CARDIOTHORACIC TRANSPLANTATION

EX VIVO GENE THERAPY PREVENTS CHRONIC GRAFT VASCULAR DISEASE IN CARDIAC ALLOGRAFTS

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Objective: We hypothesized that ex vivo hyperbaric transfection of antisense oligodeoxynucleotides for blockade of intercellular adhesion molecule-1, an important mediator of cell adhesion and T-cell co-stimulation, would reduce chronic graft vascular disease in cardiac allografts. **Methods:** PVG hearts underwent ex vivo transfection with antisense, reverse antisense intercellular adhesion molecule-1 oligodeoxynucleotide (80 μ mol/L), or saline solution at 3 atm pressure for 45 minutes at 4° C and were transplanted heterotopically into ACI recipients with or without treatment with intercellular adhesion molecule-1 (1A29) or leukocyte function associated antigen-1 (WT.1) monoclonal antibodies. Transfection efficiency was confirmed with fluorescein isothiocyanate-labeled oligodeoxynucleotides and fluorescent microscopy. Efficacy of intracellular adhesion molecule-1 blockade was assessed with the use of immunohistochemistry. Graft reperfusion injury was evaluated at 6 to 24 hours by neutrophil infiltration (myeloperoxidase [MPO]), cardiac edema (%wt/wt), and histologic injury (percent contraction band necrosis). Grafts from recipients treated with cyclosporine A (5 mg/kg per day, days 0 to 9) were scored for chronic graft vascular disease on postoperative day 90 ranging from 0 (no involvement) to 4 (>50% vascular occlusion). **Results:** Transfection was highly efficient (fluorescein isothiocyanate-labeled oligodeoxynucleotides in $48\% \pm 5\%$ of total myocardial nuclei) and effective at blocking intracellular adhesion molecule-1 expression (positive area in allografts taken on postoperative day 3 was reduced from $100\% \pm 0\%$ to $52\% \pm 14\%$, $n = 4$). Blockade with antisense oligodeoxynucleotides versus monoclonal antibodies was less effective at preventing reperfusion injury while more effective at reducing chronic graft vascular disease (score 0.98 ± 0.48 , $p < 0.05$). Reverse antisense oligodeoxynucleotides and vector control (antisense oligodeoxynucleotide infusion without pressure) groups failed to demonstrate this beneficial effect. **Conclusion:** Hyperbaric transfection of antisense oligodeoxynucleotides proved highly efficient, effective at blockade of intracellular adhesion molecule-1, and demonstrated a sequence-specific reduction in chronic graft vascular disease. This highly targeted alteration of donor organ immunogenicity may have an important future role in clinical immunosuppressive strategies. (J Thorac Cardiovasc Surg 1998;116:386-96)

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Chronic cardiac allograft rejection is a process that is thought to be due to alloantigen-dependent and -independent events¹ and results in a diffuse coronary vascular narrowing termed *chronic graft vascular disease* (CGVD). Current immunosuppressants, although effective at controlling acute alloimmune responses, have not altered the devastating impact of CGVD on long-term graft survival. Ex vivo somatic gene therapy has been proposed as a novel, highly targeted strategy of genetically manipulating donor allografts to prevent CGVD. Unlike genetic engineering applications such as cloning, somatic gene therapy has yet to prove clinically useful, primarily because of consistently poor transfection efficiency.

We^{2,3} have used hydrostatic pressure as a novel, technically simple, nontoxic vector for nuclear delivery of phosphorothioate oligodeoxynucleotides (ODN) to a variety of tissues. The consistently high transfection efficiency of this method in cardiac tissues raises the intriguing possibility of using antisense technology for cardiac allograft immunosuppression. In the present studies, phosphorothioate antisense oligonucleotides (AS ODN) that bind specifically to the target messenger ribonucleic acid (mRNA) of intercellular adhesion molecule-1 (ICAM-1) were delivered to cardiac allografts ex vivo by means of this hyperbaric vector. ICAM-1 was chosen as the antisense target because its expression is solely transcriptionally regulated⁴ and low at baseline in myocardium.⁵ Furthermore, the central role that ICAM-1 maintains in allograft pathophysiology makes it an ideal immunosuppressive target.

Specific blockade of this molecule interrupts the positive feedback cascade of ICAM-1 with its ligand, leukocyte function associated antigen-1 (LFA-1), and results in reduced neutrophil-mediated reperfusion injury⁶ and donor-specific allograft tolerance.^{7,8} It has been shown that early nonspecific inflammation from reperfusion injury up-regulates graft immunogenicity and therefore increases the rates of acute⁹ and chronic rejection.¹⁰ In addition to indirectly promoting CGVD via reperfusion injury, clinical¹¹ and experimental¹² models have revealed a second, later phase of ICAM-1 up-regulation that coincides with the histologic onset of vasculopathy, suggesting a more direct pathogenic role of ICAM-1 in development of CGVD.

Regardless of the exact mechanism of effect, we hypothesized that the ex vivo blockade of ICAM-1 in donor grafts would reduce the severity of vascu-

lopathy in a rodent cardiac allograft model of CGVD.

Methods

Drugs and agents. The sequence chosen for synthesis of the rat AS ODN (Keystone Laboratories, Inc., Menlo Park, Calif.) was modified from a previously reported⁸ mouse AS ICAM-1 ODN using information obtained from GenBank regarding the differences of rat¹³ and mouse¹⁴ ICAM-1 mRNA at the homologous 3' untranslated target region:

Mouse ICAM-1 mRNA (base No. 1755-74): 5'-ATG GTG GCC TGG GGG ATG CA-3'

AS mRNA (IP3082): 3'-TAC CAC CGG ACC CCC TAC GT-5'

Rat ICAM-1 mRNA (base No. 1768-87): 5'-ACA GTG GCC TGG GGA TGC A-3'

AS mRNA (used in our study): 3'-TGT CAC CGG ACC CCT ACG T-5'

(*bold type represents bases that differ between mouse and rat ICAM-1 mRNA and AS at this region*).

For studies to address the efficiency of transfection, ODN with a scrambled sequence was synthesized and labeled on the 5' and 3' ends with fluorescein isothiocyanate (FITC). For investigation of the true antisense mechanism of AS ODN, control reverse antisense (RAS) sequences were synthesized. LFA-1 and ICAM-1 monoclonal antibodies (MAb) were purified from the ascites of mice injected with hybridomas (WT.1 and 1A29, gift of M. Miyasaka, Osaka, Japan) and administered via the dorsal penile vein at 1.5 mg/kg per day from postoperative day 0 to day 6. This dose was confirmed to promote full saturation of their respective ligands on peripheral blood leukocytes 24 hours after administration by flow cytometric analysis. Cyclosporine A (INN: ciclosporin) was dissolved in olive oil (10 mg/ml) and administered via gavage at a dose of 5 mg/kg for 10 days after the operation to prevent acute rejection and allow for the development of CGVD in all groups.

Animals. Adult male (8 to 10 weeks old, 230 to 270 gm) PVG (RT1c) and ACI (RT1a) rats were obtained from Harlan Sprague Dawley, Indianapolis, Indiana. All animals were maintained in the animal care facilities of the Department of Cardiothoracic Surgery, Stanford University Medical Center, Stanford, California. Their environment was maintained at 21° ± 2° C with a time-regulated light period from 7:00 AM to 7:00 PM. Rats were provided water and dry food ad libitum. Periodic serologic analysis of room sentinel animals showed that all rats were free of acute viral infection. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Heart transplantation. Both donor and recipient rats were anesthetized with methoxyflurane (inhalational) and sodium pentobarbital (50 mg/kg intraperitoneally). Hearts were procured and either underwent transfection or mock

Table 1. Study groups

Group	Treatment	No.	Purpose
Experimental	Ex vivo AS ODN	25	To assess biologic effects of AS ODN transfection on CGVD
ODN control	Ex vivo RAS ODN	6	To control for non-AS (e.g., aptamer) biologic effects of ODN
Vector control	Ex vivo AS ODN without pressure	6	To verify the need for transfection to have biologic effects
Negative control	None	10	For CGVD comparison
Positive control No. 1	ICAM-1 MAb (given to recipient)	25	To compare effects of ICAM-1 blockade with AS ODN vs MAb (gold standard)
Positive control No. 2	LFA-1 MAb (given to recipient)	25	To control for endothelial activating effects of ICAM-1 MAb

transfection as described below. After incubation, hearts were grafted heterotopically into the abdomen of allogenic recipients by means of a modification of the methods described by Ono and Lindsay.^{14a} Immediately after reperfusion and daily thereafter, grafts were assessed for successful return of rhythmic cardiac contractions on a scale ranging from 0 (no contractions) to 4 (vigorous contractions). Primary failure was defined in grafts that did not achieve an immediate palpation score greater than 2. After an initial period of successful function, hearts were considered acutely rejected when palpation scores were less than 1. Grafts with either of these diagnoses were excluded from further analysis.

In vivo transfection efficiency. After donor rat thoracotomy, ice cold Stanford cardioplegic solution was applied to the heart topically and infused into the aortic root proximal to an aortic crossclamp for coronary perfusion. After complete cardioplegic arrest, the coronary arteries were perfused with FITC-tagged scrambled ODN solution (0.5 ml of 80 μ mol/L ODN in phosphate-buffered saline solution at 1 ml/min). The heart was then explanted, placed into a well of ODN solution in an ice bath at 4° C, and pressurized to 3 atm in a custom-designed pressure vessel for 45 minutes. Donor hearts treated with the above protocol without the use of pressure served as the control group. After 24 hours' reperfusion in the recipient, grafts were procured, flushed with ice-cold phosphate-buffered saline solution, and immediately snap frozen in OCT embedding compound in liquid nitrogen. After equilibration to -20° C, 6 μ m sections were taken perpendicular to the long axis of the heart from the superior, midportion, and inferior portions of the grafts. Hoechst dye was used to facilitate identification of total nuclei under fluorescent microscopy, and transfection efficiency was assessed by determining the percentage of myocardial nuclear localization of fluorescence in 5 random high-power ($\times 100$) fields chosen from each of three graft regions (superior, midportion, and inferior) for a total of 15 high-power fields per graft.

Verification of transfection efficacy. After confirmation of transfection efficiency, we then verified that the delivery of AS ODN by means of the hyperbaric method would successfully prevent ICAM-1 up-regulation in the PVG to ACI model of acute rejection. On the basis of pilot studies of ICAM-1 protein expression analyzed in control PVG

allografts by Western blot and immunohistochemistry, postoperative day 3 was chosen as the time point most appropriate for comparison with AS ODN transfected grafts. PVG grafts were transfected with AS ICAM-1 ODN or RAS ODN and heterotopically transplanted into the abdomen of untreated ACI recipients. Hearts were procured on postoperative day 3 for immunohistochemical assessment of ICAM-1, CD4, CD8, macrophage, and major histocompatibility complex (MHC) classes I and II using the method outlined in the Histostain SP kit (Zymed Laboratories, South San Francisco, Calif.). In brief, 6 μ m sections were air dried at room temperature and fixed in acetone at 20° C. Sections were rehydrated in 1% bovine albumin-phosphate buffered saline solution and then incubated with the one of the following primary antibodies (from Serotec, Westbury, N.Y.): 1A29 (ICAM-1), W3/25 (CD4), MRC OX-8 (CD8), ED1 and ED2 (macrophage), 156 and 280 (MHC class I), and 46 (MHC class II). This was followed by incubation with a biotinylated goat antimouse immunoglobulin G (Zymed Laboratories, South San Francisco, Calif.). The avidin-biotin complex was applied and diaminobenzidine tetrahydrochloride was used as the chromogen. The substitution of 1% bovine albumin-phosphate buffered saline solution for the primary antibody served as the negative (reagent) control. Rat cervical lymph nodes served as the ICAM-1 positive control. Sections from transfected and control grafts were semiquantitatively scored (0 to 3+) for antigen staining intensity by a pathologist blinded as to experimental group. Computer-assisted image analysis (C-Imaging Systems, Cranberry Twp., N.J.) was used to measure the total graft cross-sectional area positive for ICAM-1 to provide a quantitative assessment of the AS effect.

Assessment of biologic effects of ex vivo AS ICAM-1 ODN. The biologic effects of ex vivo AS ODN transfection (experimental group, $n = 25$) were compared with five additional control groups as shown in Table 1: an ODN control ($n = 6$), a vector control ($n = 6$), a negative control ($n = 10$), and two positive control groups. Rats in the positive control groups received untransfected grafts and were treated with a single injection of ICAM-1 MAb (positive control 1, $n = 25$) or LFA-1 MAb (positive control 2, $n = 25$) 1.5 mg/kg intravenously just before graft reperfusion. After successful return of heart contractions, grafts were either procured at 6, 12, and 24 hours to assess

Fig. 1. After a short postoperative course of cyclosporine A, ACI recipients were put to death on postoperative day 90 so that CGVD in the heterotopic PVG allografts could be assessed. Although grafts from both groups displayed the classic histologic findings of concentric neointimal hyperplasia inside the internal elastic lamina (*arrow*), negative control allografts were found to have a significantly greater number of vessels scoring greater than 3 as illustrated in these representative photomicrographs. (Hematoxylin-eosin, original magnifications 200 $\times$.)

reperfusion injury or the recipients were treated with a 10-day course of cyclosporine A 5 mg/kg per day via gastric gavage to promote long-term graft survival and allow for the development of CGVD. Studies of reperfusion injury analyzed three parameters of myocardial injury: (1) cardiac edema using percent wet weight assay (%wt/wt)—heart weighed before and after drying in an oven at 100 $^{\circ}$ C for 24 hours; (2) neutrophil infiltration assessed by MPO enzyme activity—total protein isolated from frozen tissue and assayed for change in absorbance at 470 nm at 1 minute after addition of guaiacol and peroxide, and (3) histologic injury as determined by the presence of contraction band necrosis (%CBN)—after initial review with a pathologist, the percentage cross-sectional area of a trichrome-stained section involved with contraction band necrosis was quantitatively determined by means of computer-assisted image analysis (C-imaging systems, Cranberry Twp., N.J.).

For CGVD analysis, grafts were removed on postoperative day 90 for sectioning after fixation in buffered formalin for 24 hours. Thin sections of paraffin-embedded samples stained with hematoxylin-eosin and Evans-van Gieson stains were examined by a pathologist (M.B.) blinded as to experimental group and assigned a CGVD score. This score was the mean score for all the individual vessels in a section and therefore represented the fact that normal and occluded vessels were often found in the same sections (i.e., displayed high standard deviations). Individual vessels were subjected to a five-point grading scale

from 0 to 4 (0 for no involvement, 1 for partial intimal involvement, 2 for concentric intimal thickening, 3 for more severe concentric involvement up to 50% luminal narrowing, and 4 for > 50% up to complete occlusion) (Fig. 1).

Immunohistochemical studies. PVG cardiac grafts were transfected with AS ICAM-1 ODN or mock transfected with saline solution for 45 minutes at 4 $^{\circ}$ C and grafted into ACI recipients treated with cyclosporin A 5 mg/kg via gavage on postoperative days 0 to 9. Grafts ($n = 3$ each time point) were then procured at 6, 12, and 24 hours and on postoperative days 30, 60, and 90. Grafts were divided so that adjacent sections were snap frozen in OCT embedding compound (Miles, Elkhart, Ind.) for immunohistochemical staining using the Histostain SP kit (Zymed Laboratories, South San Francisco, Calif.) and fixed in formalin for Evans-van Gieson staining to assess CGVD. Immunohistochemistry sections were scored (0 to 3+) in terms of both overall and specifically perivascular staining intensity for ICAM-1, CD4, CD8, macrophage, and MHC classes I and II by a pathologist blinded as to the experimental group. The immunohistochemistry and Evans-van Gieson findings of each artery and arteriole of the grafts were compared to identify any correlation between positive perivascular ICAM-1 staining (score > 1) and underlying vasculopathy (CGVD score > 2).

Statistical analysis. Transfection efficiency data (percent nuclear fluorescence) was compared between groups using the Mann-Whitney U test. The parameters of

Fig. 2. Hyperbaric transfection led to highly efficient myocyte delivery of ODN as illustrated by the large number of total myocardial nuclei (shown on the Hoechst dye stain, **B**), which demonstrate nuclear localization of fluorescence (shown under the fluorescent filter, **A**). Intracoronary infusion of FITC-ODN without application of pressure did not lead to appreciable transfection, as indicated by the failure of Hoechst dye-positive nuclei (**D**) to show nuclear localization of fluorescence (**C**). (All original magnifications 200 $\times$.)

reperfusion injury, MPO and %wt/wt, were compared by means of analysis of variance with a post hoc *t* test. Given the consistently unequal standard deviations noted in the mean areas of percent contraction band necrosis and percent ICAM-1 staining between groups, the Welch *t* test was required for comparison of these data. CGVD scores were compared between groups by means of the Mann-Whitney U test. An association between positive perivascular ICAM-1 staining of any given artery on immunohistochemical analysis (defined as a score > 1) and underlying vasculopathy of that vessel (defined as CGVD score > 2) was investigated by means of the Fisher exact test.

Results

Transfection efficiency. Hyperbaric transfection was highly efficient but non-uniform, with some but not all areas showing dense nuclear fluorescence (sections with up to 90% of nuclei) after 24 hours of reperfusion. Low-power fluorescent microscopic examination of these areas revealed the highly efficient, homogeneous nature of fluorescent nuclear localization of FITC-ODN relative to the total nuclei as shown by examining the same section under the Hoechst filter (Fig. 2, *A* and *B*). Randomly chosen high-power fields documented that a mean of $48\% \pm 5\%$ ($n = 5$) of total nuclei per graft

were successfully transfected as indicated by this nuclear localization of FITC.

Antisense efficacy. On postoperative day 3, untransfected control cardiac grafts undergoing early rejection in untreated recipients demonstrated diffuse dark red ICAM-1 immunohistochemical staining. Ex vivo AS ODN transfection significantly, although inhomogeneously, affected ICAM-1 expression at this time point, as illustrated in the representative photomicrograph of Fig. 3. By image analysis, ICAM-1 staining was reduced to $53\% \pm 14\%$ of total area in AS ODN transfected grafts ($p < 0.05$ vs $100\% \pm 0\%$ in control grafts, Student's *t* test, $n = 4$). This mean cross-sectional area was strikingly similar to the percentage of myocardium that was successfully transfected as demonstrated by the above efficiency studies. MHC class I and II expression was unchanged in transfected grafts ($n = 4$), supporting a sequence-specific effect of the AS ODN.

Effect of AS ODN on reperfusion injury. The establishment of a highly efficient transfection method with documented efficacy at blocking ICAM-1 up-regulation provided a rational basis to examine for biologic effects of AS ODN. Total

Fig. 3. On postoperative day 3, AS ODN was found to effectively block ICAM-1 expression on myocytes and prevent vascular expression (*arrow*) beyond baseline (**A**) compared with diffuse expression on both cell types at this time point in untransfected grafts (**B**) as illustrated in these representative high-power (original magnification 100 $\times$) immunohistochemistry sections. However, low-power (original magnification 20 $\times$) views reveal AS ODN blockade of ICAM-1 expression was inhomogeneous with some areas demonstrating diffuse ICAM-1 expression (**C**) as seen throughout the graft (**D**) in untreated controls (original magnification 20 $\times$).

ischemic times (from cardioplegic arrest of donor heart to return of contractions in the recipient's abdomen) ranged from 62 to 75 minutes, but there were no statistically significant differences in the mean ischemic times between groups by analysis of variance (data not shown). A single preoperative recipient dose of ICAM-1 MAb, included as a positive control, prevented the rise in MPO levels seen in negative control, untreated grafts most prominently at the early time points: 6 hours (0.02 ± 0.02 vs 0.10 ± 0.04 U/mg protein, $n = 6$, $p < 0.01$ by analysis of variance) and 12 hours (0.05 ± 0.06 vs 0.11 ± 0.04 U/mg protein, $n = 6$, $p < 0.05$) (Fig. 4, A). Cardiac edema (%wt/wt) in these grafts from recipients treated with ICAM-1 MAb followed a similar pattern, with significance reached at 6 hours ($76.30\% \pm 1.45\%$ vs $79.77\% \pm 1.32\%$, $n = 6$, $p < 0.01$) and 12 hours ($77.15\% \pm 1.29\%$ vs $79.24\% \pm 0.71\%$, $n = 6$) of reperfusion and no difference at 24 hours ($76.03\% \pm 0.89\%$ vs $76.95\% \pm 0.93\%$, $n = 6$, $p =$ not significant) (Fig. 4, B). Trends toward a significant reduction in contraction band necrosis after MAb treatment were noted at 12 hours ($10.1 \pm$

1.5 vs 12.0 ± 1.4 , $n = 5$, $p = 0.07$) and 24 hours (10.8 ± 1.4 vs 12.8 ± 1.9 , $n = 6$, $p = 0.06$) (Fig. 4, C).

Although somewhat less effective, similar reductions of cardiac parameters of reperfusion injury were noted in recipients treated with LFA-1 MAb at the 6-hour time point (MPO: 0.03 ± 0.04 vs 0.10 ± 0.04 U/mg protein, $n = 6$, $p < 0.05$; %wt/wt: 77.02 ± 2.22 vs 79.77 ± 1.32 , $n = 6$, $p < 0.05$; %CBN: $9.1\% \pm 1.8\%$ vs $11.2\% \pm 1.8\%$, $n = 6$, $p = 0.07$) and the 12-hour time point (MPO: 0.06 ± 0.02 vs 0.11 ± 0.04 U/mg protein, $n = 6$, $p < 0.05$; %wt/wt: $75.0\% \pm 1.25\%$ vs $79.24\% \pm 0.71\%$, $n = 5$, $p = 0.08$; %CBN: $10.3\% \pm 1.65\%$ vs $12.0\% \pm 2.62\%$, $n = 5$, $p = 0.06$).

Compared with the positive control groups, ex vivo AS ODN transfection was relatively less effective at preventing early reperfusion injury, displaying a reduction in parameters of reperfusion injury only at the later time points. No difference from control was noted at 6 hours and only trends toward significance were seen at 12 hours (MPO: 0.07 ± 0.02 vs 0.11 ± 0.04 U/mg protein, $n = 5$, $p = 0.08$; %wt/wt: $76.9\% \pm 2.19\%$ vs $79.24\% \pm 0.71\%$, $n = 5$,

Fig. 4. Biologic effects of AS ICAM-1 ODN at 6, 12, and 24 hours were assessed in transfected allografts by analyzing for three parameters of reperfusion injury: **A**, neutrophil infiltration using the myeloperoxidase assay, **B**, cardiac edema using the %wt/wt assay, and **C**, histologic injury using image analysis to quantitate the percent total graft area involved with contraction band necrosis. For control, three groups involving no donor or recipient treatment (negative control), recipient ICAM-1 MAb (positive control No. 1), or LFA-1 MAb (positive control No. 2) treatment were compared with the AS ODN experimental group.

$p = 0.06$; %CBN: 9.9 ± 2.23 vs 12.0 ± 2.62 , $n = 5$, $p = 0.20$). However, statistical significance was achieved in these parameters at 24 hours (MPO: 0.06 ± 0.02 vs 0.10 ± 0.04 U/mg protein, $n = 6$, $p = 0.05$; %wt/wt: 76.7 ± 1.25 vs 78.6 ± 1.46 , $n = 6$, $p < 0.05$; %CBN: $9.4\% \pm 1.03\%$ vs $12.8\% \pm 1.9\%$, $n = 6$, $p < 0.02$). There was no statistically significant difference between the parameters of reperfusion injury compared with perioperative ICAM-1 MAb at any time point.

Effect of AS ODN on CGVD. An initial 10-day course of cyclosporine A (5 mg/kg per gavage

tube) promoted long-term PVG allograft survival (i.e., >60 days) in 53 of 75 ACI recipients (70.7%). Despite prolonged survival of the vigorously beating allograft, CGVD reproducibly develops in control PVG grafts with a mean score of 1.84 ± 0.75 ($n = 17$) (Table II). Contrary to impressive effects on reperfusion injury, perioperative MAb administration had minimal benefit against chronic allograft vasculopathy. After a single perioperative dose of either MAb, CGVD scores on postoperative day 90 were reduced to 1.25 ± 1.01 , $n = 10$ (anti-ICAM-1 MAb) and

Fig. 5. Immunohistochemical studies from control (untransfected) PVG allografts between postoperative days 30 and 90 demonstrated a return to baseline expression of all antigens analyzed with the exception of a decreased but persistent perivascular expression of MHC class II, CD4, and macrophage antigens (original magnifications 200 $\times$). AS ICAM-1 ODN transfection did not alter these results (data not shown). As illustrated in the accompanying hematoxylin-eosin stains (original magnifications 200 $\times$), the persistent expression of these antigens was significantly associated with severe vasculopathy (CGVD score > 2).

1.58 ± 1.13 , $n = 10$ (anti-LFA MAb), differences which did not reach statistical significance compared with untreated controls. On the other hand, ex vivo AS ICAM-1 ODN transfection significantly reduced CGVD to 0.98 ± 0.48 , $n = 10$ ($p < 0.05$, Mann-Whitney U test). This protective effect on graft vasculature was eliminated by using an otherwise identical transfection protocol with the substitution of a control RAS ODN sequence (1.73 ± 0.98 , $n = 6$) or by merely perfusing graft coronary arteries with AS ODN without the application of pressure (1.65 ± 0.78 , $n = 6$).

Immunohistochemical studies. Immunohistochemical studies were then used to further characterize the molecular and cellular immune response in transfected versus untransfected PVG hearts in ACI recipients. At baseline (e.g., before transplantation), PVG hearts demonstrated low expression of ICAM-1 and MHC class I and II antigens with almost no CD4 cells, CD8 cells, or macrophages. Twelve hours after reperfusion, no significant effect

Table II. CGVD in AS ICAM-1-treated versus control heart grafts

Group	No.	CGVD score
Negative control	17	1.84 ± 0.75
Positive control No. 1	10	1.25 ± 1.01
Positive control No. 2	10	1.56 ± 0.96
ODN control	6	1.73 ± 0.98
Vector control	6	1.65 ± 0.78
Experimental group	10	$0.98 \pm 0.48^*$

* $p < 0.05$, Mann-Whitney U test.

of AS ODN transfection versus control was noted on ICAM-1 expression on arteries (mean score 0 vs 0, $n = 3$), arterioles (1.7 vs 2.0), capillaries (2.5 vs 2.5), veins (2.3 vs 2.5), or myocytes (0 vs 0). On postoperative day 1, however, weak ICAM-1 staining was noted on the intercalated disks of myocytes in control (mean score 0.8, $n = 5$) but not treated grafts (0, $n = 6$).

After a short course of cyclosporine A, control grafts showed a dramatic increase in the expression

Fig. 6. As shown, these representative immunohistochemistry sections procured on postoperative day 90, both AS ODN transfected (**A**, original magnification 200 $\times$) and control allografts (**B**, original magnification 200 $\times$) also demonstrated a return to baseline levels of ICAM-1 expression throughout the graft. However, specific analysis of arterial (*arrow*) expression revealed a significantly increased amount of perivascular ICAM-1 with an associated ICAM-1 positive infiltrate (*arrowhead*) in control compared with AS ODN transfected grafts.

of all antigens on postoperative day 30 (Fig. 5). By day 90, CD8 and MHC class I and II expression returned to baseline with decreased but persistent CD4 and macrophage accumulation primarily around those arteries and arterioles expressing ICAM-1 (Fig. 6). The presence of ICAM-1 around any given artery, as seen around 45% of allograft arteries (33 of 76 total vessels, $n = 17$), was significantly associated with a CGVD score > 2 ($p = 0.02$, Fisher exact test) and provided for a relative risk of vasculopathy of 2.04 (95% confidence interval: 1.1 to 3.9).

Overall, ex vivo AS ICAM-1 ODN transfection had little effect on antigen expression in PVG allografts. ICAM-1, MHC classes I and II, CD4, CD8, and macrophage antigen expression followed similar patterns to control on postoperative days 30 and 60. However, on postoperative day 90, there was a significant reduction in the number of arteries with positive ICAM-1 perivascular staining (15% vs 45%, $p < 0.02$, Fisher exact test), and there were fewer associated macrophage and CD4 infiltrates (Fig. 6). Those vessels staining positive for perivascular ICAM-1 despite ex vivo AS ODN transfection were at an increased relative risk of CGVD (2.63, 95% confidence interval: 1.1 to 6.3).

Discussion

ICAM-1, an adhesion molecule induced on myocytes and a wide variety of other cell types after cytokine stimulation, mediates major events in host inflammatory and immune responses.⁴ Ex vivo AS ODN blockade of ICAM-1 provides for a more

graft-specific, yet equally potent antiinflammatory and immunosuppressive strategy that avoids the infectious risk inherent in recipient ICAM-1 blockade using systemic MAb. In this rodent heterotopic transplant model, AS ODN transfection of donor cardiac grafts resulted in a reduction of postoperative ICAM-1 up-regulation and a significant blockade of CGVD compared with untransfected control grafts. To our knowledge, no study to date has demonstrated a biologic effect of gene therapy on the development of CGVD.

Clinical and experimental studies clearly point to an important role for ICAM-1 on the pathogenesis of CGVD. By promoting increased reperfusion injury, early (< 24 hours) ICAM-1 up-regulation indirectly plays a role in the initiation of the vascular "response to injury" phenomenon thought responsible for neointimal hyperplasia of CGVD. In addition, the correlation of late (> 30 days) up-regulation of perivascular ICAM-1 expression with development of CGVD in this and other models^{11, 12, 15} suggests a more direct role for this molecule in these vascular lesions. MAb provides a positive control group for ICAM-1 blockade in the immediate postoperative period, but because of its rapid clearance, a single perioperative dose would not be expected to influence events resulting from a late up-regulation in ICAM-1. A single ex vivo treatment with AS ODN, on the other hand, was found to be increasingly effective over the time period analyzed. For example, when the efficacy of ICAM-1 blockade by AS ODN was assessed in our studies, a difference between transfected and con-

trol groups was more clearly demonstrated in allografts at day 3 than day 1.

This difference in pharmacokinetics of AS and MAb was further illustrated by their divergent biologic effects. Because of the central role that ICAM-1 plays on neutrophil diapedesis and toxicity during reperfusion, optimized blockade of early, preexisting ICAM-1 by means of MAb prevented reperfusion injury more effectively than AS ODN. More surprising were the results of the CGVD studies. Using the same PVG to ACI model, previous studies have shown that the up-regulation of ICAM-1-mediated reperfusion injury resulted in worsened eventual CGVD.¹⁶ However, despite its optimal effectiveness at reversing this initial nonspecific injury, ICAM-1 MAb was much less effective than AS ODN at reducing CGVD. The ICAM-1 MAb used in these studies (1A29), because it is an Ig1 isotype MAb, potentially triggers recipient effector mechanisms after interaction with its endothelial target (i.e., ICAM-1). It has been proposed that initiation of these effector mechanisms may inadvertently "activate" the endothelium and promote early¹⁷ or late¹⁸ vascular injury. By targeting LFA-1, a ligand of ICAM-1 expressed on leukocytes and not endothelium, the WT.1 MAb provides a positive control for the blockade of ICAM-1/LFA-1-mediated reperfusion injury without these theoretical concerns of vascular injury. However, the reduction of reperfusion injury with LFA-1 MAb also failed to prevent CGVD. These results suggest that the anti-CGVD mechanism of donor graft AS ICAM-1 ODN treatment is not fully explained by an initial reduction in reperfusion injury-mediated immunogenicity and vascular injury. Further studies are needed to determine whether transfection leads to long-term reduction of perivascular ICAM-1 by the persistence of AS ODN or whether some change in allograft biology is triggered that provides a resistance to CGVD.

Nonsequence-specific effects of ODN transfection on gene expression have been well documented, adding complexity to hasty conclusions regarding biologic effects. The aptamer effect¹⁹ and direct antiproliferative and cytokine-inducing actions of certain ODN sequences²⁰ produce unpredictable, non-AS-related influences on biologic end points. In our studies, a true AS mechanism was supported by the lack of effect of (1) control RAS ODN on either target ICAM-1 protein expression or CGVD, (2) AS ICAM-1 ODN on the expression of other gene products potentially important for CGVD (e.g.,

MHC class I and II), or (3) AS ODN treatment without pressure (vector control group) on CGVD. Indeed, ex vivo hyperbaric delivery of AS ODN to donor cardiac grafts resulted in a 48% transfection rate. Standard viral and lipid vectors have consistently shown transfection efficiency of less than 10% of total cells.^{21,22} Prolonged allograft survival has been accomplished with the use of these more technically difficult vectors by local overexpression of factors such as T-cell growth factor- β or interleukin-10.²¹⁻²³ However, AS ODN blocks target mRNA translation only in successfully transfected cells. Immunosuppression by means of this strategy obviously depends on transfection efficiency at levels much closer to that described by the hyperbaric vector.

In conclusion, these studies document the sole use of pressure as a highly efficient vector for ODN delivery with documented efficacy of ICAM-1 blockade. Compared with ICAM-1 MAb, AS ODN was less effective at preventing the manifestations of reperfusion injury, especially at early time points, a comparison which was consistent the pharmacokinetics of these agents. Less well explained was their inverse effects on CGVD with ICAM-1 blockade with AS ODN but not perioperative MAb providing a significant preventive effect on the development of vascular lesions. Although further investigation regarding the mechanism of AS ODN effect in this model is warranted, these studies support ongoing investigations in this laboratory using the more clinically relevant nonhuman primate transplantation model. We think that this highly targeted alteration of donor organ immunogenicity may have an important future role in immunosuppressive strategies in clinical transplantation.

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