Reduction of chronic allograft nephropathy by inhibition of extracellular signal-regulated kinase 1 and 2 signaling

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Wang S, Jiang J, Guan Q, Wang H, Nguyen CY, Jevnikar AM, Du C. Reduction of chronic allograft nephropathy by inhibition of extracellular signal-regulated kinase 1 and 2 signaling. Am J Physiol Renal Physiol 295: F672–F679, 2008. First published July 9, 2008; doi:10.1152/ajprenal.90285.2008.—Chronic allograft nephropathy (CAN), the most common cause of late kidney allograft failure, is not effectively prevented by immunosuppressive regimens. Activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) via MEK mediates actions of various growth factors, including transforming growth factor (TGF)-β1, which plays a key role in CAN. Hence, we tested the therapeutic potential of MEK-ERK1/2 signaling disruption to prevent CAN. Kidneys from C57BL/6j (H-2b) mice were transplanted to bilaterally nephrectomized BALB/c (H-2k) mice. At 14 days after transplantation, the recipients were subjected to 28 days of treatment with the MEK inhibitor CI-1040. All six CI-1040-treated allografts survived, while two of seven grafts in the vehicle-treated group were lost. At the end of the experiment, the function and structure of grafts in the CI-1040-treated group were significantly preserved, as indicated by lower levels of serum creatinine or blood urea nitrogen than in the vehicle-treated group (30 ± 6 vs. 94 ± 39 μM creatinine (P = 0.0015) and 22 ± 8 vs. 56 ± 25 mM BUN (P = 0.0054)), and reduced CAN in the CI-1040-treated group compared with vehicle controls (CAN score = 4.2 vs. 10.3, P = 0.0119). The beneficial effects induced by CI-1040 were associated with reduction of ERK1/2 phosphorylation and TGFβ1 levels in grafts. Also, CI-1040 potently suppressed not only TGFβ1 biosynthesis in kidney cell cultures but also allograft immune responses in vitro and in vivo. Our data suggest that interference of MEK-ERK1/2 signaling with a pharmacological agent (e.g., CI-1040) has therapeutic potential to prevent CAN in kidney transplantation.

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panel of protein kinases using a cell-based system (7) indicated an IC50 for inhibition of MEK1 of 0.3 μM and no inhibition of any other protein kinase at 10 μM CI-1040. MEK1 and MEK2 are closely related dual-specificity kinases that are capable of phosphorylating serine/threonine and tyrosine residues of their substrates ERK1 and ERK2, the only known substrates of MEK. Thus pharmacological inhibitors of MEK can be demonstrated to block ERK1/2 activation (18). In experimental models and clinical trials, CI-1040 inhibits activation of ERK1/2 with varying levels of antitumor activity (1, 42, 46). Recently, we demonstrated that blockade of ERK signaling by PD-98059 suppresses alloimmune responses, resulting in prolongation of cardiac allograft survival (56). In the present study, we demonstrate the efficacy of CI-1040 in the reduction of CAN in renal allograft survival in a mouse model.

MATERIALS AND METHODS

Animals and cells. Male C57BL/6 (B6, H-2b) and BALB/c (H-2b) mice (25-30 g body wt; Jackson Laboratory, Bar Harbor, ME) were housed in the animal care and use facility at the University of Western Ontario. The animal experiments were conducted in accordance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal Use Subcommittee at the University of Western Ontario. Primary renal proximal tubular epithelial cells (TEC) were isolated from the kidney cortex of mice, as described previously (20).

Phenotype of TEC was confirmed by expression of E-cadherin, CD13 (alanine aminopeptidase), and CD26 (dipeptidyl peptidase), and cells were grown in K1 medium, a 50:50 mixture of DMEM-Ham’s F-12 medium (Invitrogen-GIBCO, Burlington, ON, Canada) supplemented with 10% bovine calf serum, hormone mix (5 μg/ml insulin, 34 pg/ml triiodothyronine, 5 μg/ml transferrin, 1.73 ng/ml sodium selenite, and 18 ng/ml hydrocortisone), and epidermal growth factor (EGF, 25 ng/ml; Sigma-Aldrich Canada, Oakville, ON, Canada).

Mouse models of allotransplantation. Male B6 donor mice and male BALB/c recipient mice were used for heart and kidney allotransplantations, as described previously (10, 56).

Drug treatment. CI-1040 was kindly provided by Pfizer (Ann Arbor, MI). Stock solution of CI-1040 (600 mg/ml) was prepared in DMSO (Sigma-Aldrich Canada) and diluted in vehicle containing 5% DMSO, 5% Cremophor EL (Sigma-Aldrich Canada), and 90% saline before injection. CI-1040 (100 mg·kg−1·dose−1) and vehicle alone were intraperitoneally injected twice daily. Two models of allograft were evaluated. In the cardiac allograft experiment, CI-1040 or vehicle was given to the recipient mice from the 1st day of transplantation until the end of graft rejection (nonbeating). Renal allograft recipient mice were treated with CI-1040 or vehicle from 14 days after transplantation until graft failure or for 28 days.

Determination of renal graft function and CAN histopathology. Kidney function was determined from serum creatinine and blood urea nitrogen (BUN) levels, which were measured by the Jaffe reaction method with an automated clinical analyzer (model CX5, Beckman Instruments, Fullerton, CA). Histology of CAN in kidney graft sections was assessed following the Banff 97 Working Classification of Renal Allograft Pathology (41) in a blinded fashion. Briefly, multiple sequential sections from each formalin-fixed and paraffin-embedded graft were stained with hematoxylin and eosin and periodic acid-Schiff. The severity of chronic changes in renal allograft was graded 0 (no abnormality), 1 (mild), 2 (moderate), or 3 (severe) in the following categories: 1) interstitial fibrosis, 2) tubular atrophy, 3) allograft glomerulopathy, 4) mesangial matrix increase, 5) vascular fibrous intimal thickening, and 6) arteriolar hyaline thickening. The score of each category was counted in 10-15 fields and averaged for each graft. An overall histological score was obtained by summing these individual values (maximal 18) to indicate the severity of CAN.

Mixed leukocyte reactions. Mixed leukocyte reactions (MLR) were assayed in triplicate in 96-well U-bottom microculture plates (Corning, Corning, NY). Splenocytes from B6 mice (1 × 105/well, 3,000 rad γ-irradiated) were used as stimulators, and splenocytes from BALB/c mice (2 × 105/well) were used as responders. Cultures without stimulator cells were controls for basal levels of responder proliferation. Cultures were maintained in RPMI 1640 complete medium for 4 days in 5% CO2. [3H]Thymidine (1 μCi/well) was added for the final 18 h, and incorporation of [3H]thymidine was assessed by liquid scintillation counting. Results (means ± SD) are expressed as counts per minute.

Western blot. The protein levels of phosphorylated ERK1/2 (p-ERK1/2) and TGFß1 were determined using Western blots. Briefly, whole cell lysates were homogenized in lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 1 mM DTT, and a protease inhibitor cocktail (Roche, Mannheim, Germany)], mixed with an equal volume of 2× SDS sample buffer [20 mM Tris·HCl (pH 6.8), 5% (wt/vol) SDS, 10% (vol/vol) mercaptoethanol, 2 mM EDTA, and 0.02% bromphenol blue], and then boiled for 5 min. The protein content of cell lysates was determined by Bio-Rad assay (Bio-Rad, Hercules, CA). Total protein (100–150 μg) from each sample was fractionated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, blocked with 5% fat-free milk (Carnation) in Tris-buffered saline (TBS)-Tween 20 [20 mM Tris·HCl (pH 7.6), 137 mM NaCl (TBS), and 0.1% Tween 20] for 1 h, and then probed with anti-p-ERK1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS containing 2.5% milk or anti-TGFß1 antibody (clone A75-2, BD Pharmingen) in TBS containing 5% BSA at 4°C overnight. The p-ERK1/2 or TGFß1 protein bound by the antibodies on the membrane was visualized by an enhanced chemiluminescence assay (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were reprobed using anti-ERK1/2 (Stressgen, Victoria, BC, Canada) or anti-β-actin IgG (Sigma-Aldrich Canada) antibody for total ERK1/2 or β-actin proteins and confirmation of equal protein loading. The TGFß1 protein levels were measured by densitometry and normalized with β-actin in the same blot.

Immunohistochemistry. The levels and locations of p-ERK1/2 and TGFß1 proteins in graft sections were examined by immunohistochemical staining. Briefly, after deparaffinization and rehydration, buffered formalin-fixed sections were treated with 3% H2O2 in TBS (pH 7.4) for 30 min at room temperature to quench endogenous peroxidase and then permeabilized with 0.2% Triton X-100 for 10 min at room temperature. The sections were washed with TBS and blocked with 2% normal goat serum and then incubated with the primary antibody (anti-p-ERK or anti-TGFß1 antibody, 1:200 dilution in TBS-5% normal rabbit serum) overnight at 4°C. The immune complexes of antigen and antibody on the section were detected using the secondary antibody conjugated with biotin and visualized using a 3,3′-diaminobenzidine-peroxidase substrate kit (Vector Laboratories, Burlington, ON, Canada).

Statistical analysis. Data were collected from separate experiments (≥3 experiments if results were consistent) in each study for statistical testing. Statistical significance between groups was determined using one-way ANOVA or t-test (1-tailed distribution). Graft survival between groups of transplanted animals was compared using Kaplan-Meier analysis (Statview, Cary, NC). P ≤ 0.05 was considered statistically significant.

RESULTS

Immunosuppressive activity of CI-1040 in vitro and in vivo. In a previous study, we showed that disruption of ERK1/2 signaling with PD-98059 reduces alloantigen-stimulated T cell proliferation in MLR and prolongs cardiac allograft survival in a mouse model (56). To determine whether CI-1040 has the
same effect as PD-98059 in the immunosuppression of allosternativity, we tested the immunosuppressive activity of CI-1040 in MLR, as well as in a mouse model of cardiac allograft transplantation. As shown in Fig. 1A, CI-1040 suppressed T cell proliferation in a dose-dependent manner (P < 0.01); the rate of [3H]thymidine uptake in cultures with 4 μM CI-1040 was even lower than in cultures without stimulator cells (basal levels), indicating that 4 μM CI-1040 completely inhibited alloantigen-stimulated T cell proliferation in vitro. In vivo administration of CI-1040 prolonged cardiac allograft survival, as shown by improvement of graft survival from 8.5 ± 0.58 days in the vehicle-treated control group to 13.75 ± 1.71 days in the CI-1040-treated group (log rank, P = 0.0084; Fig. 1B), which was comparable to 14.9 ± 1.1 days in cyclosporin A-treated animals (15 mg·kg⁻¹·day⁻¹ in the same combination) (56). These data imply that CI-1040, similar to PD-98059, has immunosuppressive efficacy against alloantigen-stimulated immune responses.

Inhibition of TGFβ1 biosynthesis in TEC cultures by CI-1040. In clinical biopsies, the presence of renal tubular TGFβ1 is significantly associated with grafts with CAN, as well as acute rejection, and its levels are positively correlated with development of interstitial fibrosis, tubular atrophy, and graft atherosclerosis (32, 35), indicating a pathological role of tubular TGFβ1 in the development of CAN. To test whether disruption of MEK1-ERK1/2 signaling reduces CAN, the effect of CI-1040 on TGFβ1 synthesis in TEC was examined. As shown in Fig. 2A, addition of CI-1040 resulted in inactivation of ERK1/2 phosphorylation, while the protein levels of latent TGFβ1, a precursor of bioactive TGFβ1, in these cultures were decreased. Further Western blot analysis indicated that 10 μM CI-1040, which almost completely inhibited MEK1-ERK1/2 signaling, significantly reduced (by 60–90%) latent TGFβ1 protein in TEC cultures (Fig. 2B; P = 0.004), indicating the potential antifibrotic activity of the MEK-ERK inhibitor CI-1040 by reduction of TGFβ1 biosynthesis in renal tubular cells.

Preservation of renal allograft function by CI-1040 treatment. It has been generally accepted that CAN is a principal cause of late graft function loss after the 1st yr of renal transplantation, and its progress depends on alloantigen-dependent immune responses and TGFβ (14, 21, 28). Following up our above data showing that CI-1040 suppressed the immune responses to the alloantigen, as well as TGFβ1 production in the kidney cells, we tested whether pharmacological interference of MEK1-ERK1/2 signaling with CI-1040 protected renal allograft from the development of CAN in a mouse model, in which renal allografts exhibit reduced renal function and many of the histological features of CAN seen in chronic rejection of human kidney allografts by 6 wk after transplantation (31). The survival and function of renal allografts from CI-1040-treated recipient mice were compared with those from vehicle-treated mice. All six CI-1040-treated recipients survived, whereas two of seven vehicle-treated recipients died as a result of graft failure: one on day 32 and the other on day 38. As shown in Fig. 3, at the end of the experiment, the function of grafts in CI-1040-treated recipients had been better preserved, as indicated by lower levels of serum creatinine and BUN (30 ± 6 μM and 22 ± 8 mM, n = 6) than in the vehicle-treated survivors (94 ± 39 μM and 56 ± 25 mM, n = 5, P = 0.0015 for creatinine, P = 0.0054 for BUN). These data suggest that CI-1040 may prevent chronic renal allograft dysfunction.

Reduction of CAN histopathology in CI-1040-treated renal allografts. As shown above, CI-1040 had a beneficial effect on preventing chronic renal allograft dysfunction. To further confirm the therapeutic efficacy of CI-1040 in the reduction of CAN, we examined the effect of CI-1040 on the histopathology of CAN in these grafts following Banff 97 Working Classification of Renal Allograft Pathology (41). In general, the levels of chronic/sclerosing allograft changes were reduced in the CI-1040-treated group compared with the vehicle-treated group. In a total of four grafts from the CI-1040-treated group, one was pathologically normal and the other three exhibited chronic changes, primarily in glomeruli and interstitium, that were mostly limited to an increase of mesangial matrix and interstitial fibrosis (Fig. 4, Table 1); in the vehicle-treated group, chronic changes, including severe interstitial fibrosis, glomerulopathy, and an increase in mesangial matrix, were found in every graft. Tubular atrophy and arteriolar hyalinization were seen in 80% of the grafts, and one graft exhibited...
sclerotic glomeruli and cast formation (Fig. 4, Table 1). No vascular fibrous intimal thickening was noted. As shown in Table 1, CI-1040 significantly reduced interstitial fibrosis ($P < 0.0363$), mesangial matrix increase ($P < 0.0328$), and arteriolar hyaline thickening ($P < 0.0048$). Overall, the total CAN scores were significantly higher in the vehicle-treated group (mean 10.3) than in the CI-1040-treated group (mean 4.2, $P < 0.0118$). All these data suggest that the beneficial effect of CI-1040 on prevention of renal allograft function is correlated to its effect on reduction of CAN histopathology.

Blockade of MEK1-ERK1/2 signaling and reduction of TGF-β1 levels in CI-1040-treated renal allograft tissue. To further understand the mechanisms by which CI-1040 prevented renal allograft chronic rejection, we confirmed that administration of CI-1040 at 100 mg/kg twice a day efficiently blocked MEK1-ERK1/2 signaling in renal allograft in mice. As shown in Fig. 5, ERK1/2 phosphorylation was significantly suppressed in the grafts from the CI-1040-treated group compared with the vehicle-treated group. Immunohistochemical staining revealed strong positive staining with anti-p-ERK antibody in only a few tubules in CI-1040-treated grafts, whereas ERK proteins were strongly phosphorylated in all the renal resident cells and infiltrating leukocytes in the grafts from the vehicle control group (Fig. 5A). Western blot analysis, with total ERK1/2 protein levels as controls, further confirmed this observation (Fig. 5B). As shown above, in vitro addition of CI-1040 reduced TGFβ1 synthesis in TEC cultures (Fig. 2). To demonstrate whether inhibition of MEK1-ERK1/2 signaling by CI-1040 in the grafts results in reduction of intragraft TGFβ1 production, we used immunohistological staining to examine the levels of intragraft TGFβ1 at the end of the experiment. As shown in Fig. 6, the cells that stained positive with anti-TGFβ1 antibody were located predominantly in the areas with severe leukocyte infiltration in the section of grafts from the CI-1040-treated group; staining intensity in the renal resident cells was the same as that in the control staining section. However, the strong TGFβ1 staining in much larger areas in the sections of grafts from the vehicle-treated group indicates some level of TGFβ1 staining not only in the cells in the areas with infiltrates, but also in renal tubular cells. Taken together, blockade of MEK1-ERK1/2 signaling by CI-1040 can reduce intragraft TGFβ1 production of renal allograft, particularly in the resident renal cells.
DISCUSSION

Immunosuppressive regimens have proven effective in early renal allograft survival, but not in prevention of CAN, which has become the leading cause of late graft failure. Therefore, novel therapies for CAN are needed to promote long-term graft survival. In the renal transplantation model of B6 donor mice to BALB/c recipient mice, the grafts develop pathological abnormalities after 6 wk that resemble chronic rejection in humans (30, 31), and this model has been used in preclinical study of therapy for CAN (12). Our previous study demonstrated that pharmacological intervention of ERK1/2 signaling prolongs cardiac allograft survival (56). In the present study, we have shown that this strategy also reduces CAN in the mouse model, as indicated by the beneficial effects of CI-1040 on maintenance of graft function and reduction of CAN histopathology, which is correlated with suppression of alloimmunity and a decrease in profibrotic TGFβ1 biosynthesis in TEC, as well as in graft tissue.

CAN development is a slow and multifactorial process, comprising immune-dependent and -independent factors, including acute cellular rejection, ischemia-reperfusion injury, cytomegalovirus infection, chronic inflammation, and drug nephrotoxicity (28, 51, 58). These factors together induce endothelial activation, leukocyte infiltration, and a phenotypic transition of renal resident cells (e.g., interstitial fibroblasts to myofibroblasts and transdifferentiation of TEC to myofibroblasts) (58). A recent high-density oligonucleotide microarray examination of the global expression of profibrotic and growth factors in CAN (19) indicates >1.5-fold upregulation of 212 genes, including TGFβ-induced factor, thrombospondin-1, and platelet-derived growth factor (PDGF)-C and others related to fibrogenesis and extracellular matrix deposition, such as integrins-β3 and -β6, MMP-7, -9, and -10, laminin, fibronectin, and collagen type IV. In addition, the chemokine CXCL6 and the adhesion molecules VCAM-1, activated leukocyte cell adhesion molecule, and selectin P are also upregulated, suggesting that the recruitment of leukocytes into allografts is also important in the development of CAN. Therefore, it seems that pharmacological interference of a common pathway for fibrogenesis, as well as leukocyte infiltration, may have significant therapeutic potential for prevention of CAN in patients.

TGFβ1 is a key fibrogenic cytokine involved in the fibrosis of a number of chronic diseases of the kidney and other organs, and recent evidence has shown that TGFβ1 is involved in the pathogenesis of CAN (5). Indeed, upregulation of TGFβ has been associated with CAN in a mouse model (29, 31) and in human kidney transplant recipients (6, 40, 49). Further studies indicate that TGFβ from TEC and infiltrating lymphocytes (e.g., CD8+ T lymphocytes) can contribute to CAN development (35, 43) and can be induced by numerous factors, includ-

Table 1. Histological scores for CI-1040-induced reduction of CAN

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<th>CI-1040</th>
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Score for each category was determined in >10–15 fields and averaged. CAN, chronic allograft nephropathy; CI, interstitial fibrosis; CT, tubular atrophy; CG, allograft glomerulopathy; MM, mesangial matrix increase; AH, arteriolar hyaline thickening.
The biological effects of TGF-β include the induction of matrix production and/or inhibition of matrix degradation (3), as well as EMT, a process whereby epithelial cells are transformed into myofibroblasts, and a key pathological change in the development of CAN (28, 43, 44, 49). Hence, it has been suggested that TGF-β is a critical target for prevention of CAN (28), and inactivation of TGF-β as an antifibrosis strategy has been tested in several experimental models; bone morphogenic protein-7 reverses TGF-β1-induced EMT and repairs severely damaged renal tubules in a mouse model of chronic renal injury (48), and neutralization of TGF-β activity with antibody therapy reduces renal fibrosis in chronic progressive nephritis (13) and glomerulosclerosis, including glomerular basement membrane thickening and mesangial matrix expansion in diabetic animals (48, 60).

An early study indicates that TGF-β1 production in kidney cells can be induced by growth factors (i.e., EGF and PDGF), as well as TGF-β1 (autoinduction) (53). The action of these profibrotic growth factors requires MEK1-ERK1/2 signaling in many cell types, including renal cells (17, 23, 52, 59), and disruption of the MEK1-ERK1/2 signaling pathway by a small-molecule inhibitor, PD-98059, results in a decrease in autoinduction-mediated TGF-β production (59), TEC migration, and MMP-9 production (52). Therefore, inactivation of MEK1-ERK1/2 signaling in renal allografts could reduce profibrotic TGF-β activity and synthesis, which may be generated from renal resident cells and/or infiltrates (e.g., T cells and macrophages) by stimulation of growth factors (EGF, PDGF, and TGF-β). Our data indicate that inhibition of MEK1-ERK1/2 signaling by CI-1040 reduces TGF-β1 biosynthesis in TEC cultures and intragraft levels of TGF-β1 in renal allografts, suggesting that inactivation of profibrotic TGF-β1 by inhibition of MEK1-ERK1/2 signaling may contribute, at least in part, to the efficacy of CI-1040 in the reduction of CAN.

In addition to the crucial role of TGF-β in the pathogenesis of CAN, there is some evidence that the nature of alloimmune responses, including infiltrating leukocytes, may contribute to the progression of CAN in experimental models and clinical biopsies (24, 30, 36, 58), particularly the association of the...
presence of the Th1 subset (33, 54). It is not fully understood how the responses of the alloantigen-dependent Th1 subset are related to the development of CAN, but evidence from one study suggests that alloantigen-stimulated immune response (cellular rejection) initiates early graft injury, which then leads to the late events of CAN, such as glomerular hyperfiltration, proteinuria, and glomerulosclerosis (14). It has been documented that specific inhibition of MEK-ERK1/2 signaling by PD-98059 or U-0126 reduces T cell proliferation/activation in response to T cell receptor antigen stimulation (56) and suppresses Th1 differentiation and Th1 alloimmune responses to cardiac allograft (22, 56, 57). The present study has shown the similar effects of CI-1040 in the suppression of alloantigen-stimulated T cell proliferation, as well as prevention of allograft acute rejection. These data indicate that the anti-CAN mechanisms of CI-1040 may include its immunosuppressive activity against Th1 immune responses during acute and chronic cellular rejections.

In conclusion, we demonstrate, for the first time, that pharmacological interference of MEK1-ERK1/2 signaling using CI-1040 prevents the development of CAN and that CI-1040 suppresses not only alloreactive immunity, but also profibrotic TGF-β production in kidney cells. However, the mechanisms by which CI-1040 reduces CAN in the present study are not clear. CI-1040 is a clinically used compound that has been tested as an anticancer drug; recently, it has been shown to slow the progression of polycystic kidney disease in mice (34). In addition to our study, inhibition of p38 MAPK with FR-167653 has shown similar results in the reduction of CAN, with preserved renal function and prolonged survival in a rat model (55). Therefore, small-molecule inhibitors targeting MAPK signaling, such as MEK1-ERK1/2 signaling with CI-1040, have therapeutic potential for prevention of CAN in patients with renal allografts.

GRANTS

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