Methoxyethyl-Modified Intercellular Adhesion Molecule-1 Antisense Phosphorothiateoligonucleotides Inhibit Allograft Rejection, Ischemic-Reperfusion Injury, and Cyclosporine-Induced Nephrotoxicity

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Background. The addition of phosphorothioate (PS) groups to natural phosphodiester (PD) antisense oligodeoxynucleotides (oligo) prevents their in vivo hydrolysis by nucleases allowing an RNase-dependent elimination of targeted mRNA. To further improve oligo function 2'-methoxyethyl (ME) groups were attached to selected nucleotides at the 3'-end because ME groups block RNase activity.

Methods/Results. ME modification of PS- or PD/PS-oligo targeting human intracellular adhesion molecule (ICAM)-1 mRNA significantly increased the degree and duration of the in vitro inhibitory effects without compromising selectivity and specificity. A 7-day intravenous or oral therapy with rat ME/PS-modified ICAM-1 antisense oligo extended the survivals of kidney allografts. In addition, ME/PS-modified ICAM-1 antisense oligo reduced ischemic-reperfusion injury in kidneys, as measured by glomerular filtration rate, creatinine levels, and infiltration with leukocytes. Finally, a 14-day treatment with cyclosporine (CsA)-induced nephrotoxicity in syngeneic kidney transplants correlated with both increased ICAM-1 protein expression and infiltration with leukocytes. Graft perfusion and treatment of recipients with ICAM-1 antisense ME/PS-oligo alleviated the nephrotoxic effect and decreased ICAM-1 expression and leukocyte infiltration.

Conclusions. ME/PS-modified ICAM-1 antisense oligo is very effective in inhibiting the ICAM–1-dependent mechanism of graft infiltration and tissue damage involved in allograft rejection, ischemic-reperfusion injury, and CsA-induced nephrotoxicity.

Keywords: Transplantation, Kidney, ICAM-1, Antisense oligonucleotides, Ischemia-reperfusion injury, Immunosuppression.

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Antisense technology proffers an attractive method of drug design to selectively inhibit the expression of a specific mRNA (*1*). Synthetic antisense oligodeoxynucleotides (oligos) block the expression of targeted mRNA by arresting translation, inhibiting mRNA processing, or inducing mRNA degradation (*2*). Because natural phosphodiester (PD) oligos are rapidly hydrolysed by multiple nucleases, various chemical modifications have therefore been tested to improve the oligo in vivo resistance (*3*). One of these modifications—phosphorothioate oligo (PS-oligo) with the sulphur group substituted for the nonbridging oxygen atoms in the phosphate backbone—proved resistant to endo- and exonucleases (*4*). Here, we tested in vivo a new "wing" design with 2'-methoxyethyl (ME) groups attached at the 3'-end of PS-oligo or PD/PS-oligo. An in vitro analysis suggested significantly improved affinity of ME-modified oligo to the targeted mRNA, extended half-life, and increased resistance to nucleases (*5*).

Although intracellular adhesion molecule (ICAM)-1 molecules are always expressed at a low level on the surface of many cells (*6*), interleukin (IL)-1, tumor necrosis factor (TNF), and interferon-γ markedly increase their expression on endothelial cells, smooth muscle, cardiac myocytes, and leukocytes (*6*). Multiple studies suggested that the ICAM–1-dependent mechanism of graft infiltration by leukocytes is involved in allograft rejection and ischemic-reperfusion (I/R) injury (*7*, *8*). Our experimental studies showed that a 7-day continuous intravenous (IV) infusion of 2.5 to 10 mg/kg ICAM-1 antisense PS-oligo (9125) alone delayed allograft rejection and acted synergistically in combination with cyclosporine (CsA) to extend kidney-allograft survival (*9*). Furthermore, graft perfusion with 10 mg/2 mL 9125 prevented I/R injury (*10*). On the basis of these studies, the 8 alternate-day IV infusions of human ICAM-1 antisense PS-oligo (2302; doses of 0.05, 0.5, 1.0, or 2.0 mg/kg) were added to a combined CsA/prednisone regimen as prophylaxis of acute rejection episodes in cadaveric renal allografts. Although the rates of acute rejection episodes were not improved at 6 months by 2302 therapy, the mean calculated creatinine clearance value was better, albeit not significantly, among the 2302 group (66 mL/min) than the placebo group (60 mL/min). The clinical efficacy of such therapy may be improved by development of

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oral formulation for chronic treatment and by improving the properties of the oligos.

So far, little is known about the role of ICAM-1 in the mechanism of CsA-induced nephrotoxicity characterized by 30% reduction in glomerular filtration rate (GFR) and 40% reduction in kidney blood flow (*11*, *12*). Factors associated with acute, and usually reversible, CsA toxicity appear to be related to the imbalance in multiple vasoconstrictors (catecholamines, angiotensin II, endothelin, thromboxane, and platelet activating factor) (*12*). The chronic form of CsA toxicity often causes structural changes in kidneys such as tubulointerstitial fibrosis, arteriolopathy, tubular atrophy, and glomerulosclerosis (*13*). Most likely, these changes result from oxidative stress because the urine of patients treated with CsA has high levels of free radicals derived from metabolism of CsA by cytochrome P450 (*14*, *15*). Up to now, no effective methods have been found to prevent CsA-induced nephrotoxicity.

The present experiments examined efficacy of a new ICAM-1 antisense oligo with "wing" attached ME groups. In vitro analysis showed that ME-modified human ICAM-1 antisense PS-oligo with identical nucleotide sequence displayed an increased inhibition of ICAM-1 mRNA and extended duration of such inhibitory effect in cells. ME-modified ICAM-1 antisense oligo prolonged kidney-allograft survival when delivered IV or by oral gavage. Graft perfusion prevented I/R injury. Furthermore, perfusion of graft combined with therapy of recipients with ME-modified ICAM-1 antisense oligo alleviated CsA-induced nephrotoxicity.

MATERIALS AND METHODS

Cells and Reagents

We obtained fetal bovine serum from Hyclone (Logan, UT), DMEM and Dulbecco's phosphate-buffered saline from Irvine Scientific (Irvine, CA), and Opti-MEM serum-free medium from GIBCO/BRL (Grand Island, NY). We purchased 96-well plates from Falcon Labware (Lincoln Park, NJ), human recombinant (r)TNF-α from R&D Systems (Minneapolis, MN), anti–ICAM-1 monoclonal antibody (mAb; IA29) from Seikagaka (Tokyo, Japan), DOTMA/DOPEP solution (lipofectin), biotinylated goat anti-mouse immunoglobulin (Ig)G, and β-galactosidase-conjugated streptavidin from Bethesda Research Labs (Bethesda, MD). In addition, we obtained the DNA synthesizer reagents and controlled pore glass-bound and β-cyanoethyl diisopropyl phosphoramidates from Applied Biosystems (Foster City, CA), Centrex filters from Schleicher and Schuell, Inc. (Keene, NH), zeta-probe nylon blotting membranes from BioRad (Richmond, CA), QuickHyb solution from Stratagene (La Jolla, CA), and the cDNA labeling kit, Prime-a-Gene, from Promega (Madison, WI).

Oligo Synthesis

ME-modified or nonmodified PS- and PD/PS-oligos were synthesized on a Milligen model 8800 DNA synthesizer (Millipore, Inc., Bedford, MA) using modified phosphoramidite chemistries with β-cyanoethoxyphosphoramidites and purified by column chromatography using a Millipore HC18-HA column (*16*, *17*). The purity of full-length oligos assessed by capillary electrophoresis, high-performance liquid chromatography, mass spectroscopes, and nuclear magnetic resonance imaging was at least 92% and contained less than 0.3 mol% PD linkages. The sequences and modifications of the oligos are listed in Figures 1 and 2.

Cell Culture

The human umbilical vein endothelial cells (HUVEC) and epithelial A549 carcinoma cells as well as rat L2 lung epithelial-like cells obtained from the ATCC (Rockville, MD) were grown in Ham's F12 medium supplemented with 10% fetal bovine serum. Rat aortic endothelial cells were isolated

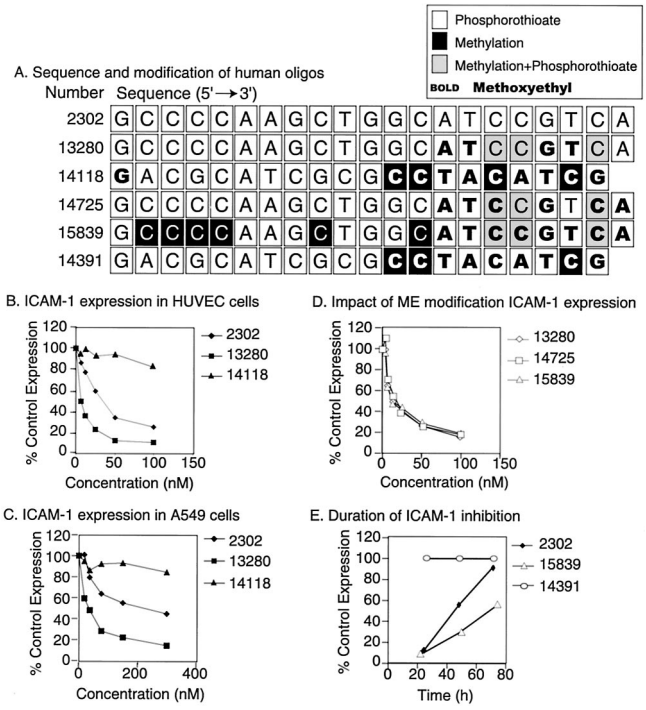


FIGURE 1. Inhibition of human intercellular adhesion molecule (ICAM)-1 by methoxyethyl (ME)-modified ICAM-1 oligodeoxynucleotides (oligo). (A) Sequence of oligos and modifications of nucleotides include phosphorothioate (*empty squares*), methylation (*black squares*), methylation and phosphorothioate (*gray squares*) and ME groups (*bold*). Cultured human umbilical vein endothelial cells (HUVEC) (B) or A549 (C) cells were treated with different concentrations of 2302, ME-modified 13280, or mismatched ME-modified 14118 delivered in DOTMA/DOPE-liposome for 48 hours before isolation of total RNA. Human ICAM-1 mRNA expression was measured by the Northern blot. In similar fashion, HUVEC cells (D) were cultured with different concentrations of 13280, 14725, or 15839 oligo (each contains distinct ME-modifications) before the measurements of ICAM-1 mRNA by Northern blot. To examine duration of the inhibitory effect, HUVEC cells (E) were cultured for 4 hours with 2302, ME-modified 15839, or mismatched ME-modified 14391 oligo delivered in DOTMA/DOPE liposome, washed, and stimulated with tumor necrosis factor (TNF)-α at 24, 48, or 72 hours. Expression of ICAM-1 mRNA was measured by Northern blot. In particular, the ICAM-1 mRNA levels were quantified by normalization to G3PDH mRNA levels in all samples. Results (B to E) are expressed as percent of ICAM-1 mRNA expression in untreated HUVEC or A549 cells.

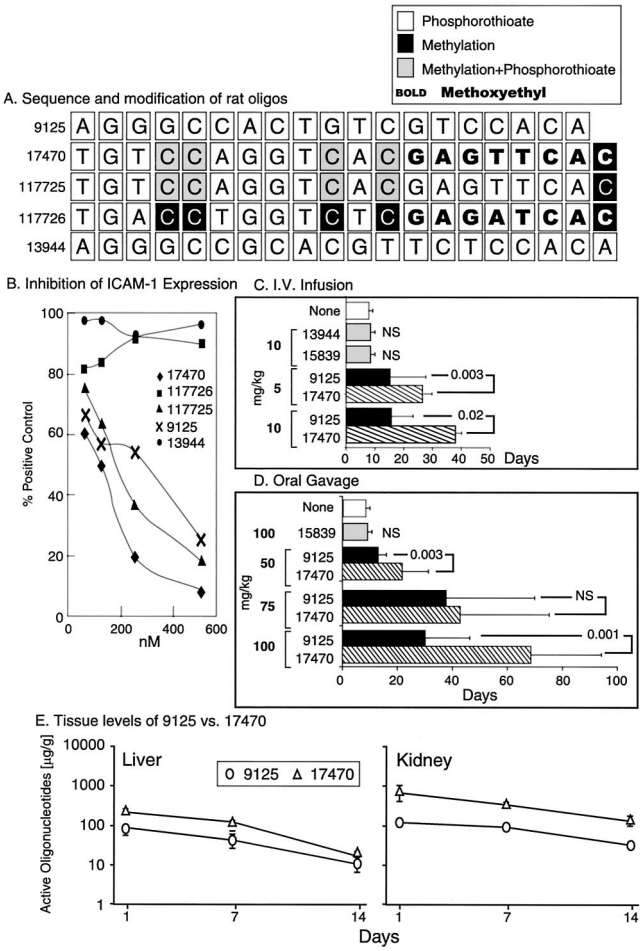


FIGURE 2. Rat ME-modified ICAM-1 antisense oligo blocks allograft rejection. (A) Sequence of oligos and modifications of nucleotides include phosphorothioate (*empty squares*), methylation (*black squares*), methylation and phosphorothioate (*gray squares*), and ME groups (*bold*). (B) Cultured rat L2 endothelial cells were treated with different concentrations of 17470, 117726, 117725, 9125, or 13944 delivered in DOTMA/DOPE liposome for 48 hours before isolation of total RNA. Rat ICAM-1 mRNA expression was measured by Northern blot; ICAM-1 mRNA levels were quantified by normalization to the G3PDH mRNA levels in all samples. Results are expressed as percent of ICAM-1 mRNA expression in untreated L2 cells. (C) Effect of a 7-day intravenous (IV) infusion by osmotic pump of 5 or 10 mg/kg 9125 (*black columns*) or 17470 (*hatched columns*) on kidney-allograft survival. Each group represents mean±SD of kidney-allograft recipients (n=4–6). (D) Effect of a 7-day daily oral gavage with 50, 75, or 100 mg/kg 9125 (*black columns*) or 17470 (*hatched columns*) on kidney-allograft survival. Each group represents mean±SD of kidney-allograft recipients (n=4–6). (E) Concentrations of intact 9125 and 17470 oligos in liver and kidneys at days 1, 7, and 14 after rats were injected daily IV for 7 days with 10 mg/kg 9125 or 17470.

from male Sprague-Dawley rats by collagenase digestion of aortic rings and plating cells onto gelatin-coated plates in the presence of endothelial cell growth medium (Clonetics, San Diego, CA). The cells plated in six-well cluster plates (75% confluence) in Opti-MEM (Gibco/BRL) were treated for 4 hours with 200 μM of each oligo in 15 μg/mL DOTMA/DOPE liposome. ICAM-1 protein expression induced by 16-hour treatment with 5 ng/mL rTNF-α was quantitated by anti–ICAM-1 (IA29) mAb and fluorescein-labeled goat anti-rat IgG Ab by flow cytometric analysis (Becton-Dickinson, Palo Alto, CA) (*16*, *18*). Results were expressed as: %=[(ICAM-1+oligo+rTNF-α)−(basal ICAM-1)]/[(ICAM-1+rTNF-α)−(basal ICAM-1)]×100.

Capillary Gel Electrophoresis

The previously described validated capillary gel electrophoresis method was used to determine oligo concentrations (*19*). Tissues were extracted using a phenol/chloroform extraction method with internal standards. Next, the extracts were processed by a strong anion exchange and reverse-phase C_{18} methods before electrokinetic injection. A P/ACE model 5010 (Beckman Coulter; Fullerton, CA) was used for gel-filled capillary electrophoresis analysis. Peaks were detected by ultraviolet absorption at 260 nm, with limits of quantitation at 0.39 μg/g. The results of three independent experiments were presented as mean±SD in a log scale.

Histology Examination and Immunostaining

Kidneys fixed in buffered 10% formalin were sectioned and stained with hematoxylin-eosin (H&E). The scores were graded blindly for tubular and glomerular changes graded as 0 (no changes); 1+ (<5%); 2+ (5–25%); 3+ (26–50%); and 4+ (>50%); and vascular changes as: 0 (none); 1+ (minimal); 2+ (mild); 3+ (moderate); and 4+ (severe). In addition, frozen sections were stained with anti-CD18 mAb (leukocytes) or anti-CD154 mAb ICAM-1. Results were evaluated by computerized analysis and thepercent positive area was measured for each group.

RNA Isolation and Analysis

Total RNA, isolated 2 hours after TNF–α-induced ICAM-1 expression by a guanidinium isothiocyanate and a cesium chloride gradient (*20*), was separated on a 1% agarose gel and transferred to nylon membranes. Blots, hybridized for 1 to 2 hours in QuickHyb solution (Stratogene, La Jolla, CA) with the rat ^{32}P-dCTP-labeled ICAM-1 cDNA probe, were washed with 0.1% sodium dodecyl sulfate. RNA expression was measured by a PhosphorImager (Molecular Dynamics; Sunnyvale, CA). Blots reprobed with a G3PDH probe were normalized for sample loading (*16*, *18*).

Animals

ACI (RT1^a) recipient and Lewis (LEW; RT1^l) donor rats (6–8 weeks old) purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) were housed in cages in a room with controlled light/dark cycles and supplied with food and water as desired. All experiments were approved by the Animal Welfare Committee and carried out according to the University of Texas Medical School Guidelines.

Kidney Transplantation

The method of orthotopic kidney transplantation was previously described (*21*). In particular, the harvested left kidney was perfused through the aorta with heparinized saline (4°C) with anastomosed recipient renal arteries and veins

using 10–0 nylon suture (Ethicon, Somerville, NJ). The native kidneys were removed on the day of transplantation. Animals that died within 3 days after transplantation were excluded from the analysis (<5%).

Evaluation of Kidney Function

Recipients were placed in metabolic cages for 24 hour to collect urine samples. Blood pressure and heart rate were monitored using a Blood Pressure Analyzer (Micro-Med; Louisville, KY). The exact urine output was measured using a Muscle Force Analyzer (Mirco-Med). Ioxehol (Omnipaque 300 mg/mL, Nycomed Inc., Princeton, NJ) was infused IV at 1,000 mg/kg loading dose (5 minutes) and at 600 mg/kg maintenance dose (90 minutes). Blood and urine samples were collected every 20 minutes to measure ioxehol blood concentrations. The GFR values were measured by the formula, GFR (mL/min)=[U (mg/mL)×[Up (mL/min)]÷[P (mg/mL)], where U is ioxehol concentration, Up is urine output, and P is blood ioxehol concentration. The iohexol concentrations were measured using the Renalyzer PRX 90 (Provalid AB, Lund, Sweden). The results are presented as mean±standard deviation; the statistical significance was calculated by the Student *t* test method, with $P<0.05$ as significant.

Cyclosporine

CsA (Sandoz, Basel, Switzerland) dissolved in Cremophor (Sigma, St. Louis, MO) was delivered by oral gavage at doses of 10 mg/kg for 14 days.

In vivo Treatment Protocols with Oligos

Recipients of kidney allografts were treated with 2.5, 5.0, or 10.0 mg/kg 9125 or 17470 dissolved in saline delivered IV for 7 days by osmotic pumps (Alzet, Palo Alto, CA) or treated with 25, 50, or 100 mg/kg 9125 or 17470 in an oral formulation vehicle delivered for 7 days by oral gavage (*22*). In an I/R model, a LEW kidney was harvested, perfused with 9125 or 17470 dissolved in 2 mL Euro-Collins, and stored at 4°C for 30 minutes before grafting to a bilaterally nephrectomized LEW recipient with standard 30-minute anastomosis time. Within 24 hours, GFR function was measured by an iohexol method. For the nephrotoxicity study, LEW rats fed (days −7 to +14) with low-salt diet (0.05% sodium, Teklad Premier, Madison, WI) received transplants with LEW kidney perfused with 17470; GFR function was measured by an iohexol method. Some recipients were treated with 10 mg/kg CsA delivered by oral gavage alone or in combination with 10 mg/kg 17470 delivered IV by osmotic pump.

Statistical Analysis

Organ allograft survival rates are presented as an median survival time±SD with comparison among groups performed by Gehan's survival test (*23*).

RESULTS

ME Modification Improves In Vitro Inhibition by Human ICAM-1 Antisense Oligo

As previously shown, 2302 PS-oligo, designed to target human ICAM-1 mRNA, specifically inhibited both ICAM-1 mRNA and protein expression in a dose-dependent fashion (*16*). Our goal was to improve the design of 2302 PS-oligo by adding ME groups to increase binding to ICAM-1 mRNA and by methylation of CpG-dinucleotides to avoid nonspecific stimulation of lymphocytes (*24–26*). Because ME groups block RNase H activity, ME groups were attached to seven nucleotides at the 3'-end (except the very last) and methylated three cytosines creating 13280 oligo (Fig. 1A). In comparison with 2302, ME-modified 13280 oligo decreased expression of ICAM-1 mRNA by three- to sevenfold in both HUVEC and A549 cells (Fig. 1, B and C) without affecting the expression of endothelial-selectin and vascular adhesion molecule-1 mRNAs (data not shown). Mismatched 14118 oligo with ME groups attached to nine nucleotides at the 3'-end and the last nucleotide at the 5'-end did not affect ICAM-1 expression (Fig. 1, B and C), documenting that ME modifications are not responsible for improved inhibitory effects. To further optimize 13280, an ME group was added to the last nucleotide at the 3'-end (14725), or in combination with methylation, added to the remaining cytosines (15839) (Fig. 1A). All three ME modified oligos were equally effective in inhibition of ICAM-1 mRNA expression (Fig. 1D) by RNase H activity (data not shown), a mechanism previously documented for 2302 (*16*). To compare the duration of the inhibitory effect, HUVEC cells were treated for 4 hours with 200 nM 2302 or 15839 and washed to remove oligos before stimulation with TNF-α for 4, 24, 48, or 72 hours (Fig. 1E). Because 4-hour exposure to 15839 inhibited ICAM-1 mRNA expression significantly longer then identical exposure to 2302, we concluded that ME modification improves not only the degree but also the duration of the inhibitory effect. In the same experiment, a 4-hour exposure to mismatched 14391 oligo with ME groups attached to nine nucleotides at the 3'-end was completely ineffective (Fig. 1E), proving that ME modifications alone did not extend the inhibitory effects.

Effect of ME-Modified 17470 on Kidney Allograft Survival

We previously documented that ICAM-1 antisense PS-oligo, 9125, delivered IV to recipients retarded rejection of kidney allografts (*9*). Now, we have targeted rat ICAM-1 mRNA with a new oligo, 17470, displaying similar PS/ME modifications as human 15839 (Fig. 1A); control 117725 oligo was made without ME groups. In vitro, 17470 (IC_{50}=156 nM) was twofold more effective than 117725 (IC_{50} of 250 nM) or 9125 (IC_{50}=335 nM) in inhibiting ICAM-1 mRNA expression in rat L2 endothelial cells (Fig. 2B). Because either a four-base mismatch of 17470 (117726) or a six-base mismatch of 9125 (13944) was completely ineffective, we conclude that 17470 is more effective than 9125, and therefore selective addition of ME groups may improve antisense activity.

In vivo, recipients of kidney allografts were treated IV for 7 days with either 9125 or 17470 (Fig. 2C). In comparison with untreated controls, treatment with either 10 mg/kg scrambled control (13944; 11.0±1.6 days; NS) or human ICAM-1 ME-modified oligo (15839; 10.5±2.6 days; NS) was ineffective. In contrast, 17470 was twofold more effective than 9125 in extending survivals of kidney allografts (Fig. 2C). Because a new oral formulation of oligo had 10% to 15% bioavailability (*27*), oral gavage with 50, 75, or 100 mg/kg 17470 documented similar twofold improvement in allograft survivals in comparison with 9125 (Fig. 2D). Because oral

gavage with 100 mg/kg ME-modified human ICAM-1 oligo (15839) was ineffective (10.2±2.5 days; NS) (Fig. 2D), we conclude that new ME-modified 17470 was more effective in vivo than 9125.

To compare the pharmacokinetics of both oligos, rats were treated for 7 days by IV injections of 10 mg/kg 9125 or 17470, and the concentrations of intact (full-size) oligos were measured in liver and kidneys (Fig. 2E). At 1, 7, and 14 day(s) after completion of treatment, the concentrations of intact 17470 produced two to threefold higher exposure in both liver and especially in kidneys in comparison with the concentrations of intact 9125. Thus, because similar results were previously reported with identical oligo sequences (*28*), we conclude that ME-modified 17470 had significantly extended the lifespan in comparison with 9125, and it is likely that ME groups contribute to improved allograft survival.

Effect of ME-Modified 17470 on I/R Injury

We have previously shown that ex vivo perfusion with 9125 inhibited ICAM-1 protein expression in kidney transplants and prevention of I/R injury (*10*). Here, kidneys were perfused with different doses of 9125 or 17470 before transplantation into syngeneic nephrectomized recipients. Controls perfused with saline alone and examined within 24 hours showed that the GFR was reduced by almost 60% in comparison with normal kidneys ($P<0.001$) (Fig. 3A). Among all tested doses, only the highest 10 mg/2 mL dose of 9125 improved kidney function (0.92±0.08 mL/min; $P<0.001$). In contrast, perfusion with 17470 at doses ranging from 4 to 10 mg/2 mL prevented I/R injury (Fig. 3A). Serum creatinine and blood urea nitrogen levels confirmed the GFR results (data not shown). Because perfusion of kidneys with 10 mg/2 mL dose of control 13944 (0.47±0.05 mL/min; NS) did not improve kidney function, we conclude that ME-modified 17470 improves prevention of I/R injury in comparison with 9125.

Effect of ME-Modified 17470 on CsA-Induced Nephrotoxicity

We also examined the impact of ICAM-1 inhibition on I/R injury in a low-salt model of CsA-induced nephrotoxicity (*29*, *30*). In comparison with normal kidneys (0.98±0.16 mL/min), the GFR value examined 14 days postgraft was significantly reduced in recipients treated with CsA (0.42±0.1 mL/min; $P<0.001$) (Fig. 3B). Pretransplant graft perfusion with 10 mg/2 mL 17470 significantly improved kidney function in recipients treated with CsA (0.8±0.1 mL/min; $P<0.001$). However, graft perfusion combined with an additional 14-day IV therapy with 10 mg/kg 17470 completely alleviated CsA-induced kidney injury (1.03±0.11 mL/min; $P<0.001$) (Fig. 3B). Thus, inhibition of ICAM-1 protein expression at graft site prevents CsA-induced nephrotoxicity.

The protection from damage produced by 17470 was clearly shown by H&E staining (Fig. 4). In comparison with normal kidneys, grafts from recipients treated with CsA showed a typical picture of nephrotoxicity: focal tubular dilation, calcification and vacuolization, signs of tubular atrophy, arterial wall thickening (<25%), increased glomerular cellularity, and interstitial fibrosis (Fig. 4, A to D). Perfusion of graft alone with 17470 not only significantly reduced I/R changes in non–CsA-treated recipients (Fig. 4E) but also reduced CsA-induced damage of tubular vacuolization, tubular atrophy, and interstitial infiltration with mononuclear cells (Fig. 4F). However, graft perfusion and treatment of recipient with 17470 prevented tubular damage and reduced graft infiltration (Fig. 4G). These results were confirmed by the immunostaining of grafts for $CD18^+$ leukocytes (Fig. 5). CsA therapy dramatically increased infiltration with leukocytes (Fig. 5D), which was significantly reduced after graft perfusion alone or combined perfusion and treatment with 17470 (Fig. 5, F and G). All these results were reflected by the pattern of ICAM-1 protein expression (Fig. 6). Thus, CsA-induced nephrotoxicity correlates with increased ICAM-1 protein expression and infiltration with leukocytes. The reduction of ICAM-1 protein expression with 17470 therapy significantly alleviates early CsA-induced nephrotoxicity.

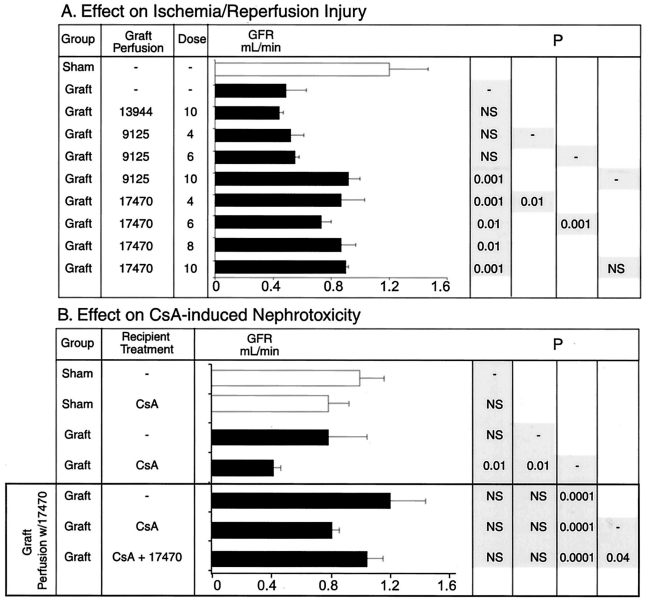
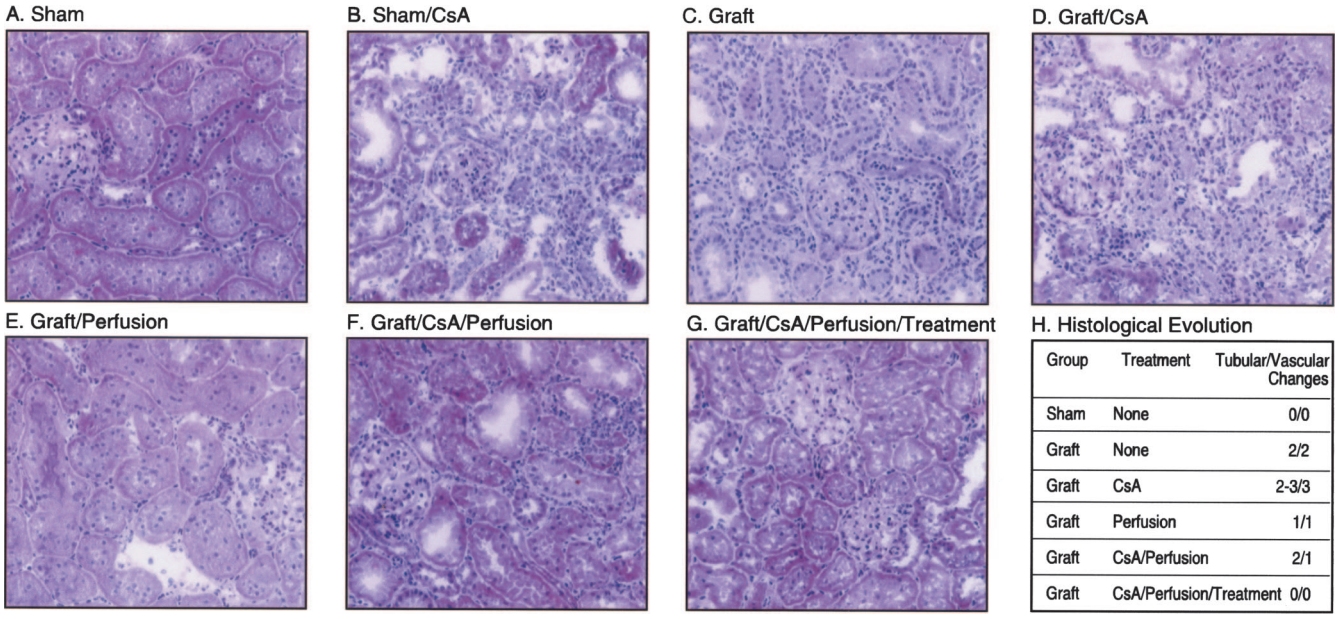


FIGURE 3. Effect of ME-modified ICAM-1 oligo (17470) on ischemia-reperfusion (I/R) injury and cyclosporine (CsA)-induced nephrotoxicity. (A) Kidneys perfused with 2 mL Euro-Collins with 13944, 9125, and 17470 were exposed to 30 minutes warm and 30 minutes cold ischemia before grafting to normal syngeneic recipients. Within 24 hours, postgrafting glomerular filtration rate (GFR) values were measured using an iohexol method. (B) Kidneys perfused with 2 mL Euro-Collins and 10 mg/2 mL 17470 were exposed to 30 minutes warm and 30 minutes cold ischemia before grafting to syngeneic recipients that were maintained on a low-salt diet (days −7 to 14). In some groups, recipients were treated with 10 mg/kg CsA delivered by oral gavage for 14 days alone or in combination with 10 mg/kg 17470 delivered IV by a 14-day osmotic pump. On day 14, GFR values were measured using an iohexol method. Results presented as mean±SD (n=5–6) with statistical significance evaluated by Student *t* test; $P<0.05$ considered statistically significant.

DISCUSSION

The most recent experiments examined ME-modified ICAM-1 antisense oligo to inhibit in vitro ICAM-1 mRNA expression as well as to block in vivo allograft rejection, I/R



Group	Treatment	Tubular/Vascular Changes
Sham	None	0/0
Graft	None	2/2
Graft	CsA	2-3/3
Graft	Perfusion	1/1
Graft	CsA/Perfusion	2/1
Graft	CsA/Perfusion/Treatment	0/0

FIGURE 4. Histology of kidneys from recipients treated with 17470 and exposed to I/R injury or CsA-induced nephrotoxicity. Kidneys perfused with 2 mL Euro-Collins and 10 mg/2 mL 17470 (perfusion) were exposed to 30 minutes warm and 30 minutes cold ischemia before grafting to syngeneic recipients maintained on a low-salt diet (days −7 to 14). Some recipients were treated with 10 mg/kg CsA delivered by oral gavage for 14 days alone or in combination with 10 mg/kg 17470 delivered IV by a 14-day osmotic pump. Hematoxylin-eosin histology was scored for tubular/arteriopathic injury using a uniform scoring method (0=no change; 1=minimal; 2=mild; 3=marked; 4=severe changes).

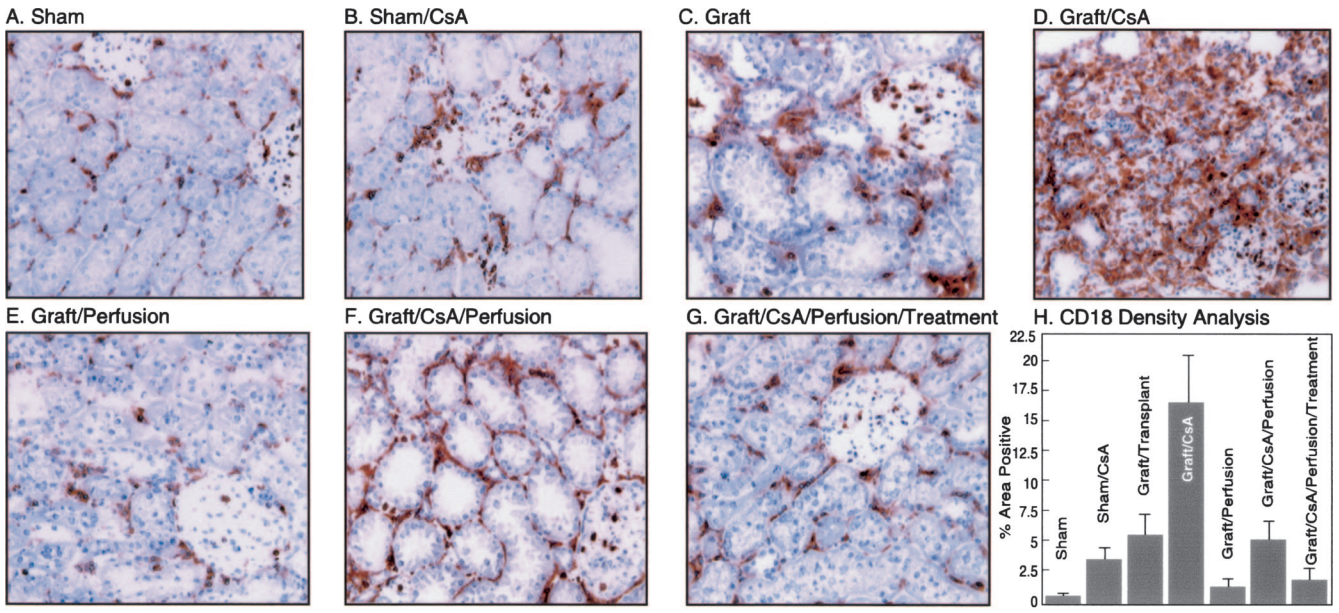


FIGURE 5. Effect of 17470 on infiltration of kidneys with $CD18^+$ leukocytes. Experimental groups as in Figures 3 and 4. Frozen sections were stained with anti-CD18 mAb. Results were evaluated by computerized analysis and the % positive area was measured for each group (n=4) and presented in panel H. For details, see Materials and Methods.

injury, and CsA-induced nephrotoxicity. Attachment of ME groups to the last eight nucleotides of human ICAM-1 antisense oligo increased its in vitro inhibitory effect on ICAM-1 mRNA expression by three to sevenfold. Our in vivo results document that ME-modified rat ICAM-1 oligo inhibits allograft rejection after IV or oral administration and reduces I/R injury and CsA-induced nephrotoxicity.

Our results show that wing attachment of ME groups antisense activity of oligos with identical sequence. Although natural PD-oligos are degraded within minutes after administration, modified PS-oligos display a average 3-day half-life (*28*). After IV injection, PS-oligos bind with low affinity to serum α2-microglobulins and albumins and are rapidly (30 minutes) absorbed into different tissues, except the brain (*31*,

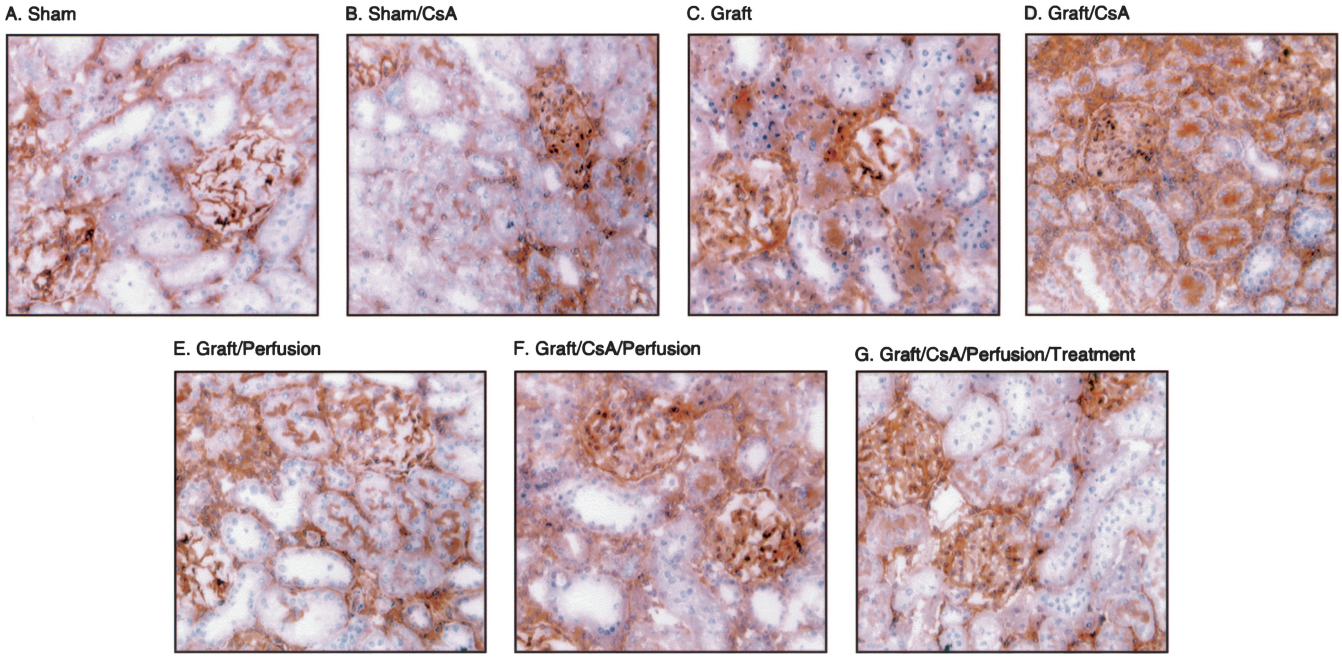


FIGURE 6. Effect of 17470 on infiltration of kidneys with $CD54^+$ ICAM-1 molecules. Experimental groups as in Figures 3 and 4. Frozen sections were stained with anti-CD54 monoclonal antibody.

32). Elimination of PS-oligos is faster in the liver (2.5 days) and much slower in the kidneys (6 days) by exo- and endonucleases. ME-modified PS-oligos display a similar distribution in tissues as PS-oligos but with a threefold improvement in half-life to 14 days (*33*). Despite differences in the nucleotide sequence, pharmacokinetic results confirmed two to threefold greater exposure in kidneys to ME-modified 17470 compared with 9125. Because in vivo results showed for the first time that a 7-day therapy either IV or orally with identical doses of 17470 produced survivals twice as long as those achieved with 9125 therapy, we conclude that ME groups may contribute in antisense activity. This is the first report showing that oral delivery of ICAM-1 antisense oligo is effective in vivo in blocking rejection.

I/R injury—the transient period of warm and cold ischemia followed by reperfusion—initiates increased expression on the cellular surface of multiple adhesion molecules, thereby increasing adhesiveness of leukocytes to graft endothelial cells (*34*, *35*). Among many different molecules, up-regulated ICAM-1 on endothelial cells facilitates the attachment of neutrophils through the leukocyte $\beta 2$ integrin complex initiating graft damage (*36*). As shown here, perfusion of kidneys with ICAM-1 antisense oligo reduced I/R injury. In addition, ME-modified 17470 oligo was effective at doses ranging from 4 to 10 mg/mL, whereas 9125 was effective only at the highest 10 mg/mL dose (Fig. 3A). A recent publication showed that identical ME-modified 17470 oligo attenuated ileitis by down-regulation of leukocyte adherence (*37*). Our studies confirmed that 17470 reduced infiltration with leukocytes in kidney transplants after I/R injury (Fig. 5).

Although the exact mechanisms of CsA-induced nephrotoxicity are not fully understood, the experimental studies revealed several clues related to CsA therapy: increased vascular resistance with decreased renal blood flow (*29*, *38*), generation of reactive free radicals with increased oxidative stress (*39*), and cytochrome P450 activation (*40*); up-regulated expression of transforming growth factor-β with profibrogenic activity (*41*); increased Ca^{2+} mobilization in smooth muscle cell; up-regulated synthesis and expression of receptors for angiotensin II (*42*); increased Fas-mediated and caspase 3-, 8-, and 9-dependent apoptosis of renal tubular cells (*43*, *44*); as well as depressed protection by both endothelial and inducible nitric oxides (*45*). Such increased vasoconstriction and apoptosis caused by CsA nephrotoxicity correlates with characteristic pathologic changes (Figs. 4 and 5). Our results revealed that CsA-induced nephrotoxicity is associated with increased ICAM-1 expression (Fig. 6) and leukocyte infiltration (Fig. 5). Inhibition of ICAM-1 protein expression by 17470 attenuated both infiltration with leukocytes and pathologic changes associated with nephrotoxicity. Clinical reports attributed increased ICAM-1 expression to both rejection and CsA-induced nephrotoxicity (*46*). Our present results suggest that CsA-induced nephrotoxicity is mediated by ICAM–1-dependent graft infiltration with leukocytes. A recently published study (*47*) designated two independent mechanisms to cisplatin-induced nephrotoxicity: first, by TNF–α-controlled inflammatory response with up-regulated IL-1, RANTES, monocyte chemotactic peptide-1, and macrophage inflammatory protein-2; and second, by ICAM–1-dependent infiltration of kidneys with leukocytes (*47*). This dichotomy stems from the fact that cisplatin-induced ICAM-1 protein elevation in kidneys was similar in normal and TNF–α-deficient mice. We postulate that although expression of ICAM-1 in kidneys may be induced by different mechanisms, direct inhibition of ICAM-1 mRNA and protein expression protects kidneys from infiltration by leukocytes and CsA-induced nephrotoxicity.

In conclusion, ME modification improves in vitro antisense activity of human ICAM-1 antisense PS-oligo. The ME groups that attached few nucleotides at the oligo end proved to be the most optimal design to increase antisense function and maintain their sensitivity to RNase H. For the first time, we have documented in vivo that ME-modified 17470 delivered IV orally blocked allograft rejection. Furthermore, the same ME-modified 17470 oligo inhibited I/R injury and medicated CsA-induced nephrotoxicity. We postulate that this new design may improve in vivo antisense efficacy. Future studies are planned to evaluate the efficacy of ME-modified ICAM-1 antisense oligo in cynomolgus monkeys before a clinical trial.

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