Ex Vivo Antisense Oligonucleotides to Proliferating Cell Nuclear Antigen and Cdc2 Kinase Inhibit Graft Coronary Artery Disease

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Background—The long-term success of cardiac transplantation is limited by graft coronary artery disease (GCAD). Antisense oligonucleotides (ASs) to proliferating cell nuclear antigen (PCNA) and Cdc2 kinase (Cdc2 k) can arrest cell cycle progression and inhibit neointimal hyperplasia. Transforming growth factor-β1 (TGF-β1) has been implicated in vascular smooth muscle cell (VSMC) activation. The role of TGF-β1 in GCAD remains unclear. We hypothesized that ASs to PCNA and Cdc2 k would inhibit VSMC proliferation and GCAD.

Methods and Results—In vitro VSMC proliferation was determined after pretreatment with AS solution or medium alone followed by angiotensin II stimulation. PVG-to-ACI rat heterotopic cardiac transplantation procedures were performed after ex vivo pressure-mediated transfection of ASs to PCNA and Cdc2k or saline alone. At postoperative days 30, 60, and 90, allografts were assessed for GCAD, percent neointimal macrophages and VSMCs, and TGF-β1 activity. AS pretreatment significantly attenuated VSMC proliferation. At postoperative day 90, percent affected arteries, percent occlusion, and intima-media ratio demonstrated severe GCAD in saline-treated allografts, whereas these parameters were significantly lower in AS-treated allografts. Percent neointimal macrophages and VSMCs was reduced in AS-treated allografts. TGF-β1 activity was increased in saline compared with AS-treated allografts and nontransplanted heart controls.

Conclusions—ASs to PCNA and Cdc2 k inhibit VSMC proliferation in vitro and reduce GCAD, percent neointimal VSMCs and macrophages, and TGF-β1 activity in vivo. TGF-β1 may play a “response to injury” role in the development of GCAD. The prevention of GCAD via AS inhibition of cell cycle regulatory genes before reperfusion may offer a useful clinical alternative to current therapeutic strategies. (Circulation. 2000;102[suppl III]:III-237-III-242.)

Key Words: transplantation ■ coronary disease ■ immunology ■ muscle, smooth ■ growth substances

Cardiac transplantation is currently the most effective treatment for end-stage heart failure. However, graft coronary artery disease (GCAD) remains the leading deterrent to the long-term success of this therapy. Although acute rejection and infection are the leading causes of death in the first year after transplantation; beyond this time period, the majority of complications and deaths are attributed to GCAD.1

GCAD consists of a broad spectrum of abnormalities that result in intimal thickening and diffuse narrowing of proximal and distal arteries. The severity of disease is not amenable to invasive therapies such as PTCA or CABG. Histopathologically, the disease is manifested by activated smooth muscle cells (SMCs) that migrate across the internal elastic membrane into the intima, where they proliferate and deposit extracellular matrix. This migration is accompanied by lipid accumulation and an influx of macrophages and T-lymphocytes. Finally, the intima expands while the media is attenuated. Over time, these changes may progress to a histological picture more typical of nontransplantation coronary artery disease, that is, fatty, atheromatous plaque formation and calcification complicated by fissuring and thrombus formation.2

The development of GCAD involves both antigen-dependent and-independent molecular mechanisms. Nonspecific inflammation related to reperfusion injury activates endothelial cells with the consequent expression of vascular adhesion molecules. The developing cellular milieu, which consists of endothelial cells, SMCs, and immune system cells, generates a variety of cytokines and growth factors that propagate the proliferative response. In particular, transforming growth factor-β1 (TGF-β1) has been implicated in the pathogenesis of atherosclerotic lesions via several possible mechanisms.3 In many in vitro and in vivo models, TGF-β1 appears to inhibit cellular proliferation. However, TGF-β1 has also been shown to participate in extracellular matrix production and SMC migration and proliferation.4,5 The role of TGF-β1 activity in GCAD remains unclear.

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The proliferative response, which is the final common pathway of a variety of stimuli of neointimal hyperplasia, is mediated by cell cycle proteins such as proliferating cell nuclear antigen (PCNA) and Cdc2 kinase (Cdc2 k). PCNA is integrally involved in the S phase of the cell cycle, acting as an accessory protein that allows DNA polymerase to replicate the DNA of a cell. Cdc2 k, also known as p34cdc2 or Cdk 1, acts in the propagation from G2 into mitosis through phosphorylation of important effector molecules. Because of their important roles in cellular proliferation, PCNA and Cdc2 k have been targeted in other vascular disease models. Phosphorothioate antisense oligonucleotides (ASs) that correspond to the promoter regions of the PCNA and the Cdc2 k gene transcripts have been transfected into vascular tissues and shown to preserve endothelial function and inhibit neointimal hyperplasia. Although various transfection vectors have been used in these studies, recent work in our laboratory and others has demonstrated the high efficiency and efficacy of pressure-mediated oligonucleotide transfection of arteries and cardiac allografts.

In the current study, we examined the roles of PCNA and Cdc2 k in our previously published model of GCAD. With AS technology, we demonstrated the importance of PCNA and Cdc2 k in the proliferative response of vascular SMCs (VSMCs) in culture and in the development of GCAD in vivo. Furthermore, we assessed the contributions of VSMCs and macrophages to the neointimal lesions in treated and untreated allografts. Finally, we link the activity of TGF-β1 to this disease process and suggest possible mechanisms of its involvement in GCAD.

**Methods**

**Drugs and Agents**

The rat PCNA AS sequence was 5′-GAT-CAG-GCG-TGC-CTC-AAA-3′, and the rat Cdc2 k AS sequence was 5′-GTC-CTC-CAT-AGT-TAC-TCA-3′. These ASs are complementary to the 5′ initiation codon regions of the respective mRNAs and have been previously shown in our laboratory and others to have a sequence-specific effect both in vitro and in vivo. In addition, previous work has demonstrated that the inhibition of both PCNA and Cdc2 k, but not one or the other, is necessary for cell cycle arrest. The ASs were synthesized in the Protein, Amino Acid, Nucleotide Facility at Stanford University School of Medicine with a phosphorothioate backbone and dissolved in 0.9% sodium chloride (normal saline) solution at a concentration of 80 μmol/L each.

Cyclosporin A was dissolved in olive oil at a concentration of 7.5 mg/mL and administered to heart transplant recipient rats via gavage at a dosage of 7.5 mg·kg⁻¹·d⁻¹ for 10 days (postoperative days 0 to 9). We have previously shown this dosage to be sufficient to block 90% of transplanted hearts acutely reject and are excluded from the study. The hearts that survive to 30, 60, and 90 days exhibit GCAD that is morphologically similar to the human disease without the attendant acute rejection or vascular lesions seen in other models.

**Donor Heart Treatment and Transplantation**

Treatment groups consisted of AS-treated and saline-treated allografts analyzed at postoperative day 30, 60, and 90 for GCAD. Donor (PVG) and recipient (ACI) rats were anesthetized with 1 to 2% methoxyflurane inhalation followed by intraperitoneal pentobarbital (40 mg/kg). Donor animals were systemically heparinized (50 mg/kg IV) before organ procurement. Stanford cardioplegia solution (3 mL) was infused into the donor heart proximal to an aortic cross-clamp followed by 1.0 mL of normal saline or a 80 μmol/L solution of AS to both PCNA and Cdc2 k.

Hearts were removed from the donor animals immediately after the infusion of AS or normal saline treatment and placed inside a customized pressure chamber in a 4°C bath of the respective treatment solution. The chamber pressure was increased at a rate of 60 psi/min to the desired treatment pressure of 78 psi (1 atm=14.7 psi). Depressurization was performed at a rate of 5 psi/min so that total pressure incubation time equaled 45 minutes. This ex vivo transfection technique has been optimized for rat heart transplantation and is based on the work by Dzau et al in vascular tissues. Donor cardiac grafts were then transplanted heterotopically into the abdomen of the recipient rats according to a modification of the technique described by Ono and Lindsey. Recipient animals were maintained on inhalation methoxyflurane anesthesia as needed. End-to-side anastomoses were performed from the donor ascending aorta to the recipient abdominal aorta and from the donor pulmonary artery to the recipient inferior vena cava with 8-0 Prolene. Recipients

**Cell Culture**

Primary rat VSMCs (donated by Philip Tsao, Stanford University) were derived from normal Sprague-Dawley rat thoracic aortas through enzymatic digestion as previously described. Cells were maintained in DMEM (GIBCO BRL) with 10% newborn calf serum (GIBCO BRL) and incubated at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Studies were conducted on confluent passage 4 cells. In vitro AS transfection was achieved with the nontoxic FuGENE 6 transfection reagent (Boehringer-Mannheim Bioc hemicals). Briefly, 3 μL FuGENE was added to 50 μL serum-free DMEM and incubated at room temperature for 5 minutes. The diluted FuGENE was then added to ~6 μg AS and incubated at room temperature for 15 minutes. At the indicated time point, 5 μL FuGENE/AS complex or FuGENE/medium was added to VSMCs in 100 μL medium in a 96-well plate (resulting in a final AS concentration of 1.2 μmol/L).

**Cellular Proliferation Assay**

VSMCs were added to wells of a 96-well plate at a density of 3000 cells per well, in triplicate for each of 3 conditions. After settling overnight, cells were exposed to FuGENE/medium (groups A and B) or FuGENE complexed to AS to both PCNA and Cdc2 k (group C). At 24 hours after FuGENE exposure, cells were washed 3 times with PBS. Group A cells served as a baseline and were allowed to grow in normal medium (DMEM, 10% NCS). Group B and C cells were supplemented with normal medium containing recombinant human angiotensin II (100 nmol/L; Phoenix Pharmaceuticals, Inc), which is a known potent inducer of VSMC proliferation. Cells were then allowed to proliferate for 48 hours. Cellular proliferation was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazium bromide (Sigma Chemical Co) as previously described.
were treated with cyclosporin A as described earlier, and graft function was assessed with daily palpation. On a scale ranging from 0 (no contractions) to 4 (vigorous contractions), any grafts with a score of <3 at the time of harvest was considered to be acutely rejecting or rejected and excluded from the study.

Morphometric Analysis of GCAD

Transplanted hearts were procured at postoperative days 30, 60, and 90. Transverse sections (2 to 3 mm thick) that included both ventricles and the interventricular septum were fixed in 10% buffered formalin for 24 hours. Thin (5-μm) sections of paraffin-embedded samples were stained with elastin-van Gieson stain to delineate the internal elastic membrane. One section per allograft was analyzed in a blinded fashion with computer-assisted image analysis (C-Imaging Systems), including on average at least 2 large epicardial, 2 medium-sized intramyocardial, and 2 small intramyocardial arteries per graft (mean±SD 7.1±1.7 arteries per AS-treated allograft, 7.9±3.1 arteries per saline-treated allograft; P=NS).

To assess the severity of disease, each artery was assessed for percent intimal proliferation (percent occlusion, also described as “cross-sectional luminal stenosis”), defined as the area of the intima divided by the entire area inside the internal elastic membrane (intimal area plus luminal area), and for intima-media ratio, defined as intimal area divided by media area (area between internal elastic membrane and external elastic membrane).

As in the human disease, the incidence of GCAD in a given cardiac allograft in our model has a wide variability on an artery-to-artery basis. To document this intra-animal variability of GCAD, heart sections were assessed for the incidence of disease based on the observation that normal arteries of native rat hearts demonstrate an intimal proliferation of up to 10% (data not shown). The percent of arteries affected by GCAD was determined by counting the number of arteries in a given heart section that had an intimal proliferation of >10%.

Neointimal VSMC and Macrophage Immunohistochemistry

Immunohistochemistry with mouse anti-rat macrophage antibody (ED-1; Serotec) and anti-α-actin SMC monoclonal antibody (Sigma Chemical Co) was performed in 5-μm paraffin-embedded sections with an established technique based on an avidin-biotinylated horseradish peroxide complex (Vectastain Elite ABC kit; Vector Laboratories). Arteries to be scored were selected in a manner similar to that described here for GCAD morphometry (mean±SD 6.0±2.3 arteries per AS-treated allograft, 5.8±0.8 arteries per saline-treated allograft; P=NS). The area stained specifically with ED-1 or α-actin SMC was assessed by measurement of the area encompassed by pixels of the color intensity of immunopositive cells and tabulated as the percentage of the total pixels of the neointimal area which was detected in sequential sections stained for elastin.

Reverse Transcription–Polymerase Chain Reaction Semiquantification of TGF-β1–Induced Early Gene mRNA Transcripts

TGF-β1–induced mRNA transcripts (TIEG) is a recently described Sp-1–like transcription factor that serves as an indicator of TGF-β1 activity.19 Left ventricular specimens (weight ~100 mg) were taken from the cardiac allografts at the time of harvest of the 90-day time point animals. Total RNA was isolated with the RNAgents Total RNA Isolation System (Promega Corp). Reverse transcription (RT)–polymerase chain reaction (PCR) was performed with the Access RT-PCR System (Promega Corp) under conditions previously described.19 Primers for the reactions were TIEG 5′, CAT-CTG-TAG-GTC-TTC-TCA-GCC; TIEG 3′, CTG-CTG-AAG-AGA-ATC-CAG-G (cDNA length 348 bp); β-actin (total RNA standard) 5′, TTG-TAA-CCA-CTT-GAG-AGT-GAG-GCA; and 3′, GAT-CAT-CTT-GTC-GTT-GCC-G (cDNA length 750 bp). The RT-PCRs were RT, 45 minutes at 48°C; RT inactivation and RNA/cDNA/primer denaturation, 5 minutes at 94°C; 35 cycles of PCR amplification: denaturation, 45 seconds at 94°C, annealing,
GCAD Immunohistochemistry
Postoperative day 90 lesions were characterized by quantifying the percent neointimal area of SMCs and macrophages, respectively. The results of this analysis, shown in Figure 4, indicate increased macrophage (ED-1) and SMC-actin area within the intima of the saline-treated group compared with the AS-treated group. For macrophages, this difference is statistically significant (AS 0.07 ± 0.24%, saline 0.64 ± 1.6%, P = 0.04). For SMCs, the difference is not statistically significant; however, a trend is apparent (AS 0.83 ± 1.99%, saline 1.19 ± 1.96%, P = 0.46).

TGF-β1-Induced Early Gene Reverse Transcription–Polymerase Chain Reaction
Because of its association with atherosclerotic disease and with cell cycle regulation, we investigated TGF-β1 activity in our model. TGF-β1-induced early gene is a recently characterized Sp-1-like zinc finger protein that serves as an indicator of TGF-β1 activity. Figure 5 demonstrates TGF-β1-induced early gene reverse transcription–polymerase chain reaction amplification products in postoperative day 90 hearts and in normal heart controls. The bottom row depicts amplification products of β-actin mRNA as a standard. In the top row, the first 3 lanes represent normal, nontransplanted PVG hearts; the middle 3 lanes represent saline-treated hearts; and the right 3 lanes represent AS-treated hearts. The band of interest is a 348-bp product that is clearly seen in the saline-treated hearts. In the normal and AS-treated hearts, little or no amplification product is detected.

Conclusions
From these data, we conclude that increased expression of PCNA and Cdc2 k may be important in the development of neointimal proliferation in this model of GCAD. In addition, AS blockade of these cell cycle regulatory proteins effectively prevents both in vitro VSMC proliferation and in vivo...
of treatment that have been suggested include the use of TGF-\(\beta\) lowering agents, 22 which have delayed disease progression. Because PCNA and Cdc2 k are transcriptionally regulated, they represent ideal targets for AS technology. In addition to playing discrete roles in the progression of the cell cycle, these 2 genes may also be critically involved in cell cycle checkpoints, at which points cells may phenotypically change, undergo apoptosis, or experience mitotic catastrophe.6 Such changes are likely to occur in a variety of cell types on reperfusion and early function of cardiac allografts, and these phenotypic changes may have a significant impact on the later-stage vascular biology. Our work with fluores-

Figure 4. Postoperative day 90 immunohistochemistry. Immunopositive areas stained for macrophages (ED-1) and VSMCs (SMC-actin) were quantified as a percent of the total neointimal area with computerized image analysis. Saline-treated allograft neointimal areas trended toward higher VSMC content and had significantly higher macrophage content than AS-treated allografts. Error bars indicate 1 SD.

incidence and severity of GCAD. Furthermore, increased TGF-\(\beta\) activity is associated with neointimal hyperplasia in this model.

 Currently, retransplantation is the only definitive therapy for GCAD, and recurrence cannot be prevented. Other forms of treatment that have been suggested include the use of calcium channel blockers, 20 ACE inhibitors, 21 and lipid-lowering agents, 22 which have delayed disease progression. In addition, PTCA, CABG, and varying immunosuppression protocols have been suggested; however, none of these treatments have led to significant improvements in survival. In the present study, another avenue that is explored is gene therapy, specifically, the use of ASs toward cell proliferative genes as a preventative strategy before reperfusion of the allograft.

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Figure 5. TIEG RT-PCR. At postoperative day 90, amplification products of TIEG mRNA (top row) serve as an indicator of TGF-\(\beta\) activity. This band is clearly seen in the saline-treated hearts (middle lanes), whereas little or no amplification product is detected in the normal (left 3 lanes) and AS-treated (right 3 lanes) hearts.

The association of TGF-\(\beta\) with GCAD seen in the present study complements data from previous research and suggests further avenues of investigation. Koglin et al23 have shown that TGF-\(\beta\)-deficient mice are less able to suppress immune system cytokine expression than are TGF-\(\beta\)-competent mice. In addition, in vitro studies have suggested that TGF-\(\beta\) can arrest cell growth via activation of cell cycle inhibitors24 or via inhibition of the function of cell cycle–regulatory proteins such as Cdc2 k.25 Finally, Bobik et al26 suggested that TGF-\(\beta\) contributes to atherosclerotic lesions by stimulating extracellular matrix production, inhibiting SMC proliferation, and activating macrophages. These data suggest that the increased TGF-\(\beta\) activity seen in our model of GCAD represents a “response to injury” phenomenon. It is possible that in the saline-treated allografts, TGF-\(\beta\) is upregulated to counteract the proliferative response and paradoxically causes further intimal thickening via stimulation of extracellular matrix deposition. In the AS-treated allografts, on the other hand, the cascade of cyclical events that lead to the proliferation and activation of various cell types may be curtailed, with little or no activation of the TGF-\(\beta\)-feedback loop. Future investigations into what cell types express TGF-\(\beta\) at which time points in the development of the disease may further elucidate this process.

In summary, ASs to PCNA and Cdc2 k inhibit VSMC proliferation in vitro and prevent GCAD in vivo. TGF-\(\beta\) activity is linked to the process of GCAD and may represent a response-to-injury phenomenon. This novel technique of ex vivo single-administration AS-mediated gene therapy may be a useful clinical tool in the prevention of GCAD.

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