Endothelial Healing in Vein Grafts
Proliferative Burst Unimpaired by Genetic Therapy of Neointimal Disease

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Background—Although inhibition of neointimal hyperplasia by cell cycle gene blockade therapy results in improved endothelial cell function in experimental vein grafts, little is known either about endothelial healing immediately after vein grafting or about the effect of this therapy on the healing process.

Methods and Results—Scanning electron microscopy demonstrated an immediate decrease in vein graft endothelial cell density associated with vein graft wall stretch, followed by a return to baseline by postoperative day 3. En face detection of bromodeoxyuridine incorporation confirmed a rapid endothelial proliferation by 48 hours. Despite inhibition of underlying vascular smooth muscle cell proliferation, E2F decoy oligonucleotide did not inhibit either endothelial bromodeoxyuridine incorporation or the return to baseline cell density. This differential response to E2F decoy was also observed in human umbilical vein endothelial cell culture, which resisted the E2F decoy inhibition of cell growth that was observed in human umbilical artery smooth muscle cells, despite evidence for nuclear localized delivery of the oligonucleotide into both cell types. Furthermore, the reduction of E2F binding activity seen in a nuclear gel shift assay of cultured smooth muscle cells was not observed in endothelial cells.

Conclusions—These results suggest a burst of graft endothelial cell proliferation that allows a rapid restoration of cell density in the monolayer. Additionally, there is a selective effect of E2F decoy gene therapy on target smooth muscle cells with sparing of this endothelial healing.

Key Words: endothelium gene therapy bypass surgery veins

Autologous vein remains the most widely used bypass conduit for the treatment of occlusive coronary and peripheral vascular disease, although failure rates in these grafts remain as high as 30% and 50% at 5 and 10 years, respectively.1,2 Vascular smooth muscle cell (VSMC) proliferation and neointima formation provide wall thickening that relieves the increased wall stress brought on by exposure to the high-pressure arterial circulation. These activated VSMC, along with dysfunctional endothelial cells, render the graft highly susceptible to accelerated atherosclerosis.3–5

Endothelial cell dysfunction, including reduction in endothelial cell nitric oxide synthase activity, decreased vasorelaxation, and increased expression of proinflammatory cell adhesion molecules, has been well documented in both experimental models of vein grafting and in human clinical specimens.6,7 Our group has demonstrated that inhibition of neointima formation by blockade of cell cycle regulatory gene expression leads to a significant improvement in endothelial cell function.8 Whereas the endothelium of genetically engineered vein grafts may be spared chronic activation by the paracrine influences of the underlying neointimal cells, acute endothelial healing remains an important component of the response to the injury associated with vein graft harvest and implantation. Little is known, however, about the acute healing response of the endothelial cell monolayer to the grafting procedure or the effects of cell cycle inhibitory therapy on that response.

Progression through the cycle of DNA synthesis and cell division is coordinated through a complex network of cell cycle regulatory genes and in part is regulated by the E2F family of transcription factors.9 Cell cycle gene blockade with the use of an E2F decoy oligonucleotide (ODN) that bears the consensus binding sequence for the E2F transcription factor and that prevents interaction of the factor with the promoters of target genes has been shown to inhibit neointimal hyperplasia in a model of vein grafting. This approach instead induces an alternative pathway of adaptive vein graft remodeling involving medial hypertrophy that does not predispose the vessel to occlusive disease.10

In the present study, we characterized the acute healing response of the vein graft endothelial cell monolayer to the grafting process. Additionally, we explored the effect of cell cycle gene blockade treatment, with the use of the E2F decoy ODN, on the healing response.
Methods

Oligonucleotides
The double-stranded E2F decoy phosphorothioate ODN was custom synthesized (Keystone-Biosource) with the sequence 5′-CTAGATTTCCCCGG-3′ annealed to 5′-GATCCGCGGAAAAT-3′, containing the E2F consensus binding sequence of the human c-myc promoter. The scrambled ODN, used as a control, has the following sequence: 5′-TCCAGCTTCGTAGC-3′ annealed to 5′-GCTAGTACGAAGC-3′. ODN was dissolved at a concentration of 40 μmol/L in normal saline solution. Scrambled ODN labeled with either fluorescein-isothicyanate (FITC) or biotin at the 3′-end of one strand was used for fluorescence or light microscopic analysis of ODN distribution after transfection, respectively.

Vein Graft Model and Ex Vivo Transfection
Jugular vein–to–carotid artery interposition grafting was performed in New Zealand White rabbits (weight, 3 to 3.5 kg) as previously described.10 Briefly, a “no-touch” technique was used to harvest a 2.5-cm segment of vein, which was either left in normal saline at ambient pressure or treated with pressure-mediated delivery of ODN for 10 minutes at a nondistending pressure of 300 mm Hg.10,11 The vein graft was then anastomosed with 7-0 polypropylene sutures. The double-stranded E2F decoy phosphorothioate ODN was custom synthesized (Keystone-Biosource) with the sequence 5′-CTAGATTTCCCCGG-3′ annealed to 5′-GATCCGCGGAAAAT-3′, containing the E2F consensus binding sequence of the human c-myc promoter. The scrambled ODN, used as a control, has the following sequence: 5′-TCCAGCTTCGTAGC-3′ annealed to 5′-GCTAGTACGAAGC-3′. ODN was dissolved at a concentration of 40 μmol/L in normal saline solution. Scrambled ODN labeled with either fluorescein-isothicyanate (FITC) or biotin at the 3′-end of one strand was used for fluorescence or light microscopic analysis of ODN distribution after transfection, respectively.

Häutchen Preparation
Endothelial preparations of pressure-fixed vessels (100 mm Hg with 10% formalin for 10 minutes) were prepared with bromodeoxyuridine (BrdU) immunohistochemical staining (Zymed), as previously described.12 Briefly, flattened specimens were dehydrated and glued endothelial surface down onto 10% parlodion–coated glass slides. The vessel wall and subendothelium were peeled away, and the endothelial cells were applied to 5% gelatin–coated (Difco Laboratories) glass slides. The parlodion was dissolved, and slides were rehydrated for light microscopy. At time points beyond 1 to 2 days, Häutchen preparations failed to yield an endothelial monolayer because of the adherence of nonneointimal cells to the parlodion glue.

Silver Staining and Scanning Electron Microscopy
Vessels were harvested at 1, 3, 7, and 14 days after operation and pressure fixed. Silver stained (0.3% silver nitrate over 20 seconds), pressure fixed. Sliver stained (0.3% silver nitrate over 20 seconds), opened, and dehydrated samples underwent critical point drying with liquid CO2 and sputter coating with a gold-palladium alloy. The preparation was examined with the use of an Amray 1000A scanning electron microscope. Silver staining of the endothelial cell borders showed no areas of denudation. Because of the acute increase beyond the anastamosis, whereas the remainder of the graft noted to have endothelial cell loss only in the first 1 to 2 mm after implantation and was compared with ungrafted vein and carotid artery. At day 1, blue staining was present only at the anastamoses and was absent at days 3 and 7, whereas the body of the graft was devoid of staining at all time points (data not shown).

BrdU Incorporation and Immunohistochemistry
BrdU was administered at 18 hours (100 mg/kg SC and 30 mg/kg IV) and 12 hours (30 mg/kg IV) before harvest. BrdU labeling (Zymed) was carried out on either 5-μm frozen sections or with the use of the Häutchen preparation. BrdU-labeled and total nuclei were counted in 8 fields per sample at ×400.

Western Blot Analysis
Whole graft lysates were separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Sciences). Protein concentrations were determined by the Bradford method (Biorad). The membrane was incubated with a polyclonal antibody for proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology Inc) for 1 hour and developed by the ECL system (Amersham Life Sciences). Signal intensities were quantified (NIH Image 1.52), and the results were expressed in arbitrary units per microgram of protein.

Endothelial and Smooth Muscle Cell Cultures, ODN Treatment, and Growth Assays
Human umbilical vein endothelial cells (HUVEC) and umbilical artery smooth muscle cells (HUA/SMC) were grown according to the supplier’s recommendations (Clonetics Corporation). Confluent cells were synchronized in basal medium for 48 hours. The cells were restimulated with growth media for 24 hours and pulse-labeled with [3H]thymidine for 4 hours. [3H]thymidine incorporation (cpm) was measured in cell lysates as described.13 Where indicated, ODN (5 μmol/L) were added to the culture 24 hours before serum stimulation. Transfection efficiency was assessed by fluorescent microscopy of cells transfected with FITC-labeled ODN.

Electrophoretic Mobility Shift Assay
Nuclear extracts were prepared as previously described.14,15 Under similar conditions, the isolation of nuclear extract has not disrupted the sequence specific binding of phosphorothioate oligonucleotides to nuclear protein.16 A peak of E2F binding activity was observed at 6 hours in preliminary experiments. Purified monoclonal E2F-1 antibody (PharMingen) was used in supershift assays as previously described.14,15

Statistical Analysis
All results are expressed as mean±SEM. One-way ANOVA was used to compare differences between groups. A value of P<0.05 was considered to indicate a statistically significant difference, with Bonferroni correction for multiple comparisons.

Results

Endothelial Cell Healing
Vein graft endothelial barrier function was assessed macroscopically at days 1, 3, and 7 after grafting, with Evans blue dye administered intravenously (20 mg/kg in normal saline solution) 10 minutes before graft harvest, and was compared with ungrafted vein and carotid artery. At day 1, blue staining was present only at the anastamoses and was absent at days 3 and 7, whereas the body of the graft was devoid of staining at all time points (data not shown).

The interposition of a vein into an arterial vessel leads to an acute distention of the vein graft and consequently a significant increase in surface area. Harvested vein segments, all 2.5 cm in length, were perfusion-fixed, opened lengthwise, and laid flat for measurement of surface area. A statistically significant change in the surface area of the graft compared with the ungrafted vein was observed at day 1 (291±17 mm2 versus 183±16 mm2, respectively, P<0.05). Further increase in graft surface area occurred up to day 7, at which time a maximum area of 361±39 mm2 was reached (n=4 for each time point).

Scanning electron microscopy was performed on the endothelial surface after silver staining at 1, 3, 7, and 14 days after implantation and was compared with ungrafted vein and carotid artery (n=4 for all groups). At day 1, the grafts were noted to have endothelial cell loss only in the first 1 to 2 mm beyond the anastamosis, whereas the remainder of the graft showed no areas of denudation. Because of the acute increase in surface area, the cell densities of the day-1 grafts were significantly decreased (1819±61 cells/mm2) compared with those of the ungrafted veins (2587±140 cells/mm2) (P<0.05). Cell density at day 3 was 2897±103 cells/mm2 (P<0.05 when compared with day 1). Endothelial cell density plateaued at 3338±157 cells/mm2 at day 7 at a level...
slightly below that seen in the contralateral carotid artery (3409 ± 133 cells/mm²). The number of endothelial cells present on the graft at each time point could be calculated by multiplication of the cell density by the graft surface area. The ungrafted vein was found to have $4.7 \times 10^5$ cells per 2.5-cm graft, whereas day-1 grafts had $5.3 \times 10^5$ cells ($P < 0.05$). However, by day 3, the number of cells had increased to $9.6 \times 10^5$ at day 7 and $1.2 \times 10^6$ at day 14 ($P < 0.05$ when compared with day 1 and the ungrafted vein). These data collectively suggest that a burst of endothelial proliferation occurs in the vein graft between postoperative days 1 and 3 and that an equilibrium of cell number is reached by day 7 (Figure 1).

To further document this endothelial proliferative response, en face immunohistochemical staining of endothelial cells labeled with BrdU was performed by means of the Häutchen technique ($n = 3$ for all time points). A labeling index of <1% was noted in the ungrafted vein, whereas an index of 6.6 ± 4.3% was observed 1 day after grafting. At day 2, however, the BrdU labeling index had dramatically increased to 71.8 ± 2.7% (Figure 2). Tissue from animals not treated with BrdU and sections of BrdU-treated tissue stained with nonspecific IgG isotype antibody served as negative controls.

**Effect of E2F Decoy ODN Treatment on Graft Endothelial Cell and VSMC Proliferative Response**

Using a previously described, ex vivo, nondistending, pressure-mediated delivery of ODN to the vein graft wall, we first documented efficient uptake of FITC-labeled ODN in both endothelial cells and VSMC (Figure 3, A and B). The effective delivery of functional E2F decoy ODN was further confirmed by Western blot analysis for PCNA in whole vascular graft 4 days after transfection. Our findings demonstrated that E2F decoy ODN treatment yielded a sequence-specific inhibition of PCNA upregulation in the vessel wall that is predominantly composed of VSMC (Figure 3C and Table). Grafts harvested 7 days after implantation, a time when VSMC proliferation is known to be at its peak, underwent assessment of BrdU incorporation in medial cells on 5-μm cross sections. The findings confirmed that E2F decoy ODN treatment inhibited medial VSMC proliferation. Grafts treated with the E2F decoy ODN had a significantly lower labeling index compared with either vehicle or scrambled ODN-treated grafts (Table).

Given the dependence of endothelial healing on the burst of proliferative activity in the early postoperative period, we examined endothelial healing in grafts once again treated with either E2F decoy or a control scrambled sequence ODN. The increase in cell density observed in vehicle-treated grafts at each time point was essentially unchanged in vehicle and scrambled ODN-treated as well as E2F decoy ODN-treated vessels ($P = 0.4$) (Figure 4A). This observation was further confirmed with BrdU labeling of endothelial monolayers with the Häutchen technique (Figure 4B). To document the successful delivery of ODN into the endothelial cells, we examined the transfection of grafts with biotinylated ODN.
Our result confirmed nuclear localization of labeled ODN in >75% of cells by streptavidin-peroxidase staining of isolated vein graft endothelial monolayer (Figure 5).

Response of Endothelial Cells and VSMC In Vitro to E2F Decoy ODN Treatment
Having demonstrated a differential response to E2F decoy ODN treatment, we sought to test the hypothesis that normal endothelial healing in ODN-treated grafts reflects an ability of endothelial cells to mount a proliferative response despite the presence of E2F decoy ODN in a cell culture system. Despite a similar degree of nuclear localization of FITC-labeled ODN in the HUASMC and HUVEC cultures (70 to 80% in both), HUASMC proliferation was inhibited by E2F decoy ODN treatment (41.7 ± 4.4% inhibition versus vehicle-treated control, P < 0.001), whereas this treatment did not affect the proliferation of HUVEC (1.9 ± 5.6%, P > 0.5) (n = 6 per treatment group). By using the electrophoretic mobility shift assay, we were able to confirm an effective reduction in E2F binding activity after serum stimulation in HUASMC treated with E2F decoy that was not seen in scrambled ODN-treated cells (Figure 6). In HUVEC, however, there was no significant reduction of serum-induced E2F binding activity after E2F decoy treatment. Supershift with E2F-1 monoclonal antibody and competition with unlabeled probe confirmed the predominant role of this E2F isoform and the

<table>
<thead>
<tr>
<th>Groups</th>
<th>PCNA, Mean Density/μg Protein</th>
<th>BrdU Labeling Index, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ungrafted vein</td>
<td>0 (n=6)</td>
<td>ND</td>
</tr>
<tr>
<td>Vehicle</td>
<td>24.13 ± 3.88 (n=6)</td>
<td>25.1 ± 1.7 (n=3)</td>
</tr>
<tr>
<td>E2F decoy ODN</td>
<td>0.79 ± 0.79† (n=6)</td>
<td>8.3 ± 2.8† (n=3)</td>
</tr>
<tr>
<td>Scrambled ODN</td>
<td>17.87 ± 4.76 (n=6)</td>
<td>24.9 ± 2.3 (n=3)</td>
</tr>
</tbody>
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PCNA indicates proliferating cell nuclear antigen; BrdU, Bromodeoxyuridine; and ODN, oligonucleotides. Values are mean ± SEM. *P < 0.05 for E2F decoy ODN vs vehicle* and scrambled ODN† groups. ND indicates not determined.
specificity of E2F binding activity, respectively (data not shown).

**Discussion**

Cell cycle inhibition achieved through intraoperative transfection of therapeutic ODN into rabbit vein grafts has been shown to redirect vein graft biology away from neointimal hyperplasia and toward medial hypertrophy as an adaptive response to the hemodynamic stress of the arterial circulation. The integrity of the endothelium is believed to play an important role in the prevention of vascular proliferative diseases such as atherosclerosis, which is responsible for the majority of bypass vein graft failures. Despite traumatic and biological injury to the endothelium during vein grafting, including acute stretch, several groups have documented an intact vein graft endothelium by postoperative day 7. In this study, we sought to characterize this endothelial cell healing response during the first week after experimental vein grafting and measure the effect of treatment with E2F decoy ODN.

We observed a rapid, synchronized burst of endothelial proliferative activity in response to acute stretching of the native vein graft wall. This response was documented both by electron microscope analysis of cell density and cell number as well as immunohistochemical analysis of BrdU incorporation. This rapid proliferative response was uniformly distributed throughout the length of the vein graft and occurred despite the apparent preservation of cell-cell contact on electron microscopic analysis of silver-stained tissue specimens. Previous studies have begun to examine the morpho-

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**Figure 4.** Endothelial cell response to E2F decoy ODN treatment. A, Scanning electron microscopy; B, Häutchen BrdU labeling index (n=3 for each group). POD indicates postoperative day.

**Figure 5.** Nuclear localization of ODN. Light microscopy photomicrograph of Häutchen preparation demonstrating nuclear localization of biotinylated ODN in vein graft endothelial cells at time of grafting (magnification ×400).
logical and biological responses of endothelial cells to physical strain on the cell membrane. Cyclic stretch of endothelial cells in culture, as well as other cell membrane perturbations, has been associated with cell cycle stimulation and a rapid increase in adenylate cyclase activation. In this study, the decline of endothelial cell proliferation back to baseline occurred within a time frame of several days and was associated with the restoration of normal endothelial cell density.

Interestingly, this rapid and synchronized endothelial proliferative response in the graft was not hindered by efficient nuclear delivery of E2F decoy ODN, despite the simultaneous blockade of cell cycle progression in VSMC within the same vessel. This differential response of endothelial cells and VSMC was further confirmed in studies of cultured cells that revealed an endothelial cell resistance to E2F decoy-mediated cell cycle arrest. We speculate that the properties that lead to this differential response, which has also been suggested by previous studies involving antiproliferative ODN treatment of denuded arteries, may include differences in ODN metabolism or in the ability of endothelial cells to respond to ODN-mediated gene blockade by further augmenting upregulated expression of cell cycle regulatory proteins. Additionally, cyclin E–driven pathways of cell cycle activation have been described that are relatively independent of E2F activity; under certain conditions, cells have been shown to progress through S, G2, and M phases in the absence of E2F transactivation. It is not known how common this type of cyclin E–dependent cell cycle progression may be in different cell types. As demonstrated in this study, however, endothelial cells appear to be programmed to support a burst of proliferative activity that are not available to smooth muscle cells. Finally, there are numerous members of the E2F family of transcription factors that play differing stimulatory and inhibitory roles in cell cycle regulation. Differential expression of these factors in different cell types might result in a varied response to decoy treatments. The profiles of E2F family member expression in endothelial cells and VSMC have not yet been characterized, and further investigation into these profiles, along with an analysis of the decoy’s affinity for different family members, may shed further light onto the mechanism of this differential response to the ODN therapy.

In any event, E2F decoy ODN treatment of vascular grafts inhibits VSMC proliferation and activation but spares the endothelium, thereby allowing normal endothelial healing. The improved endothelial cell function observed in previous studies at 4 to 6 weeks after operation probably is related to the inhibition of neointimal hyperplasia and to a subsequent decrease in local concentrations of cytokines and other proinflammatory molecules that are released by activated VSMC and leukocytes within the neointimal layer. The differential responses of endothelial cells and VSMC to E2F decoy observed in this study may have important implications for the therapeutic blockade of cell cycle progression in treating postangioplasty restenosis, native arterial atherosclerosis, and transplantation vasculopathy. Future studies elucidating differences in the molecular machinery regulating proliferation in endothelial cells and VSMC may contribute significantly to our understanding of normal vascular homeostasis and disease.

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