GFP, IL8RA-GFP, or IL8RB-GFP or cotransduced with IL8RA-GFP and IL8RB-GFP were cultured in 100-mm culture dishes until >80% cells expressed GFP (1.5×10⁶ cells per dish). Cells were washed with 0.9% saline, collected with a cell scraper, dispersed by gentle pipetting, concentrated with centrifugation at 100g, and resuspended in 1.5 mL normal saline. Each rat was transfused 3 times with ECs (0.5×10⁶ cells/500 μL) through a femoral venous catheter at 1, 3, and 5 hours after carotid artery injury. Control rats received a corresponding volume of normal saline (vehicle-control). After carotid artery injury, rats (n=6 per group) were randomly divided into 5 groups that received saline (vehicle-control; group 1), ECs transfused with AdNull-GFP (AdNull-EC; group 2), ECs transfused with IL8RA-GFP (IL8RA-EC; group 3), ECs transfused with IL8RB-GFP (IL8RB-EC; group 4), or ECs cotransfused with IL8RA-GFP and IL8RB-GFP (IL8RA/RB-EC; group 5).

**Effects of IL8RA-, IL8RB-, IL8RA/RB-, and AdNull-ECs on Inflammation and Remodeling in Injured Arteries**

Rats were euthanized for assessment of attachment of transfused ECs to the injured endoluminal surface by confocal microscopic analysis of GFP cells (30 minutes after EC transfusion). Proinflammatory mediator mRNA expression was measured by real-time quantitative reverse transcriptase–polymerase chain reaction (24 hours after EC transfusion). Infiltration of neutrophils (myeloperoxidase staining), monocyte/macrophages (ED1 staining), and transduced ECs (GFP staining) was assessed by immunohistochemistry (24 hours after EC transfusion), and reendothelialization was measured by Evans blue staining (14 days after EC transfusion). Neointima formation was determined by quantitative morphometric analysis of elastin-stained arteries (28 days after EC transfusion). The contralateral unjured left common carotid arteries were used as controls.

**Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis of Inflammatory Mediators**

RNA was extracted from arteries, reverse transcribed to cDNA, and amplified by polymerase chain reaction with specific primers (Table I in the online-only Data Supplement) for TNF-α, monocyte chemotactic protein-1 (MCP-1), P-selectin, cytokine-induced neutrophil chemoattractant-2 (CINC-2β; equivalent to human IL-8), vascular cell adhesion molecule-1, IL-1α, IL-6, and IL-10 and quantified with the iCycler (Applied Biosystems). Levels of specific mRNAs were normalized by use of ribosomal protein S9 mRNA because expression of this housekeeping gene has been shown to remain stable in the rat carotid artery injury model.

**Immunohistochemical Analysis of Neutrophil, Monocyte/Macrophage, and Transfused EC Infiltration Into Injured Carotid Arteries**

One day after EC transfusion, formalin perfusion–fixed carotid arteries were harvested and embedded in paraffin. The avidin-biotin peroxidase immunohistochemical technique was used to detect GFP-labeled IL8RA-, IL8RB-, IL8RA/RB-, and AdNull-transduced ECs.
neutrophils, and monocytes/macrophages in paraffin-embedded sections of carotid arteries with a Vector Laboratories kit (Biotechnology). Transfused ECs, neutrophils, and monocytes/macrophages were recognized by specific primary antibodies against GFP, myeloperoxidase (Santa Cruz Biotechnology), and ED1 (Serotec), respectively.

Evans Blue Staining for Reendothelialization
Balloon-injured carotid arteries harvested at 14 days after injury were examined for reendothelialization. Thirty minutes before death, rats received an intravenous injection of Evans blue dye (0.5 mL of 0.5% in saline, Fisher, catalog No. 23860) and then perfused with 500 mL of 1× PBS. Carotid arteries were harvested and photographed, and the area of denuded endothelium was identified by blue staining. The ratio of area stained blue (no endothelium) to total common carotid artery length (from aortic arch to bifurcation of internal and external carotid arteries) was calculated.

Morphometric Analysis of Neointima Formation
Neointima-to-media ratios were calculated from 5-μm cross sections of the injured carotid arteries at 28 days after injury. Morphometric analysis of representative cross-sectional photomicrographs of injured carotid arteries was performed with a computer-based Biosquant II Morphometric system. At least 3 elastin-stained sections from the middle third of each vessel were examined, and the measurements were averaged for statistical analysis. The cross-sectional surface areas of the vessel within the external elastic lamina, within the internal elastic lamina, and within the lumen were measured. Neointima formation in the injured artery was expressed as the ratio of neointima (internal elastic lamina area minus lumen area) to media (external elastic lamina area minus internal elastic lamina area).

Statistical Analysis
In each in vivo experiment, rats were age matched to minimize individual differences. Results were expressed as mean±SEM. Statistical analysis was carried out with the SigmaStat statistical package (version 3.5). The primary statistical tests were 2-way ANOVA and 1-way ANOVA. When the overall F test result of ANOVA was significant, a multiple-comparison Tukey test was applied. Student t test was used in 2-mean comparisons. Differences were reported as significant when P<0.05.

Results
Adenoviral Transduction Increases IL8RA and IL8RB Expression in ECs But Does Not Attenuate EC Proliferation, Migration, or Apoptosis
Confocal fluorescence microscopic analysis showed that IL8RA and IL8RB were overexpressed in the endoplasmic reticulum and on the cell membrane but not in the nucleus of the IL8RA- and IL8RB-transduced ECs (Figure 1A–1C). GFP was expressed in the nuclei and perinuclear endoplasmic reticulum in IL8RA-, IL8RB-, and AdNull-transduced ECs (Figure 1D–1F). IL8RA and IL8RB were undetectable when AdNull-ECs were probed with anti-IL8RA or anti-IL8RB antibodies (data not shown), indicating that endogenous IL8RA and IL8RB levels were very low in these cells.

Adenoviral transduction did not alter baseline or IL8-stimulated EC proliferation, apoptosis, or migration (comparison between AdNull-ECs and vehicle-ECs [without adenoviral transduction]). There were no statistically significant differences in mean values of proliferative activity between experimental groups (Figure 1G). IL8 dose dependently (20–500 ng/mL) enhanced migration of vehicle-control, AdNull-, IL8RA-, IL8RB-, and IL8RA/RB-ECs. IL8-stimulated cell migration was significantly greater in IL8RA/RB-ECs (2-way ANOVA, P<0.05) compared with the other 4 experimental groups (Figure 1H). Baseline apoptotic activity was undetectable in all 3 cell types, and IL8 treatment did not alter apoptosis in any cell type (data not shown). Thus, overexpression of IL8RA and/or IL8RB did not attenuate basal or stimulated proliferation, migration, or apoptosis of transduced ECs.

Attachment of IL8RA-, IL8RB-, and IL8RA/RB-ECs to endothelial monolayers was time dependently greater than that of AdNull-ECs (2-way ANOVA, P<0.05; Figure 2A). Furthermore, IL8RA/RB-ECs dose dependently inhibited the binding of neutrophils to endothelial monolayers (Figure 2B). In an additional experiment, we showed that neutrophils could also inhibit the binding of IL8RA/RB-EC to endothelial monolayers (Figure II in the online-only Data Supplement).

Figure 1. A through F. Confocal fluorescence micrographs showing overexpression of the interleukin-8 receptors IL8RA or IL8RB in rat aortic endothelial cells (ECs). ECs were transduced with adenoviral vectors carrying (A and D) IL8RA, (B and E) IL8RB genes, or (C and F) AdNull (ECs transduced with the empty adenoviral vector) with a green fluorescent protein (GFP) marker. Arrowheads indicate the expression of IL8RA or IL8RB on the EC membrane. The expression of IL8RA, IL8RB, and GFP was controlled by separate cytomegalovirus promoters. D through F, Immunofluorescence stain showing expression of GFP in the same ECs. C, DAPI staining of the nuclei of cells in D. G, Proliferative activity (assessed by actual cell counts) of adenosine-transduced ECs overexpressing IL8RA, IL8RB, or both RA and RB (IL8RA/RB). Vehicle (Veh) control is ECs without adenoviral transduction. AdNull control is ECs transduced with empty adenovirus. H, Migratory activity (assessed by Boyden chamber technique for 12 hours) of ECs overexpressing IL8RA, IL8RB, or both IL8RA and IL8RB. The bottom wells contain various concentration of IL-8. Results are mean±SEM; n indicates wells or chambers. Two-way ANOVA was used to analyze the data in G and H. *P<0.05, IL8RA/RB vs other groups in H.

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and IL8RA/RB-EC transfusion (0.5×10^6 cells, intravenously), indicating that ECs equipped IL8RA and IL8RB infiltrated into the injured arteries (Figure 2C). In rats transfused with AdNull-ECs, only a few GFP-positive ECs could be found in adventitia (Figure 2D). Furthermore, transfused IL8RA/RB-ECs attached to the endoluminal surface of carotid arteries as early as 30 minutes after balloon injury and EC transfusion (Figure 2F).

**ECs Transduced With IL8RA, IL8RB, and IL8RA/RB Inhibit Inflammation in Injured Carotid Artery**

Real-time quantitative reverse transcriptase–polymerase chain reaction analysis of 24-hour–injured and control uninjured carotid arteries from rats transfused with AdNull-ECs or vehicle (no EC transfusion) showed that all inflammatory mediators measured (TNF-α, MCP-1, P-selectin, CINC-2β, vascular cell adhesion molecule-1, IL-1β, IL-6, and IL-10) were expressed at very low levels in uninjured vessels and increased markedly after injury (Figure 3). IL8RA–, IL8RB–, and IL8RA/RB-EC transfusion resulted in significant reductions in mRNA levels of the inflammatory cytokine TNF-α, the adhesion molecule P-selectin, and the chemokines MCP-1 and CINC-2β compared with vehicle- and AdNull-EC groups. The reductions of TNF-α, MCP-1, P-selectin, and CINC-2β by IL8RA/RB-EC treatment were the greatest among the 3 groups. Expression of vascular cell adhesion molecule-1, IL-1β, IL-6, and IL-10 mRNA in injured carotid arteries was not altered by IL8RA/RB-EC treatment and did not differ significantly from levels in injured arteries of AdNull-EC– and vehicle-treated rats (data not shown).

Immunohistochemical staining demonstrated large numbers of myeloperoxidase–positive neutrophils and ED1+ monocytes/macrophages in the adventitial domains of 24-hour–injured arteries from vehicle-treated and AdNull-EC–treated rats; neutrophil and monocyte/macrophage numbers were greatly reduced by IL8RA–, IL8RB–, and IL8RA/RB-EC treatment (Figure 4). The number of neutrophils in adventitia of injured carotid artery was negatively correlated with the number of GFP-labeled ECs and positively correlated with the number of monocytes/macrophages (Figure III in the online-only Data Supplement). Infiltrating neutrophils and monocytes/macrophages could not be detected in the media of injured arteries in any treatment group.

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** A, The attachment rates of interleukin-8 receptors IL8RA, IL8RB, and IL8RA/RB endothelial cells (ECs) to an in vitro endothelial monolayer were faster than that of ECs transfused with empty adenoviral vector (AdNull-EC). Endothelial monolayer was stimulated with tumor necrosis factor-α (TNF-α; 10 ng/mL) for 16 hours before AdNull, IL8RA, IL8RB, or IL8RA/RB ECs (with green fluorescent protein [GFP] green color) applied to the monolayer. The asterisk indicates that the slopes of the lines of IL8RA, IL8RB, and IL8RA/RB ECs were steeper (P<0.05) than that of AdNull-ECs. The attachment rates were not statistically different among the IL8RA, IL8RB, and IL8RA/RB groups. Results are mean±SEM; n indicates wells per time point. Two-way ANOVA was used to analyze the data in A. B, IL8RA/RB-ECs inhibited binding of neutrophils to the in vitro endothelial monolayer in a dose-dependent manner. The endothelial monolayer was stimulated with TNF-α (10 ng/mL) for 16 hours before various concentrations of IL8RA/RB-ECs (GFP color) were applied to the monolayer. Neutrophils (labeled with CellTracker CM-Dil, red) were added 30 minutes after the application of IL8RA/RB-ECs and incubated for additional 1 hour before cell (red and green) counting in the same well. Results are mean±SEM; n indicates wells per time point. Two-way ANOVA was used to analyze the data in B. C, Representative confocal fluorescence micrographs (×100) of GFP-labeled AdNull-ECs in the injured rat carotid artery 24 hours after injury and cell transfusion. D, Representative GFP immunofluorescence-stained micrograph (×400) showing accumulation of GFP-labeled IL8RA/RB-ECs in the injured rat carotid artery (especially in the adventitia and vasa vasorum) 24 hours after balloon injury of the carotid artery and transfection of IL8RA/RB-ECs. Transfused ECs were immunostained with GFP primary antibodies. D, Representative GFP immunofluorescence–stained micrograph (×100) of GFP-labeled AdNull-ECs in the injured rat carotid artery 24 hours after injury and cell transfusion. E, Representative confocal fluorescence micrographs (×400) showing GFP-labeled IL8RA/RB-ECs attached to the endoluminal surface of balloon-injured rat carotid artery 30 minutes after transfection of IL8RA/RB-ECs. The injured artery was opened longitudinally to expose the endoluminal surface and washed 3 times with PBS before the micrograph was taken.
ECs Transduced With IL8RA/RB Accelerate Reendothelialization of Injured Carotid Artery

To elucidate the effects of IL8RA/RB-EC transfusion on reendothelialization, Evans blue staining of 14-day–injured and uninjured carotid arteries of rats transfused with IL8RA/RB-ECs, AdNull-ECs, or vehicle (no EC transfusion) was performed. Arteries from rats treated with IL8RA/RB-ECs had smaller areas of blue staining relative to arteries from rats that received AdNull-ECs or vehicle (Figure 5A). Uninjured contralateral carotid arteries were virtually negative for Evans blue staining (data not shown). Quantitative assessment showed that transfusion with IL8RA/RB-ECs resulted in a 2-fold reduction in the expression of chemokines and adhesion molecules in injured and contralateral uninjured control arteries at 24 hours after injury. Each rat received femoral venous transfusions of ECs (0.5 × 10^6 cells per injection in 500 μL saline) at 1, 3, and 5 hours after carotid artery injury. mRNA levels measured by real-time quantitative reverse transcriptase–polymerase chain reaction were first normalized with ribosomal protein S9 (RpS9) mRNA to correct for differences in total RNA loading and then standardized to the mean value of the respective vehicle–uninjured group, which was assigned a value of 1. The vehicle group received balloon injury of the right carotid artery but did not receive EC transfusion. Results are mean ± SEM; n indicates number of rats. One way ANOVA was used to analyze the data. *P < 0.05 vs respective uninjured groups. #P < 0.05 vs respective vehicle and AdNull-EC groups. MCP-1 indicates monocyte chemotactic protein-1; CINC-2β, cytokine-induced neutrophil chemoattractant-2β (equivalent to human IL-8).

Figure 3. Effects of interleukin-8 receptor IL8RA, IL8RB, IL8RA/RB, or AdNull endothelial cell (EC) transfusion on mRNA expression of chemokines and adhesion molecules in injured and contralateral uninjured control arteries at 24 hours after injury. Each rat received femoral venous transfusions of ECs (0.5 × 10^6 cells per injection in 500 μL saline) at 1, 3, and 5 hours after carotid artery injury. mRNA levels measured by real-time quantitative reverse transcriptase–polymerase chain reaction were first normalized with ribosomal protein S9 (RpS9) mRNA to correct for differences in total RNA loading and then standardized to the mean value of the respective vehicle–uninjured group, which was assigned a value of 1. The vehicle group received balloon injury of the right carotid artery but did not receive EC transfusion. Results are mean ± SEM; n indicates number of rats. One way ANOVA was used to analyze the data. *P < 0.05 vs respective uninjured groups. #P < 0.05 vs respective vehicle and AdNull-EC groups. MCP-1 indicates monocyte chemotactic protein-1; CINC-2β, cytokine-induced neutrophil chemoattractant-2β (equivalent to human IL-8).

Figure 4. Effects of interleukin-8 receptor IL8RA, IL8RB, IL8RA/RB, or AdNull endothelial cell (EC) transfusion on neutrophil and monocyte/macrophage infiltration into adventitia of injured carotid artery 24 hours after transfusion of ECs overexpressing IL8RA, IL8RB, or both IL8RA/RB. Neutrophils and monocytes/macrophages were immunostained with myeloperoxidase and ED1 primary antibodies, respectively. A and B, Representative micrographs showing the myeloperoxidase-positive and CD11c+ cells in adventitia, respectively. C and D, Bar graphs showing neutrophil and monocyte/macrophage densities in adventitia of injured carotid arteries of rats transfused with AdNull, IL8RA, IL8RB, or Ad-IL8RA/RB ECs. The vehicle group received balloon injury but did not receive EC transfusion. Results are mean ± SEM; n indicates number of rats. One way ANOVA was used to analyze the data. *P < 0.05 vs respective vehicle and AdNull-EC groups.
an ≈2-fold increase in reendothelialization at this time point (Figure 5B).

**ECs Transduced With IL8Rs Decrease Neointima Formation Injured Carotid Artery**

Morphometric analysis showed that at 4 weeks after balloon injury, the neointima-to-media area ratios of injured carotid arteries were significantly smaller in rats transfused with ECs that overexpress IL8RA and/or IL8RA (Figure 6A). Treatment with IL8RA-, IL8RB-, and IL8RA/RB-ECs reduced neointima-to-media area ratios by 80%, 74%, and 95% (compared with the AdNull-EC group), respectively (Figure 6B). The mean values of medial areas were not statistically significantly different among the treatment groups (Figure IV in the online-only Data Supplement). The vasoprotective effects of transfusion with ECs transduced with both IL8RA and IL8RB were greater than those of transfusion with ECs transduced with either IL8RA or IL8RB alone. As an additional control, we found that transfusion with rat aortic smooth muscle cells transduced with IL8RA and IL8RB genes (1.5×10^6 cells) did not inhibit neointima formation (Figure 6).

**Discussion**

This study has shown for the first time that intravenous transfusion of ECs transduced with IL8RA and/or IL8RB results in a significantly reduced inflammatory response, enhanced reendothelialization, and diminished neointima formation in balloon-injured rat carotid arteries. These results support our hypothesis that ECs that overexpress IL8RA

and/or IL8RB mimic the behavior of neutrophils that target and adhere to injured tissues, thus minimizing the inflammatory response to injury, accelerating reendothelialization, and inhibiting subsequent neointima formation (Figure 7). Targeting delivery of ECs equipped with the homing device (IL8RA and RB receptors) to the site of arterial injury, as demonstrated in this study, provides a novel strategy for the prevention and treatment of cardiovascular diseases.

Cell-based therapies for vascular and cardiac diseases have proliferated over the past decade but have had limited success and only moderate benefit in clinical application. The major hurdles for successful cell therapies are cell-type selection (progenitor versus differentiated cells), time for cell delivery (short- versus long-term treatment), cell delivery mode (direct into tissue versus peripheral administration), and targeted delivery of cells to damaged tissue to maximize therapeutic effects. The present study demonstrates an innovative, relatively noninvasive strategy to overcome the above hurdles. We have shown that acute intravenous transfusion of healthy differentiated adult rat ECs overexpressing selective neutrophil IL8RA and IL8RB receptors within hours after endoluminal injury of rat carotid artery effectively inhibits inflammatory mediator expression, inflammatory leukocyte infiltration, reendothelialization, and neointima formation in injured vessels.
IL-8, a member of the CXC chemokine subfamily, is a major chemoattractant/activator for neutrophils and has been implicated in a variety of inflammatory diseases. A large variety of cell types, including endothelial and epithelial cells, monocytes, T lymphocytes, neutrophils, and fibroblasts, express IL-8 mRNA and protein, and expression of IL8 is significantly increased in the setting of acute cell/tissue injury. In particular, IL8 plays a crucial role in recruiting neutrophils to sites of injury (eg, adhesion and transendothelial migration) to sites of blood vessel injury. Il8-overexpressing IL8RA and/or IL8RB mimic the behavior of neutrophils that target and adhere to the sites of injury. Proteolytic cleavage of collagen by matrix metalloproteinases generates an acetylated tripeptide, N-acetylated proline-glycine–proline (Pro-Gly-Pro [PGP]), which mimics key sequences of IL-8, stimulates IL8RA and IL8RB, and prolongs influx of neutrophils. We postulate that IL8RA/RB-ECs compete with the binding of neutrophils to IL8 and/or the collagen fragment (Pro-Gly-Pro) expressed in injured tissue and thus inhibit neutrophil-induced inflammatory responses (eg, infiltration of monocytes/macrophages and expression of proinflammatory cytokines) and accelerate healing of the injured tissue. IEL indicates internal elastic lamina; EEL, external elastic lamina; and VSMC, vascular smooth muscle cell.

In a more long-term setting, IL8 increases the adhesion of neutrophils to the endothelium and induces their transendothelial migration, initiating vascular dysfunction and vascular diseases, including atherosclerosis, aortic aneurysm formation, and hypertension. Activated neutrophils exert potent cytotoxic effects through the release of proteolytic enzymes and proinflammatory effects through the upregulation of cytokines such as TNF-α, MCP-1, and P-selectin, which induce recruitment of monocytes to areas of injury. Monocyte-derived macrophages then produce additional cytokines and growth factors necessary for fully developed inflammation. These processes induce myofibroblast transformation, proliferation, and neovascularization, leading to tissue repair, fibrosis, and scar formation. Thus, blockade of initial neutrophil adhesion and infiltration has the potential for downregulating interactions between neutrophils and injured tissues, effectively attenuating inflammatory responses to tissue injury.

The acute inflammatory response to endoluminal vascular injury plays an important role in determining the extent of subsequent neointima formation and vascular remodeling. Our previous studies showed that medial smooth muscle cells (SMCs) are activated immediately after endoluminal arterial injury via a combination of EC denudation and direct mechanical trauma and release a variety of chemoattractants (eg, CINC-2β [equivalent to human IL-8] and MCP-1), which reach the adventitial and periadventitial space by diffusion through the vessel wall and via the circulation (vasa vasorum). Adventitial cells are activated within hours of endoluminal vascular injury and recruit inflammatory leukocytes (neutrophils initially and monocyte/macrophages later) to the adventitia in the early phase of the injury response. The influx of neutrophils triggers the inflammatory reaction by secreting cytokines (eg, TNF-α, IL-1, IL-6) and contributes to tissue injury by releasing proteases (eg, collagenase, elastase, and myeloperoxidase). Release of these mediators from neutrophils stimulates further neutrophil/macocyte, smooth muscle cell, and adventitial fibroblast activation, as well as smooth muscle cell and adventitial fibroblast proliferation and migration into neointima. We have shown that inhibition of these early proinflammatory responses with estrogen partially attenuates neointima formation in balloon-injured rat carotid arteries. In the present study, transfection of IL8-RA/RB-ECs significantly attenuated expression of proinflammatory mediators and completely inhibited neointima formation in injured carotid artery, supporting our hypothesis that IL8RA/RB-ECs compete with neutrophils at the site of injury and protect injured tissues from inflammatory damage. In the late phase of the vascular injury response, reendothelialization of the damaged vessel limits the extent of vascular remodeling. We and others have demonstrated that SMC proliferation and neointima formation can be inhibited by facilitating reendothelialization at sites of balloon injury, supporting the hypothesis that interventions that accelerate reendothelialization inhibit the vascular injury response. Regeneration and proliferation of ECs after vascular injury were long believed to be a local process regulated by adjacent ECs in the intact uninjured intima. This dogma was challenged by recent evidence that circulating endothelial progenitor cells or bone marrow–derived stem cells promote endothelial repair by seeding themselves on the damaged vascular surface and participating in reendothelialization.
alization and repair after vascular injury.20–29,31 Our current data show enhanced reendothelialization of the injured carotid artery in rats receiving transfection of IL8RA/RB-ECs compared with AdNull- and vehicle-treated rats, suggesting that overexpression of IL8RA and IL8RB in ECs enhanced reendothelialization by increased the homing of ECs to injured arteries.

Interestingly, IL8 shares neutrophil chemotactic properties with a specific collagen-derived tripeptide fragment, N-acetylated proline-glycine–proline (acPGP), and expression of acPGP in injured tissue has been reported to induce neutrophil chemotaxis in murine models.22–32 acPGP is a proteolytic cleavage product of collagen by matrix metalloproteinases (eg, matrix metalloproteinase-8 and -9) and prolyl endopeptidase, a key proteolytic enzyme involved in the inflammatory process. acPGP has a 3-dimensional structure very similar to that of IL8 and acts as a potent neutrophil chemoattractant through binding to IL8RA and IL8RB on neutrophil membranes.22,32–33 Levels of matrix metalloproteinases and proteases and generation of acPGP correlate closely with the level of inflammation in tissue, indicating that structural proteins (eg, collagen or elastin) can regulate innate immunity by prolonging the influx of neutrophils into injured tissue.22 We postulate that acPGP also acts as a local ligand that attracts the adhesion of IL8RA/RB-ECs to the damaged endoluminal surface and adventitial tissue of the injured carotid artery (Figure 7).

Conclusions
Our data demonstrate that transfection of ECs overexpressing IL8RA and IL8RB inhibits neointima formation in balloon-injured carotid arteries. Because transfused IL8RA/RB-ECs were present both on the injured endoluminal surface and in the adventitia, these results support our hypothesis that inhibition of neointima formation is mediated by both decreasing the neutrophil-mediated proinflammatory response and accelerating reendothelialization of arteries with endothelial injury. We also demonstrated that inhibition of inflammation and neointima formation was greater after IL8RA/RB-EC treatment than after IL8RA-EC or IL8RB-EC treatment, indicating that IL8RA-II8RB heterodimers have better functionality than either IL8RA or IL8RB homodimers in transduced ECs. The present study has significant clinical application. Equipped with the homing device, ECs will mimic the behavior of neutrophils that target and adhere to injured tissue and thus will inhibit inflammation and accelerate tissue repair. Targeted delivery of ECs to sites of cardiovascular injury provides a novel therapeutic strategy for cardiovascular disease.

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Disclosures
None.

References
Clinical Perspective

The endothelium is a functional component of the cardiovascular system that plays an important role in health and disease. Injury or dysfunction of endothelium can lead to many forms of cardiovascular disease, and the injured endothelium can serve as a therapeutic target in the prevention and treatment of cardiovascular disease. The cell-based therapies have been shown to alleviate tissue injury and organ dysfunction in animal models of cardiovascular disease. However, clinical trials with cell therapy for patients with cardiovascular disease have yielded inconsistent results and modest benefit overall. A major unsolved problem for cell-based therapy is how to home transplanted cells to damaged organs to improve their survival and to enhance tissue repair and organ function. We have developed an innovative strategy to overcome this hurdle by intravenously transfusing rat endothelial cells overexpressing the neutrophil interleukin-8 (IL-8) receptors IL8RA and IL8RB into rats with experimental endoluminal injury of the carotid artery. We have shown that, equipped with the IL8R homing device, endothelial cells mimic the behavior of neutrophils and compete with the binding of neutrophils to IL-8 overexpressed in injured vessels, thus inhibiting the neutrophil-induced inflammatory response and accelerating reendothelialization and vascular repair. This targeted delivery of endothelial cells to sites of cardiovascular injury provides a novel therapeutic strategy for cardiovascular disease. The results of the present study open the door to new therapeutic applications of cell therapy in patients with cardiovascular injury.
Endothelial Cells Overexpressing Interleukin-8 Receptors Reduce Inflammatory and Neointimal Responses to Arterial Injury
Dongqi Xing, Peng Li, Kaizheng Gong, Zhengqin Yang, Hao Yu, Fadi G. Hage, Suzanne Oparil and Yiu-Fai Chen

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SUPPLEMENTAL MATERIAL

Supplemental Methods and Materials

Plasmid and Adenoviral Vector Generation

ECs that overexpress IL8RA and/or IL8RB were generated using adenoviral vectors that contain human IL8RA and/or IL8RB cDNAs and the green fluorescent protein (GFP) gene using the AdEasy™ Adenoviral Vector System (Stratagene)¹⁰ as summarized in Supplemental Figure 1. Full length human IL8RA (IL8 receptor alpha, Cat# MGC-40015, ATCC) and IL8RB (IL8 receptor beta, Cat# MGC-46215, ATCC) cDNAs in pCMV-SPORT6 vectors were purchased from American Type Culture Collection (ATCC). The shuttle vector pAdTrack-CMV which has the GFP gene controlled by the CMV promoter was purchased from Addgene (Cat# 16405). To construct the recombinant vectors, pAdTrack-IL8RA-GFP or pAdTrack-IL8RB-GFP, the IL8RA or IL8RB cDNAs were cut out from pCMV-SPORT6 host vectors by SalI/XbaI or KpnI/XbaI, respectively. The isolated IL8RA or IL8RB cDNAs were then subcloned into pAdTrack-CMV plasmids at the corresponding restriction sites. Expression of IL8RA, IL8RB and GFP is controlled by separate CMV promoters. An empty vector pAdTrack-Null-GFP that does not contain IL8RA or IL8RB cDNAs was also generated and served as a control. The pAdTrack-IL8RA-GFP, pAdTrack-IL8RB-GFP, and pAdTrack-Null-GFP plasmids were subcloned into pAdEasy-1 adenoviral backbone in BJ5185 cells and then amplified in XL10-Gold ultra-competent cells following the manufacturer’s instruction (AdEasy™ Adenoviral Vector System, Stratagene Cat# 240010). The positive recombinant DNAs (pAd-IL8RA-GFP-, pAd-IL8RB-GFP and pAd-Null-GFP) were harvested and purified using the Qiagen® Plasmid Purification System.

The Ad-293 cells were used to generate adenoviral vectors containing the IL8RA or IL8RB cDNAs. Briefly, Ad-293 cells were plated at 7-8 x 10⁵ cells per 60-mm tissue culture dish in 10% FBS DMED medium 24 hrs prior to transfection. The ~70% confluent cells were
transfected with Pac I-linearized pAd-IL8RA-GFP, pAd-IL8RB-GFP or pAd-Null-GFP recombinant DNAs using Lipofectamin LTX and PLUS transfection reagents from Invitrogen (Cat# 15338-100) and cultured in 10% FBS DMED medium for 7-10 days. When most of the cells became GFP+, the primary adenovirus was harvested by four-rounds of freezing/thawing. The adenoviral stocks were stored at -80°C until use.

**Cell Migratory Activity and Chemotaxis assays.**

EC migratory and chemotactic activity was assayed in a 96-well modified Boyden chamber (Millipore, Billerica, MA) using IL8RA, IL8RB, IL8RA/RB, AdNull and vehicle (not transduced) ECs. To test the IL8 dependence of EC chemoattractant activity, the bottom wells of the chamber were filled with endothelial growth medium contained 0, 20, 100, or 500 ng/ml of IL8. A polyvinylpyrrolidone-free polycarbonate filter plate with 3-µm pores was placed over the samples, and 100 µl of the EC suspension (2 x 10⁶ cells/ml) were placed into the upper wells. The chambers were incubated in humidified air with 5% CO₂ at 37°C for 12 hrs. The upper portion was then removed, and four photomicrographs (200x) per well were digitally recorded using an Olympus IX70 microscope and Perkin-Elmer Ultraview image capture equipment. Cell counts were made from these images.

**Preparation of Activated Neutrophils**

Human myeloid leukemia HL-60 cells (ATCC, Manassas, VA) were maintained in Iscove's modified medium (ATCC) supplemented with 10% fetal calf serum, 50 µg/ml streptomycin, 2 U/ml penicillin, and 2 mM L-glutamine. For differentiation, cells (3 x 10⁵/ml) were incubated in the presence of 1.3% (vol/vol) DMSO for 4~6 days (Newburger PE) before using for transduced EC and neutrophil competition study.
### Supplemental Table 1. Selective sense and antisense PCR primers for real-time quantitative RT-PCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sense Sequence</th>
<th>Primer Antisense Sequence</th>
<th>PCR Product (bp)</th>
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<td>TGACTTTCTGTCTGGGTG</td>
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<tr>
<td>TNF-α</td>
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<td>MCP-1</td>
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<td>VCAM-1</td>
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<tr>
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<tr>
<td>IL-6</td>
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<tr>
<td>Ribosomal Protein S9</td>
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<td>CGAACAATGAAAGATGGG</td>
<td>192</td>
</tr>
</tbody>
</table>

**CINC-2β** - cytokine induced neutrophil chemoattractant-2-beta (equivalent to human IL-8).

**TNF-α** - tumor necrosis factor-alpha; **MCP-1** - monocyte chemotactic protein-1; **VCAM-1** - vascular cell adhesion molecule 1; **IL-1β** - Interleukin-1 beta; **IL-6** - Interleukin-6; **IL-10** - Interleukin-10
Online Supplemental Figure 1: Schematic illustration of the generation of rat aortic endothelial cells (ECs) that over-express IL8RA or IL8RB. Human IL8RA and IL8RB cDNAs (2,026 and 2930 bp, respectively) in the pCMV-SPORT6 plasmid were purchased from ATCC and subcloned to the shuttle plasmid pAdTrack-CMV (not shown here) containing green fluorescent protein (GFP) as a marker. The Pme I-linearized plasmid was transformed into BJ5183 cells (not shown here) carry the backbone of the replication deficient adenovirus vector. (A) Cells with the recombinant DNA were selected and amplified in XL10-Gold ultracompetent cells, which allowed for insert stability. (B) The Pac I-linearized adenoviral proteins necessary for replication. The adenovirus was subsequently amplified. After 7-10 days, the adenovirus was harvested from lysed Ad293 cells, and (C) used to transduce ECs, causing them to overexpress the IL8RA, IL8RB, or both (double transduction). pAd-Null-GFP is the empty vector used as a control.

Online Supplemental Figure 2: Neutrophils inhibited binding of IL8RA/RB-ECs to endothelial monolayer in vitro. Endothelial monolayer was stimulated with TNF-α (10 ng/ml) for 16 hrs before various concentrations of neutrophils (labeled with CellTracker CM-Dil, red color) applied to the monolayer. IL8RA/RB-ECs (GFP color) were added 30 min after the application of neutrophils and incubated for additional 1 hr before cell (red neutrophils and green ECs) counting in the same well. Results are means±SEM; n=wells/per group.
Online Supplemental Figure 3: (A) Negative correlation of infiltrated neutrophils (MPO immunohistochemical stained) and transfused ECs (GFP immunohistochemical stained) in adventitia of balloon injured rat carotid arteries 24 hrs post injury. Samples included rats transfused with IL8RA, IL8RB, IL8RA/RB, or AdNull ECs. (B) Positive correlation of infiltrated monocytes/macrophages (ED-1 immunohistochemical stained) and neutrophils (MPO stained) in adventitia of balloon injured rat carotid arteries 24 hrs post injury. Samples included rats transfused with IL8RA, IL8RB, IL8RA/RB, or AdNull ECs.
Online Supplemental Figure 4: Medial area of injured right carotid arteries at 4-wk post balloon injury. Results are means±SEM. (n)=number of rats