c-Jun Regulates Shear- and Injury-inducible Egr-1 Expression, Vein Graft Stenosis after Autologous End-to-Side Transplantation in Rabbits, and Intimal Hyperplasia in Human Saphenous Veins*

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Coronary artery bypass graft failure represents an unsolved problem in interventional cardiology and heart surgery. Late occlusion of autologous saphenous vein bypass grafts is a consequence of neointima formation underpinned by smooth muscle cell (SMC) migration and proliferation. Poor long term patency and the lack of pharmacologic agents that prevent graft failure necessitate effective alternative therapies. Our objective here was to evaluate the effect of targeted inhibition of the bZIP tran-
scription factor c-Jun on intimal hyperplasia in human saphenous veins and vein graft stenosis after autologous end-to-side transplantation to carotid arteries following permanent ligation (7) and balloon angioplasty (8). Here we evaluated the effect of targeted inhibition of c-Jun in the initiation of intimal hyperplasia and vein graft stenosis after autologous end-to-side transplantation to carotid arteries. We also explored the role of c-Jun in the initiation of injury- and shear-inducible transcriptional networks.

These data demonstrate that strategies targeting c-Jun may be useful for the prevention of vein graft stenosis. Control of one important shear-responsive transcription factor by another indicates the existence of transcriptional amplification mechanisms that magnify the vascular response to cell injury or stress through inducible transcriptional networks.

Coronary artery bypass grafting (CABG)2 remains one of the most widely used surgical means of treating coronary heart disease after heart attack (1, 2). Venous graft occlusion is the major cause of recurrent ischemia in patients after CABG. Because of difficulties in obtaining arterial graft material and the relative ease of harvesting long vascular segments, the saphenous vein remains the most widely used graft. Fitzgibbon et al. (3) found that patency rates of saphenous vein grafts in patients undergoing CABG 1, 5, and 10 years after surgery were 78%, 65, and 57%, respectively. Although statins improve graft patency by ~24–35%, the search for a better pharmacologic agent for the prevention of intimal hyperplasia and vein graft stenosis after autologous end-to-side transplantation remains an important therapeutic consideration and necessitates the search for effective alternative therapies (6).

EXPERIMENTAL PROCEDURES

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EXPERIMENTAL PROCEDURES

Culture of Vein Grafts in Vitro and DNAzyme Intervention—Segments of human saphenous vein ∼8–10 cm in length were obtained during bypass surgery from the Cardiothoracic Surgical Unit at Royal Prince Alfred Hospital, Sydney with informed consent. The vein was placed in sterile saline and kept at room temperature; it was promptly transported from the hospital to the University of New South Wales where the vein was cut into small segments, ∼1 cm in length and cultured in RPMI 1640 (Invitrogen) supplemented with 20% FBS. Veins were randomly allocated to 4 groups: untreated (fresh isolated), vehicle alone, those treated with c-Jun DNAzyme (Dz13), or Dz13Scr, in which the hybridization arms were scrambled but containing the same 10–23 catalytic core as Dz13. Transfections with FuGENE 6 (Roche Applied Science) were performed with either 0.4 μmol/liter DNAzyme, or mock (vehicle alone) on Day 0 and Day 7. Veins were grown for 14 days with a daily change of culture medium. On Day 14, veins were placed into 10% forma-
lin and fixed at 4°C overnight. Veins were cross-sectioned and stained by Miller’s elastin and van Gieson staining.

Construction of Adenovirus-c-Jun—Murine c-Jun cDNA was cloned from mouse mRNA by RT-PCR using primers (forward, 5′-ACG GAC TCT TGT ACT GCA A-3′; reverse, 5′-TCT CGT TGG CCC CTC AGC-3′) and ligated into a shuttle plasmid (Qiagen PCR cloning kit). c-Jun cDNA was then transferred into the Ad-Easy expression system (Stratagene) (9), and Adeno-c-Jun was produced after digestion by PacI and amplification in HEK 293 cells.

SMC Culture Conditions, Proliferation, and Injury Assays—SMCs isolated from rat and rabbit aortae were obtained mycophenolase-free at P1 from Cell Applications, Inc. (San Diego, CA). Cells were grown in Waymouth media with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO₂. In proliferation assays, SMCs in 96-well plates were transfected with Adeno-c-Jun with or without DNAzyme (using FuGENE 6) and arrested for 24 h in medium, excluding serum, then exposed to medium containing 5% FBS at 37°C for 72 h. Cells were harvested by trypsin, and the suspension was counted by automated Coulter counter. In injury assays, SMCs were seeded into 6-well plates, transfected with Adeno-c-Jun and DNAzyme (multiplicity of infection of 10, unless otherwise indicated) in serum-free medium, then scraped using a P1000 pipette tip. After 48 h, the cells were washed with PBS prior to photomicroscopy.

Fluid Shear Stress Model and Immunocytochemistry—SMCs were seeded on Flexcell slides precoated with 10% FBS culture media. After confluence, cells were starved overnight. DNAzymes Dz13scr, ED5 (DNAzyme adenovirus), or ED5SCR with 0.4 μm of Dz13 were transfected with JNKII (JNK pathway inhibitor), PD98059 (MEK/ERK pathway inhibitor), and PD98059 with HA1076 (JNK inhibitor) at the concentration of 20 μM. After treatment with DNAzymes or pharmacologic inhibitors, the cells were transfected into the Streamer Shear Stress Device (Flexcell International, Hillsborough, NC) filled with Dulbecco’s modified Eagle’s medium/0.5% FBS, and shear stress was applied to the cells with the aid of a computer-controlled peristaltic pump. Immunocytofluorescence staining was performed to detect c-Jun, early growth response-1 (Egr-1), or MMP-2. Briefly, the cells were firstly washed by PBS twice and fixed by 3% formaldehyde for 30 min. The fixed cells were incubated with primary c-Jun (Santa Cruz Biotechnology) and Egr-1 (Cell Signaling) antibody overnight. The secondary fluorescein isothiocyanate (FITC)-conjugated antibody was added to the cells for 1 h. Finally, cells were stained with DAPI for nucleic visualization. The results were carefully observed under fluorescence microscope. FITC⁺ cell percentages were calculated based on the ratio of positively staining cells and the total cell number indicated by DAPI from at least five random chosen fields of view.

RT-PCR, Western Blotting, ELISA, and Zymography—For RT-PCR analysis, total RNA was extracted using the RNeasy Mini Kit (Qiagen). After assessment of RNA concentration, constant amounts of RNA were combined with RT-PCR reagent (Qiagen one-step RT-PCR kit) prior to amplification (10, 11). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. For Western blot analysis, cells were washed twice with PBS and lysed in radioimmune precipitation assay buffer (12, 13). Lysates were resolved by SDS-PAGE on 4–15% gels, and proteins were transferred to polyvinylidene difluoride membranes and blocked for 30 min in blocking buffer (Tris-buffered saline, pH 7.6, 0.05% Tween, and 5% non-fat dry milk). c-Jun (H-79, Santa Cruz Biotechnology) proliferating cell nuclear antigen (PCNA, PC10, Santa Cruz Biotechnology), MMP-2 (4D3, Santa Cruz Biotechnology), and Egr-1 (44D5, Cell Signaling) antibodies were added to the membranes for 1 h, prior to washing and addition of secondary antibody peroxidase conjugate. The membrane was washed and incubated with ECL chemiluminescence substrate (Pierce) prior to exposure. For MMP-2 ELISA, the culture media were harvested and centrifuged, and supernatants were assessed for soluble MMP-2 by commercial ELISA (R&D Systems, Minneapolis, MN). The supernatants containing equivalent units of protein were also run on SDS-PAGE gels, probed with an anti-murine antibody (R&D Systems, Minneapolis, MN) and fixed at 4°C overnight. Veins were cross-sectioned and stained by fluorescence microscopy.
transfection in veins, conduits were incubated in 200 μl of transfection solution containing 500 μg of FITC-Dz13 at 37 °C for 30 min. The vein was rinsed in PBS and snap frozen with OCT in liquid nitrogen. Blocks were sectioned and fixed in 95% ethanol. Following DAPI nuclear staining, the slides were imaged under fluorescence microscopy. At 28-day post-transplantation, the proximal and distal carotid segments were cannulated and the vein was perfusion-fixed under distending pressure with 10% formalin in PBS, pH 7.4. Grafts were fixed in 10% formalin (Ambion) overnight and stored at ~80 °C until immunohistochemical analysis.

RESULTS

**c-Jun DNAzymes Attenuate Intimal Hyperplasia in Human Saphenous Veins**—Saphenous vein graft failure after CABG is strongly associated with SMC proliferation and migration (15). To determine the effect of c-Jun knockdown on intimal hyperplasia in human vein grafts, we incubated freshly isolated human saphenous vein explants with Dz13, a catalytic oligodeoxynucleotide (DNAzyme) targeting c-Jun (16). In this explant model, the intima of the vessel thickens
spontaneously after several weeks in culture (9). Dz13 attenuated neointima formation within 2 weeks compared with the vehicle control (Fig. 1, A and B). Veins treated with Dz13scr, a DNAzyme containing an intact catalytic domain in which the RNA-hybridizing arms are scrambled, had no effect (Fig. 1, A and B). Immuno- histochemical analysis revealed that Dz13, but not Dz13scr, suppressed both c-Jun and PCNA expression in these vessels, which stained positive for alpha-SM actin, suggesting c-Jun may be associated with intimal hyperplasia in human saphenous veins (Fig. 1C).

FIGURE 4. Dz13 suppresses adenovirus c-Jun-inducible SMC wound repair. A, rat SMCs grown in 6-well plates were transduced with adenovirus-c-Jun or adenovirus-LacZ and transfected with 0.4 μmol/liter Dz13 or Dz13scr in serum-free medium. The cells were scraped with a sterile P1000 pipette tip and left for 48 h prior to photomicroscopy. B, representative images at 200×. Dashed lines denote the wound edge. The data are representative of at least two independent experiments performed in triplicate.

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SMC Proliferation Is Increased after Transduction with Adeno-c-Jun—We next determined the effect of overexpressing, rather than knocking down, c-Jun on SMC growth. c-Jun overexpression was achieved using the replication-defective adenovirus Ad-Easy system. c-Jun protein levels were barely detectable in growth-quiescent rat aortic SMCs (Fig. 2A), consistent with poor c-Jun expression in uninjured carotid arteries (7). Within 24 h, however, Adeno-c-Jun transduction increased c-Jun expression in SMCs without affecting levels of β-actin (Fig. 2A). c-Jun overexpression also induced PCNA expression (Fig. 2A), complementing our findings with Dz13 in Fig. 1C. We assessed levels of MMP-2 in transduced SMC cells. Exogenous c-Jun stimulated MMP-2 expression within 24 h (Fig. 2A). SMCs transduced with adenovirus-c-Jun also underwent accelerated proliferation within 3 to 4 days compared with non-transduced cells, although the effect was not dose-dependent at the multiplicity of infection examined (Fig. 2B).

DNAzyme Targeting c-Jun Suppresses Adeno-c-Jun-inducible SMC Wound Repair—A single linear scrape of SMC monolayers in culture initiates a reparative response involving proliferation and migration from wound edge and regrowth in the denuded zone. We hypothesized that Adeno-c-Jun would accelerate this response to injury and that Dz13 would inhibit the effect of exogenous c-Jun. Adeno-c-Jun indeed stimulated this reparative response to injury (Fig. 4A and B). Moreover, Dz13 inhibited this increase, whereas the Dz13scr had no effect (Fig. 4A and B).

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c-Jun DNAzymes Inhibit Vein Graft Stenosis after Autologous End-to-Side Transplantation in Rabbits—The preceding findings using adenovirus to increase SMC levels of
c-Jun and Dz13 to knockdown c-Jun, demonstrate that SMC growth and wound repair in vitro are tightly controlled by this transcription factor. Dz13 has previously been used in animal models of arterial injury (7, 8), but it has not been deployed in models of autologous vein transplantation. Using the E2F decoy approach of intra-operative, ex vivo oligonucleotide delivery, we incubated rabbit jugular veins in a solution containing Dz13 or Dz13scr then grafting the vein into the carotid artery of the same animal end to side, as is performed in standard CABG. Intra-operative, ex vivo delivery of Dz13 was indicated by green fluorescence visualized in the graft following delivery of FITC-labeled Dz13 (Fig. 5A) in vehicle containing the commercial transfection agent FuGENE 6. This model results in a thickened neointima within the graft 4 weeks after anastomosis (Fig. 5, B and C). Dz13 attenuated intimal thickening (Fig. 5, B and C), whereas neointima formation after treatment of the veins with Dz13scr did not differ from the vehicle control (Fig. 5, B and C). Immunohistochemical analysis of cross-sections revealed that c-Jun, PCNA, and MMP-2 were poorly and overall charge-matched, sequence-specific negative control. To provide further evidence for the causal role of c-Jun in vein graft neointima formation, we performed further experiments in rabbit vessels, independently of Dz13. We transduced jugular vein segments ex vivo with adenovirus-c-Jun (10^{10} pfu/ml) over 30 min, prior to end-to-side transplantation into carotid arteries (as used for Dz13/Dz13scr in Fig. 5, B and C) and assessed the extent of intimal thickening after 28 days. Fig. 5F demonstrates that Adeno-c-Jun significantly potentiates neointima formation in the vessels compared with its control (Adeno-LacZ). Immunohistochemical staining of the rabbit tissue provides supportive data. Adeno-c-Jun increased c-Jun immunoreactivity in the veins (Fig. 5F, middle upper), together with the expression of the c-Jun-dependent gene, MMP-2 (Fig. 5F, middle lower), and the mitogenic marker, PCNA (Fig. 5F, middle center). These in vivo data with Adeno-c-Jun complement our findings in the same model with Dz13 and provide compelling evidence for c-Jun playing a causal role in vein graft thickening.
Induction of c-Jun in SMCs Exposed to Fluid Shear Stress Is ERK1/2- and JNK-dependent and p38-independent—The process of intimal thickening as a prelude to bypass graft stenosis may be initiated by changes in fluid biomechanical forces sensed by the cells in the graft (18). For example, conduits undergo an acute static-to-shear transition after the reperfu-
Moreover, because arterial shear forces are considerably higher than those in veins, venous conduits experience biomechanical forces not previously sensed by veins. SMCs represent the most prominent cellular element of the intimal lesion (19), and immunohistochemical analysis by our group (Fig. 6A) and others (20, 21) indicates that SMCs, rather than endothelium, can make contact with flowing blood in human vein grafts. It is therefore possible that the SMC response to flow may influence shear-dependent thickening in the de-endothelialized vein graft.

Immunocytofluorescence analysis revealed that c-Jun is poorly expressed in cultured growth-quiescent SMCs but is induced by laminar shear stress (10 dynes/cm²) within 1 h (Fig. 6B and C). The induction of c-Jun by shear stress was completely abrogated by Dz13 (Fig. 6B), whereas Dz13scr had no effect (Fig. 6B). To determine kinase(s) mediating shear-inducible c-Jun expression, we exposed SMCs to pharmacologic inhibitors of MEK-ERK1/2 (PD98059), JNK (JNKII inhibitor), or p38 (SB202190) prior to exposure to shear. Fig. 6B clearly demonstrates that the induction of c-Jun by shear is ERK1/2- and JNK-dependent and is p38-independent.

Shear- and Injury-inducible Egr-1 Expression Is Controlled by c-Jun—To provide additional insights on the molecular pathways regulated by c-Jun in stressed SMCs. Accordingly, we performed microarray analysis to identify differently expressed c-Jun-dependent genes in SMCs 24 h after transduction with Adeno-c-Jun and Adeno-LacZ. Using 44K Agilent GeneChip Arrays, we found that c-Jun increased the expression of 607 genes, and reduced that of 180 genes by at least 2-fold after this time. Among the genes induced by c-Jun was the zinc finger transcription factor, Egr-1, which increased 6-fold. Egr-1 is an immediate-early gene, which, like c-Jun, plays a master regulator role by switching on the expression of many pathophysiologically important genes in numerous models of cardiovascular pathology (22). We demonstrated over a decade ago that Egr-1 is induced by shear in vascular endothelial cells (23). However, the transcriptional pathway responsible for its induction by shear is unknown, as is whether Egr-1 is activated in other cell types by shear stress. The time course of c-Jun and Egr-1 induction by shear stress revealed that the peak of c-Jun expression precedes that of Egr-1 (Fig. 6D). Intriguingly, shear-induced Egr-1 expression was inhibited by Dz13 (Fig. 6E). In contrast, c-Jun-induced c-Jun expression was inhibited by ED5, a DNAzyme that targets Egr-1 (Fig. 6F).

These findings were not confined to shear stress. We found that shear-induced Egr-1 expression was completely inhibited by Dz13 (Fig. 6F). In contrast, injury-induced c-Jun expression was completely inhibited by ED5 (Fig. 6F). We further demonstrated that Dz13 suppressed Egr-1 expression in rabbit autologous bypass transplants (Fig. 6G), in which intimal thickening was inhibited by Dz13 (Fig. 5, B and C). Moreover, Egr-1 levels were increased in veins transduced with Adeno-c-Jun (Fig. 6G). These studies thus demonstrate that inducible c-Jun expression is ERK1/2- and JNK-dependent and p38-independent and that c-Jun controls Egr-1.

DISCUSSION

CABG disease represents an unsolved problem in interventional cardiology and heart surgery. Late occlusion of autologous saphenous vein bypass grafts is a consequence of neointima formation underpinned by SMC migration and proliferation. The beneficial effects of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors on saphenous vein bypass grafts independent of their cholesterol-lowering properties have been attributed to their ability to reduce intimal hyperplasia by suppressing SMC invasion and proliferation (24). The present study offers several novel advances over prior work. First, the present study provides the first ever demonstration that Dz13 inhibits neointima formation in venous tissue. All previous models involving Dz13 have only used arteries, and findings from arteries cannot necessarily be extrapolated to veins (25). Second, the end-to-side venoarterial autologous bypass transplantation model used here in rabbits is analogous to the anastomosis commonly performed in human CABG. Agents that inhibit neointima formation in rat vessels do not necessarily perturb intimal thickening in higher species or humans. Table 1 illustrates that drugs such as heparin, carve-
**c-Jun, Egr-1, and Vein Graft Stenosis**

**TABLE 1**

Agents that inhibit neointima formation in rat vessels do not necessarily perturb intimal thickening in higher species or humans

| Drug          | Species | Model or clinical setting                                      | Effect on Ni | Reference |
|---------------|---------|-----------------------------------------------------------------|--------------|-----------|
| Heparin       | Human   | Coronary artery stenting with heparin-coated stents (clinical) | No effect    | (41)      |
| Baboon        | Human   | Aortoiliac bypass grafting with heparin-coated ePTFE graft     | Inhibition   | (42)      |
| Pig           | Rat     | Coronary artery restenosis                                      | No effect    | (43)      |
| Rat           | Carotid artery restenosis                                 | Inhibition   | (43)      |
| E2F decoy     | Human   | Intraoperative _ex vivo_ vein graft (clinical)                   | No effect    | (44)      |
| Carvedilol    | Rabbit  | Jugular vein to carotid artery interposition vein graft         | Inhibition   | (45)      |
| Carvedilol    | Rat     | Carotid artery restenosis                                      | No effect    | (46)      |
| Candesartan   | Human   | Restenosis after coronary stenting                              | No effect    | (47)      |
| Candesartan   | Rat     | Carotid artery restenosis                                      | Inhibition   | (48)      |
| Cilazapril    | Human   | Restenosis after coronary stenting                              | Inhibition   | (49)      |
| Rapamycin     | Human   | Restenosis after coronary stenting                              | Inhibition   | (50)      |
| Rapamycin     | Rat     | Carotid artery restenosis                                      | Inhibition   | (51–53)   |
| Paclitaxel    | Human   | Restenosis after coronary stenting                              | Inhibition   | (54)      |
| Paclitaxel    | Rat     | Carotid artery restenosis                                      | Inhibition   | (55)      |

Dilol, candesartan, and edifoligide inhibit intimal thickening in rat arteries but fail as inhibitors of vein graft failure or restenosis in humans. Sirolimus (rapamycin) and paclitaxel (taxol) are of course notable exceptions.

This study demonstrates that Dz13 can inhibit restenosis in human (Fig. 1, A and B) and rabbit (Fig. 5, B and C) venous tissue. c-Jun, is an attractive target for interventional strategies aimed at improving the long term survival of vein grafts, for several reasons. First, this study demonstrates that c-Jun is expressed with PCNA in SMCs of femoral and saphenous veins used for CABG. These findings are supported by independent studies that reported by others that c-Jun mRNA is up-regulated in stented grafts by microarray analysis. c-Jun is poorly expressed in normal arteries but dramatically induced after scraping injury. Moreover, c-Jun overexpression potentiates SMC proliferation. Finally, c-Jun overexpression after scraping injury increases MMP activity thereby causing matrix degradation, SMC migration, intimal thickening and is the predominant gelatinase regulating early vein graft remodeling (37).

Localized “anti-gene” therapy, rather than classic gene therapy, in the surgically isolated vessel at the time of harvest _ex vivo_ provides an alternative and potentially safer means to treat transplantable grafts. This approach has distinct strategic advantages for the inhibition of graft failure for several reasons. First, conduits are generally free of disease at the time of bypass and are, therefore, amenable to genetic manipulation. Second, manipulation and irradiation of the vessel is already a routine part of the surgical procedure. Third, _ex vivo_ treatment of the graft would reduce the propensity of systemic side effects. Finally, SMC hyperplasia in graft failure, like restenosis after percutaneous transluminal coronary angioplasty or stenting, occurs early after injury, which suits acute single administration (38). The safety and feasibility of treating human vein grafts _ex vivo_ with oligo-
nucleotides was demonstrated in the PREVENT trials using foldigide, a double-stranded synthetic phosphorothioated oligonucleotide bearing the E2F binding site first described by Dzau et al. in 1995 (39). PREVENT provides important proof-of-principle clinical evidence of the practicability of gene-manipulative treatment of grafts ex vivo prior to implantation. It also suggests the likelihood of even greater efficacy using more potent inhibitors and approaches to established or new targets. Recent studies have shown that topical pretreatment of vein grafts with the ERK1/2 inhibitor U0126 effectively reduces neointimal formation after vein graft arterIALIZATION (40). Local delivery of c-Jun-targeting agents, alone, or in combination with other strategies, provides a new opportunity to improve vein graft patency following coronary artery or peripheral vascular bypass surgery.

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ADDITIONS AND CORRECTIONS

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c-Jun regulates shear- and injury-inducible Egr-1 expression, vein graft stenosis after autologous end-to-side transplantation in rabbits, and intimal hyperplasia in human saphenous veins.

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The original versions of Figs. 1, 5, and 6 contained incorrect micrographs.

In Fig. 1C, the “Dz13scr/Elastin” panel contained the same micrograph as the “Dz13/Elastin” experimental condition. A replacement micrograph for the “Dz13scr/Elastin” panel from a replicate experiment performed at the time of the original experiment is provided.

The “fresh isolate/PCNA” and “fresh isolate/MMP-2” micrographs in Fig. 5D, also used in Fig. 5F to represent the base-line condition, contained the same image. Replacement micrographs from a replicate experiment performed at the time of the original experiment are provided for the two panels in Fig. 5, D and F. The panels labeled “Adeno-LacZ/c-Jun” and “Adeno-LacZ/PCNA” in Fig. 5F were reversed.

The same micrograph used to represent the “vehicle” condition in Fig. 6C was also used in Fig. 6B as the “no shear” condition. A replacement micrograph from a replicate experiment performed at the time of the original experiment is provided for the “no shear” panel in Fig. 6B.

The corrections do not affect the interpretation of the results or the conclusions of the original paper.

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