CD40 expression on graft infiltrates and parenchymal CD154 (CD40L) induction in human chronic renal allograft rejection

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Background. CD40-CD154 (CD40L) costimulatory signaling plays a pivotal role in the effector mechanisms of transplant graft rejection. In animal models, CD40-CD154 blockade induces long-term graft acceptance concurrent with an absence of chronic rejection (CR) lesions. Given the critical importance of CD40-CD154 interactions in the development of chronic transplant allograft rejection, the relevance of in situ CD40 and CD154 expression was assessed in human chronic renal allograft rejection.

Methods. The expression of CD40, CD154, CD68, and T-cell receptor (TCR)α/β was analyzed by immunohistochemistry. Serial cryostat sections of snap-frozen core renal allograft biopsies were obtained from 30 renal transplant patients. Biopsy specimens received diagnoses of CR (N = 23) according to the Banff classification and were compared with controls (N = 7) consisting of stable allografts and normal kidney tissue.

Results. Striking CD40 staining of graft cellular infiltrates (P = 0.016) was observed in renal allografts with CR compared with controls. The CD40+ cellular infiltrates in CR were predominantly TCRα/β+ T cells and some CD68+ macrophages. These findings were contrasted by the low-level CD40 expression detected in glomeruli and tubules of CR and controls. However, glomerular induction of CD154 was observed in CR allografts (P = 0.028) as compared with controls. CD154 immunoreactivity was demonstrated on glomerular endothelial, epithelial, and mesangial cells. Moderate CD154 expression was detected on tubular epithelial cells, and only weak CD154 immunoreactivity was observed on the infiltrates in isolated CR cases.

Conclusion. In human chronic renal allograft rejection, CD40 is expressed on graft-infiltrating cells of the T cell and macrophage compartments. CD154 expression is induced on glomerular and tubular epithelial cells during CR, demonstrating another novel source of CD154 expression. The data substantiate the potential contributory role of an interaction between CD40+ graft-destructive effector T cells and macrophages with CD154+ renal allograft parenchymal cells in the development of chronic renal allograft rejection.

Kidney transplantation (KTx) has become an established therapeutic procedure for end-stage renal disease (ESRD). Chronic rejection (CR) remains a major cause of late graft failure [1]. The underlying pathogenetic mechanisms of chronic allograft rejection remain ill-defined. Worse yet, only limited data are available in human allograft situations. However, several experimental systems have been established that enable studies of renal, cardiac, and vascular allografts to be performed in animal models of CR [2–4]. Current concepts of CR obtained from such experimental data implicate a multifactorial pathogenesis that involves both alloantigen-dependent and -independent mechanisms [3, 5–8].

The important role of costimulatory signaling in the alloimmune response is well documented. Effector cell activation is tightly regulated by signaling mechanisms following Ag-specific T-cell activation: signal 1 through engagement of the T-cell receptor (TCR) with the peptide-major histocompatibility complex on the antigen-presenting cell and signal 2 through interactions of co-stimulatory accessory molecules. Recent evidence has implicated the CD28/B7 and the CD40/CD154 (CD40L) pathways in allograft rejection. Inhibition of the CD28/B7 pathway prolongs allograft survival [9, 10]. More interestingly, however, are recent reports of the induction of long-term graft acceptance concurrent with an absence of CR lesions by simultaneously blocking both CD28/B7 and CD40/CD154 pathways [11–14]. Given the critical role CD40/CD154 interactions may play in chronic allograft rejection, in situ CD40 and CD154 expression was assessed in chronic rejecting allografts following human KTx.
Table 1. CD40 and CD154 staining intensity scores of chronic rejecting renal allografts

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<th>CD40 staining intensity score</th>
<th>CD154 staining intensity score</th>
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a No available data
b No glomeruli and vascular structures identified on H&E slide
c No available H&E slides for CAN score evaluation
d Blank space denotes no corresponding structures identified on frozen-sections for analysis

METHODS

Renal allograft specimens

Kidney biopsies obtained from 30 KTx patients at the University of Kentucky Transplant Center were collected and immediately snap frozen in methylbutane. Immunohistochemical analysis was performed on the kidney allograft biopsies obtained from KTx patients with CR as the experimental group (N = 23). Six stable renal allograft biopsies diagnosed as no rejection from KTx patients and one normal kidney obtained at autopsy served as the controls (N = 7). The diagnosis of CR and no rejection was based on criteria used as part of the Banff classification [15, 16]. Histological data of the experimental group are presented as chronic allograft nephropathy scores based on the Third Banff Conference on renal allograft pathology (Table 1) [16]. The individual chronic allograft nephropathy score was obtained by the sum of each lesion quantitation scores of transplant glomerulopathy (cg), interstitial fibrosis (ci), tubular atrophy (ct), and vascular changes (cv). Twenty-two of the KTx patients were treated with the cyclosporine A-based immunosuppressive regimen, which included azathioprine and methylprednisolone. Three KTx patients received the FK506-based regimen, which also included azathioprine and methylprednisolone. Five KTx patients were treated with azathioprine and methylprednisolone but without either cyclosporine A or FK506. Renal allograft biopsies in the CR group were obtained late in the post-transplant period (mean days post-transplant, 2378; range 167 to 8725; Table 1), and indication for biopsy included a slowly rising serum creatinine concentration. Stable renal allograft biopsies in the control group consisted of post-transplant protocol biopsies.

Immunohistochemistry

CD40 and CD154 expression was analyzed by immunohistochemistry on serial cryostat sections of snap-frozen kidney tissue. Immunostaining was performed using an indirect immunoperoxidase procedure with the Vectastain Elite ABC Kit (Vector, Burlingame, CA, USA). Kidney biopsies were embedded in optimal cutting temperature (OCT) compound and snap frozen in dry ice/methylbutane during sampling. Five-micrometer cryostat sections were mounted on glass slides. Following paraformaldehyde fixation, endogenous peroxidase was blocked using 0.3% H2O2/methanol, and the slides were washed in phosphate-buffered saline (PBS). For CD154 staining, quenching of endogenous peroxidase was performed by incubation in glucose oxidase/glucose/sodium azide solution for one hour, followed by PBS washing [17]. Tissue sections were incubated with 5% horse serum for one hour, followed by blocking of endogenous biotin with the Avidin/Biotin blocking kit (Vector). The slides were then incubated overnight with monoclonal antibodies (mAbs) directed against human CD40 (clone mAb89, mouse IgG1, diluted 1:150; Immunotech, West-
In each structural compartment was assessed. The follow-

- CD40

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by two independent observers (A.S.G. and B.L.M.). The To determine the phenotype of CD40

- Data analysis

Serial anti-CD68 and anti-TCR

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biotin-horseradish peroxidase and PBS wash, sections (1.076

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England Nuclear, Boston, MA, USA) according to the Moderate CD40 staining of proximal tubules and a few

- tyramide signal amplification (discussed later in this article) prior to incubation with chromogen. Chromogenic reaction was developed using aminoethyl carbonate (AEC) and was counterstained with Mayer’s hematoxylin. Serial sections of snap-frozen renal allograft biopsies from the CR group were stained with anti-CD68 (clone EMB11, mouse IgG1, diluted to 1:200; Dako, Carpinteria, CA, USA) as a marker for macrophages and anti-TCRα/β (clone BF1, mouse IgG1, diluted 1:100; Endogen, Woburn, MA, USA) as a marker for T cells, as described earlier here, to identify the phenotype of the CD40+ graft infiltrates in the CR group.

Tyramide signal amplification

Cryostat sections for CD154 and TCRα/β staining were subjected to a tyramide signal amplification step using a commercially available amplification kit (New England Nuclear, Boston, MA, USA) according to the manufacturer’s specifications. After incubation in avidin-biotin-horseradish peroxidase and PBS wash, sections were incubated for 10 minutes in biotinyl tyramide solution and washed in PBS. Slides were then incubated in streptavidin-horseradish peroxidase for 30 minutes followed by a PBS wash. Chromogen was added and counterstained as described earlier.

Data analysis

Immunohistochemical sections were assessed blindly by two independent observers (A.S.G. and B.L.M.). The staining intensity was semiquantitatively scored (0 = no staining, 1 = weak, 2 = moderate, 3 = strong), and the staining distribution (percentage estimate of positivity) in each structural compartment was assessed. The following specific compartments were assessed: glomeruli, tubules, vasculature, infiltrates, and fibroblasts in areas of interstitial fibrosis. A statistical analysis comparing the mean staining intensity was performed using the Student’s t-test. The correlation of CD40 and CD154 staining distribution with staining intensity of every structural compartment in each individual biopsy was analyzed by linear regression. All statistical analyses were performed using SigmaStat® software. A P value of less than 0.05 was considered significant.

RESULTS

CD40 expression

Moderate CD40 staining of graft infiltrates was detected in CR (1.938 ± 0.306, mean staining intensity score ± SEM, P = 0.016; Tables 1 and 2; Figs. 1 and 2), which contrasted to the absence of CD40 expression on the scanty infiltrates present in control specimens. The estimated mean distribution (± SEM) of CD40 expression on graft cellular infiltrates in CR allografts was at 60% (± 7%) and ranged from 10 to 100%. The extent of CD40 staining on graft infiltrates of CR correlated with the CD40 staining intensity scores (R = 0.854, P < 0.001). CD40+ infiltrates were seen around areas of CD154+ tubules and glomeruli (discussed later here). Moderate CD40 staining of proximal tubules and a few distal tubules was similarly demonstrated in both CR (1.076 ± 0.198) and controls (1.500 ± 0.373). Only sparse glomerular CD40 expression in isolated cases of CR (0.287 ± 0.111) and controls (0.333 ± 0.167) was detected. No CD40 immunoreactivity was detected on fibroblasts in areas of interstitial fibrosis and in the vasculature.

Serial anti-CD68 and anti-TCRα/β staining of chronic rejection specimens

To determine the phenotype of CD40+ graft-infiltrating mononuclear cells in CR allografts, sequential staining with anti-CD68 and anti-TCRα/β mAbs were performed on serial sections of eight CR specimens. The CD40+ cellular infiltrates were predominantly TCRα/β+ with a mean (± SD) estimated distribution of 73% (± 5) (range 10 to 50%, P < 0.001) compared with only 27% (± 5, range 50 to 90%), which were CD68+ (Fig. 2).

<table>
<thead>
<tr>
<th>Table 2. Mean staining intensity scores of CD40 immunoreactivity in chronic rejecting human kidney allografts and controls</th>
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<td>CR (N = 23)</td>
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<td>Controls (N = 7)</td>
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<td>CR (N = 23)</td>
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<td>Controls (N = 7)</td>
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*Mean staining intensity ± SEM

P = 0.001 vs. controls

Fibroblasts in areas of fibrosis were assessed in CR that were not present in controls

P = 0.028 vs. controls

brook, ME, USA) and CD154 (clone TRAP1, mouse IgG1, diluted to 1:200; Pharmingen, San Diego, CA, USA). Tissue sections were washed in PBS buffer and subsequently incubated with biotinylated horse anti-mouse IgG and avidin-biotin-horseradish peroxidase. Sections for CD154 staining were subjected to tyramide signal amplification (discussed later in this article) prior to incubation with chromogen. Chromogenic reaction was developed using aminoethyl carbonate (AEC) and was counterstained with Mayer’s hematoxylin. Serial sections of snap-frozen renal allograft biopsies from the CR group were stained with anti-CD68 (clone EMB11, mouse IgG1, diluted to 1:200; Dako, Carpinteria, CA, USA) as a marker for macrophages and anti-TCRα/β (clone BF1, mouse IgG1, diluted 1:100; Endogen, Woburn, MA, USA) as a marker for T cells, as described earlier here, to identify the phenotype of the CD40+ graft infiltrates in the CR group.

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Fig. 1. CD40 expression on mononuclear cellular infiltrates in representative chronic rejection (CR) allograft biopsy of patient 2. AEC, Mayer’s hematoxylin, magnification ×400.

Fig. 2. Serial anti-CD68 and anti-TCRα/β immunostaining demonstrating CD40+ T cells and macrophages. Intense CD40 expression on graft cellular infiltrates in a representative chronic rejection (CR) allograft biopsy (A, magnification ×100; D, magnification ×400). Sequential staining of corresponding serial sections with anti-TCRα/β (B, magnification ×100; E, magnification ×400) and anti-CD68 mAbs (C, magnification ×100; F, magnification ×400). AEC, Mayer’s hematoxylin.
CD154 expression
Enhanced CD154 expression was detected in the glomeruli of CR (1.853 ± 0.274, mean intensity ± SEM, \( P = 0.028 \); Tables 1 and 2), which contrasted to that of the controls (0.6714 ± 0.376). CD154 immunoreactivity was detected on glomerular endothelial, mesangial, and epithelial cells of almost all CR specimens (Fig. 3). The extent of CD154 staining in glomerular structures correlated with the staining intensity scores of CD154 (\( R = 0.689, P < 0.001 \)). A positive trend was observed in the moderate tubular CD154 immunoreactivity (1.429 ± 0.283; Fig. 4), mostly seen in atrophic and intact proximal tubules and in a few distal tubules in CR compared with controls (0.778 ± 0.290). Only weak CD154 expression in the vasculature and infiltrates was detected in three isolated cases of CR, which was absent in all control cases. No CD154 staining was detected on fibroblasts in fibrotic areas of CR.

DISCUSSION
The CD40 receptor belongs to the tumor necrosis factor receptor superfamily and is expressed on a variety of cell types, including B cells, T cells, monocytes/macrophages, dendritic cells, and parenchymal cells [18–21]. In chronic renal allograft rejection, the findings presented demonstrate CD40 expression on graft infiltrating cells of the macrophage and T-cell compartments. Denton et al have shown CD40 expression on infiltrating leukocytes in acute rejecting human renal allografts [22]. These data are in contrast to the rare CD40 expression by control groups reported using normal human kidney tissue and nonrejecting kidney allografts [23, 24]. The observed tubular expression of CD40 may play a yet undefined role in kidney function, as it has been demonstrated to influence cell growth and survival. The data presented are in line with previous studies demonstrating the constitutive CD40 expression on tubular epithelial cells in culture and in normal kidney, inflammatory renal diseases, and acute rejecting renal allografts [22, 23, 25].

The finding of enhanced CD154 expression predominantly in renal parenchyma of kidney allografts manifesting histologic evidence of CR extends the broad cellular expression pattern of CD154. CD154, the ligand for CD40, was initially thought to be expressed exclusively on activated T cells. Recent reports, however, have documented a wide cellular distribution of CD154 expression on human dendritic cells [26], B cells [26], endothelial
cells, renal tubular epithelium [23], smooth muscle cells, and macrophages of atherosclerotic lesions [27], as well as acute rejecting heart allografts [28]. In human chronic renal allograft rejection, CD154 is induced not only on glomerular microvascular endothelial cells but on glomerular epithelial, mesangial cells, and tubular epithelial cells as well. The observed de novo CD154 expression during CR stands in contrast to the relative absence of CD154 expression in nonrejecting kidney allografts and normal kidney tissue. Zheng et al have recently reported the up-regulation of CD154 gene transcripts in acute rejecting human renal allografts [24]. In accordance with their findings of low-level CD154 gene activation in nonrejecting renal allografts and normal kidney, these data support and confirm their findings at the protein expression level of a lack of CD154 expression in the absence of rejection and in immunologic quiescence. The minimal CD154 expression detected on the minor graft infiltrates, albeit seen only in cases of CR, suggests a different important source of CD154 in CR other than activated T cells alone. The marked de novo expression of CD154 on glomerular and tubular structures in chronic rejecting renal allografts extends the available current data relative to the broad cellular distribution of CD154 and its potential importance in the pathogenesis of chronic kidney graft rejection.

A stimulation-dependent soluble form of CD154 (sCD154) exists and, like the membrane-bound CD154,
possesses functional biological properties [29, 30]. Activated T cells have been reported to release sCD154 in vitro through intracellular proteolytic processing that parallels the kinetics of cell surface CD154 expression [29, 31]. The observed CD154 immunoreactivity on glomerular and tubular epithelial cells therefore implicates the potential immunolocalization of sCD154 bound to CD40 on renal parenchymal cells during CR. However, the data presented refute the argument of a potential cross-reactivity with α-CD154 mAb. False immunoreactivity against shed CD154 bound to CD40 is unlikely, as only minimal CD40 expression was observed on the same cells, and strongly contrasts with the enhanced CD154 expression on glomerular and tubular structures. Furthermore, the known concomitant membrane CD154 expression and sCD154 release strongly argue that sCD154 is being released in the relative absence of surface CD154 expression on infiltrating mononuclear cells during CR. The sparse CD40 expression observed in glomeruli and tubules could be due to a CD154-mediated negative regulatory mechanism. sCD154 down-regulates CD40 expression of bystander cells in an autocrine and paracrine manner in a cytokine-like fashion [29]. Given the enhanced glomerular and tubular CD154 expression, it is

Fig. 4. Strong CD154 immunoreactivity in proximal tubules of a chronic rejection (CR) biopsy (A, magnification ×100; B, magnification ×400). (C) The incubation of a serial section of the same biopsy (A, B) with isotype-matched Ab as negative control (magnification ×100). (D) The absence of CD154 immunoreactivity in proximal tubules of a control renal allograft (magnification ×100). AEC, Mayer’s hematoxylin.
probable that concomitant sCD154 release by these same cells may act to repress or inhibit the expression of CD40.

An important consequence of CD40 interaction on antigen-presenting cells with CD154 on graft-specific CD4+ T cells involves regulatory mechanisms crucial to the destructive process of graft rejection. These include the potentiation of antigen-presenting cell function and T-cell priming, activation, and differentiation through up-regulation of cell surface costimulatory molecules, adhesion molecules, and provision of critical inflammatory mediators and cytokines that all contribute to graft rejection pathology. Of interest, however, is the possibility of a bidirectional modulation of CD40-CD154 signaling mechanisms that may contribute to the destructive effector mechanisms of CR. Both CD40 and CD154 have been reported to be coexpressed in human cardiac allografts on both allograft endothelial cells and graft-infiltrating T lymphocytes during acute rejection [28] and in atherosclerotic lesions [27]. In line with these findings, these data demonstrating CD40 expression on recipient effector cells and CD154 induction on donor allografts implicate a direct cross-talk between CD40+ T cells and macrophages with CD154+ glomerular and tubular epithelial cells.

The effects of CD40 ligation include not only the induction of major histocompatibility complex and costimulatory molecule B7 expression, but also the provision of inflammatory cytokines and chemokines by mononuclear cells [23, 27, 32]. A possible CD40 cross-linking on CD40+-infiltrating graft lymphocytes and macrophages by the de novo expressed CD154 on glomerular and tubular epithelial cells could therefore induce the production of inflammatory mediators known to be elaborated during CR. The sustained intragraft release of these potentially graft destructive mediators may play a critical role in the pathology of chronic allograft rejection. The administration of CD154, which leads to CD40 ligation, induces potent biological effects on T cells and monocytes promoting optimal activation, proliferation, and cytokine production [33–35]. The production of these mediators by CD40+ graft infiltrates is likely, as these cells have been demonstrated to produce inflammatory cytokines following direct CD40 engagement [23, 27, 32].

In support of this hypothesis, the disruption of CD40/CD154 interactions that leads to the prevention of graft rejection is associated with the inhibition of proinflammatory and T helper type 1 (Th1) cytokines [11, 36]. Of critical importance, moreover, are recent reports attributing CD40 signaling in preventing apoptosis [37]. CD40 engagement promotes the viability of mononuclear cells through antiapoptotic mechanisms [32, 37]. Recently, CD40-mediated signaling has been reported to activate nuclear factor-κB (NF-κB) [38], thereby inducing its known antiapoptotic properties through the induction of antiapoptotic genes [39]. In fact, the antiapoptotic effects of CD40-mediated signaling through CD154 treatment have been directly attributed to NF-κB activation, which is associated with IkB degradation [40].

Studies of chronic allograft rejection pathology have emphasized the transplant vasculopathy lesions that are common to all solid organ allografts. In CR of the allograft kidney, characteristic glomerular and tubular changes occur. Current concepts of CR obtained from experimental data provide evidence for a central role of inflammatory mediators, which include cytokines and chemokines, and growth factors in several CR allograft models [2–8], and are clearly observed in human renal allografts as well [41–44]. In fact, selective therapeutic intervention preventing the development of CR is associated with the down-regulation of these inflammatory mediator responses [45]. It is, therefore, tempting to speculate that a consequence of CD40-CD154 interaction not only leads to provision of critical inflammatory mediators like proinflammatory cytokines and chemokines by allograft-specific cellular effector cells, but, also by renal epithelial cells of the allograft. In addition to its role as a cognate ligand in CD40-mediated events, current data suggest that direct CD154 signaling may independently contribute to signal-transducing events through a direct costimulatory receptor function [46]. In fact, directly engaging CD154 leads to cytokine production [47, 48]. This assumption would not be surprising as glomerular and tubular epithelial cells are known to synthesize cytokines and chemokines directly in the ensuing stages of chronic renal allograft rejection [49].

Evidence has already been presented demonstrating the importance of blocking the CD40/CD154 costimulatory pathway in the induction of long-term allograft survival and prevention of chronic allograft rejection in animal models [11, 13, 14]. The novel data presented in this study of de novo CD154 expression on renal allograft parenchyma and graft-infiltrating CD40+ T cells and macrophages during CR may help elucidate the destructive mechanisms of CD40/CD154 interactions that may contribute to the development of human chronic renal allograft rejection.

NOTE ADDED IN PROOF


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REFERENCES


33. Fanslow WF, Clifford KN, Seaman M, Alderson MR, Spriggs...


