Infiltration of immune cells into the renal interstitium is characteristic of chronic inflammatory kidney diseases. CD4+ T cells and platelets express CD40 ligand (CD40L) and are reported to mediate proinflammatory events in renal proximal tubular epithelial cells (RPTEC) via interaction with CD40. In other cell types, CD40 signals can also induce protective genes. Here, human RPTEC were treated with sCD40L to ligate CD40, and a significant increase in the generation of proinflammatory reactive oxygen species was found; however, CD40-activated cells did not undergo apoptosis. This suggests that CD40 signals may simultaneously induce antiapoptotic genes for cytoprotection of RPTEC. Heme oxygenase-1 (HO-1) expressed in RPTEC may simultaneously induce antiapoptotic genes for cytoprotection of RPTEC. Heme oxygenase-1 (HO-1) expressed in RPTEC serves as a protective gene, but it is not known whether it is regulated by CD40. Next, RPTEC were transiently transfected with a full-length HO-1 promoter-luciferase construct and were treated with sCD40L. CD40 ligation was found to significantly increase HO-1 promoter activity. By electrophoretic mobility shift assay, it was confirmed that CD40 signaling induced the transcriptional activation of HO-1 through the binding of NF-κB to its promoter. By Western blot analysis, a marked increase in HO-1 protein expression following CD40 ligation was also found. These observations are of clinical significance because it was found that CD40 and HO-1 are induced in expression in vitro in inflamed rejecting kidney biopsies and co-expressed in renal tubules. Therefore, ligation of CD40 in RPTEC promotes both inflammatory and anti-inflammatory processes. Regulating the balance between these two events may be of importance in the prevention of tubular injury associated with renal disease.

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Infiltration of immune cells into the renal interstitium is characteristic of chronic inflammatory kidney diseases. CD4+ T cells and platelets express CD40 ligand (CD40L) and are reported to mediate proinflammatory events in renal proximal tubular epithelial cells (RPTEC) via interaction with CD40. In other cell types, CD40 signals can also induce protective genes. Here, human RPTEC were treated with sCD40L to ligate CD40, and a significant increase in the generation of proinflammatory reactive oxygen species was found; however, CD40-activated cells did not undergo apoptosis. This suggests that CD40 signals may simultaneously induce antiapoptotic genes for cytoprotection of RPTEC. Heme oxygenase-1 (HO-1) expressed in RPTEC serves as a protective gene, but it is not known whether it is regulated by CD40. Next, RPTEC were transiently transfected with a full-length HO-1 promoter-luciferase construct and were treated with sCD40L. CD40 ligation was found to significantly increase HO-1 promoter activity. By electrophoretic mobility shift assay, it was confirmed that CD40 signaling induced the transcriptional activation of HO-1 through the binding of NF-κB to its promoter. By Western blot analysis, a marked increase in HO-1 protein expression following CD40 ligation was also found. These observations are of clinical significance because it was found that CD40 and HO-1 are induced in expression in vitro in inflamed rejecting kidney biopsies and co-expressed in renal tubules. Therefore, ligation of CD40 in RPTEC promotes both inflammatory and anti-inflammatory processes. Regulating the balance between these two events may be of importance in the prevention of tubular injury associated with renal disease.
Oxidative stress conditions provoke cellular responses, principally involving the transcriptional activation of genes encoding proteins that participate in the defense against oxidative tissue injury (30-32). Exposure of mammalian cells to oxidative stimuli induces heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme degradation (33-35). Excess of free heme, which is released from hemeproteins under such stress conditions, may constitute a major threat because it catalyzes the formation of ROS (30,36). HO-1 converts heme to biliverdin, carbon monoxide, and iron. Among the three HO isoforms described to date, HO-1 is highly inducible by oxidative stress (30,33,37). In various tissue injury models, including an inflammatory renal injury model, induced HO-1 has been shown to confer protection (38,39). Moreover, it has been shown that renal tubuli are dependent on intrinsic HO-1 production for their survival under oxidative stress (40). However, the mechanism by which the immune response can induce HO-1 expression in RPTEC is poorly understood. This is important, as the induction of HO-1 might confer a protective phenotype to RPTEC to protect tubular cells from ongoing immune-mediated injury.

In this study, we evaluated the effect of CD40-mediated signaling on HO-1 expression in RPTEC. Although CD40 signals induce ROS, the cells fail to undergo apoptosis, likely as a result of a coincident ability of CD40 to induce HO-1. We found that CD40 signals regulate HO-1 expression at the transcriptional level involving an NF-κB-induced pathway. Our observations suggest that CD40 activation of RPTEC can stimulate both pro- and anti-inflammatory signals that may be critical either to the progression or to the attenuation of renal inflammatory processes. Understanding how to promote protective mechanisms clearly has therapeutic implications in the treatment of renal disease.

Materials and Methods

Reagents

Soluble CD40L (sCD40L) was obtained from Y.M. Hsu of Biogen (Cambridge, MA) or from Ancell (Bayport, MN) and was used for all assays at 3.0 μg/ml. Hydrogen peroxide (H₂O₂), DNase-I, and vitamin C (Ascorbic acid) were purchased from Sigma Chemicals (St. Louis, MO).

Cell Culture

Human RPTEC were purchased from Clonetics (Walkersville, MD) and were cultured in complete epithelial medium (REGM BulletKit; Clonetics), as supplied, according to recommended instructions. The cells were subcultured and used at passages 3 to 6. All of the cells used in these studies were assessed by FACS analysis for constitutive cell surface expression of CD40 such that they are responsive to stimulation by sCD40L.

Plasmid

A human HO-1 promoter-luciferase construct was obtained as a gift from J. Alam (Alton Ochsner Medical Foundation, New Orleans, LA). The plasmid pHHO4luc was constructed by cloning the promoter fragment from the human HO-1 gene (bp -4067 to 70 relative to transcription start site) into the luciferase reporter gene vector pSKeluc. The pcDNA3-HO-1 overexpression plasmid was obtained as a gift from Miguel P. Soares (Beth Israel Deaconess Medical Center, Boston, MA) (41).

Flow Cytometry

Cell suspensions were incubated with primary antibody, washed, and incubated with a secondary FITC-conjugated antibody at 4°C for 30 min as described (14). The stained cells were washed and fixed in 1% paraformaldehyde and analyzed by FACScan (Becton-Dickinson, Oxford, CA).

Detection of ROS Generation

After CD40 ligation/H₂O₂ treatment, the cells were stained with a redox-sensitive dye dichlorodihydrofluorescein diacetate (H₂DCFDA; 10 μmol/L; Molecular Probes, Eugene, OR) for 5 min according to the manufacturer’s protocol. The generation of ROS was visualized by fluorescence microscopy. The amount of ROS generated was determined by computer-assisted fluorescence densitometric analysis (24).

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling (TUNEL) was done using the ApoAlert DNA Fragmentation Assay kit (BD Biosciences, San Diego, CA) according to the manufacturer’s protocol. Briefly, after CD40 ligation/H₂O₂ treatment, the cells were fixed with 4% ice-cold formaldehyde for 25 min and washed with PBS. Subsequently, they were permeabilized with chilled 0.2% Triton X-100 and subjected to TUNEL for visualization by fluorescence microscopy. The cells for positive control were treated with DNase-I (0.5 μg/ml; 10 min).

Western Blot Analysis

Protein samples were mixed with 2× sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 4% SDS, and 0.0025% bromophenol blue), boiled, and run on 10% polyacrylamide gel with Tris-glycine-SDS running buffer (Bio-Rad, Hercules, CA). Size-separated proteins were transferred to a polyvinylidene difluoride membrane (NEN Life Sciences Product Inc., Boston, MA) at 60 volts for 1 h. The membranes were blocked with 5% milk in PBS-Tween 20 and coated with anti-HO-1 (Calbiochem, San Diego, CA). As an internal control, the membranes were coated with anti-β-actin antibody (Sigma Chemical). After three washes, the membranes were finally incubated with peroxidase-linked secondary antibody and the reactive bands were detected by chemiluminescence (Pierce, Rockford, IL). Expression was quantified by densitometry using an Alpha Imager 2000 system (Alpha Innotech, San Leandro, CA). The signals were standardized to the expression of the internal control (β-actin).

Transfection and Luciferase Assay

RPTEC (2.5 × 10⁶ cells) were transfected with either the HO-1 promoter-luciferase construct or the HO-1 overexpression plasmid using the Effectene transfection reagent (Qiagen, Studio City, CA), according to the manufacturer’s protocol. A 1:25 ratio of DNA to Effectene was used for all of the experiments. For luciferase assays, cells were harvested 24 to 48 h after transfection, and luciferase activity was measured using a standard assay kit (Promega, Madison, WI). Transfection efficiency was determined by co-transfection of the β-galactosidase gene under control of cytomegalovirus immediate early promoter and by measurement of β-galactosidase activity. We analyzed the average results of three independent experiments.
Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from RPTEC as described (42). Cells were washed in cold PBS and suspended in a buffer that contained 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 10 μg/ml aprotinin, 3 mM dithiothreitol, and 0.1 mM PMSF for 15 min on ice. Cells then were lysed with 0.5% Nonidet P-40, and the pellets were resuspended for 25 min in a buffer that contained 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 3 mM dithiothreitol, and 0.1 mM PMSF. The samples then were centrifuged at 14,000 rpm for 10 min, and clear supernatants that contained the nuclear proteins were collected and stored at −70°C until use.

Electrophoretic mobility shift assay (EMSA) was performed as described previously (42). Briefly, an EMSA binding reaction mixture (25 μl) was prepared with 20 mM HEPES (pH 8.4), 100 mM KCl, 20% glycerol, 0.1 mM EDTA, 0.05% Nonidet P-40, and 1 μg of BSA. Nuclear protein extract and 200 ng of poly(dA-dT). poly(dA-dT) were added to the mixture for 10 min at room temperature before addition of approximately 0.1 ng of radiolabeled oligonucleotide probe. After 20 min of incubation at 4°C, samples were run on 7% acrylamide gel in 1× TAE (40 mM Tris acetate and 1 mM EDTA) buffer. For quantification by densitometry, signals were standardized to the intensity of the free probe.

Radiolabeled oligonucleotides used in EMSA studies were two PCR-generated fragments of human HO-1 promoter, a 230-bp product (bp −362 to −132, relative to transcription start site) that contained a NF-kB binding site, and a 175-bp product (bp −150 to 24, relative to transcription start site) that contained an AP-2 binding site. The unlabeled NF-kB consensus oligonucleotide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry

Three cores of renal allograft biopsy tissue were obtained for diagnostic purposes after renal transplantation. One core was frozen, and the other two cores were processed by routine formalin fixation and histology for pathologic diagnosis. After the clinical diagnosis was completed, a portion of the remaining frozen tissue was used in this study. All allograft biopsies used in this study had evidence of cellular rejection by histopathology. Normal renal tissue used in this study was obtained from kidneys that were excised for neoplasia. A portion of each specimen was embedded in OCT (Tissue Tek; Miles Diagnostics, Elkhart, IN), snap-frozen in liquid isopentane/liquid nitrogen, and stored at −70°C until use. The protocol for tissue collection was approved by the Human Research Committee at the Children’s Hospital Boston and the Brigham and Women’s Hospital (Boston, MA).

Cryostat 4-μm-thick sections then were examined by immunohistochemistry, using either a single- or a double-labeling technique. The frozen specimens were fixed in acetone and immunostained as described (15). Briefly, for single labeling, the sections were incubated with either mouse anti-human CD40 (G28.5, a gift from Bristol Myers Squibb, Princeton, NJ) or rabbit anti-human HO-1 (Santa Cruz Biotechnology). After a thorough washing, the sections were incubated with a species-specific horseradish peroxidase–conjugated secondary antibody. All specimens were developed in 3-amino ethylcarbazole and were counterstained in Gill’s hematoxylin. For double labeling, the sections were incubated with primary antibodies alone or in combination. After a thorough washing, we used species-specific secondary antibodies conjugated either with FITC or rhodamine. Controls involved incubation of the sections with mouse and/or rabbit IgG in place of the primary antibodies. The sections were mounted in Vectashield mounting medium that contained DAPI (Vector Laboratories, Burlingame, CA) and visualized under fluorescence microscope.

Results

CD40–CD40L Interactions Promote Generation of ROS in RPTEC

By FACS analysis, we observed that our cultured RPTEC express a significant amount of cell surface CD40 (Figure 1A), which is similar to that reported in vivo in human kidneys (43). To evaluate the pathophysiologic function of CD40 on RPTEC in vitro, we first evaluated whether CD40 ligation generates ROS. We treated confluent cultures of cells with sCD40L for time intervals from 2 to 18 h. Our findings were that sCD40L time-dependently increased the formation of endogenous ROS, as observed by immunofluorescence using the redox-sensitive dye H₂DCFDA (Figure 1B). ROS generation increased from 6 h and was maximum (eight-fold increase) at 18 h. As a positive control, the cells were treated with 500 μM/L exogenous H₂O₂ for 15 min, which also produced significant amounts of ROS. Basal low levels of ROS were persistently detected in untreated cells and served as a negative control.

ROS are important regulators of apoptosis. We next wished to examine whether the amount of ROS generated during CD40 ligation could mediate apoptotic signals in renal epithelium. To this end, we first determined that treatment with a low concentration of H₂O₂ (250 μM/L) promoted a similar fold induction of ROS (approximately eight-fold) in RPTEC, as observed in cells that were treated with sCD40L for 18 h. We found that this similar fold induction of ROS promoted a significant increase in apoptosis in RPTEC after 8 h as observed by TUNEL assay (Figure 2, A and B). Next, to analyze whether CD40 ligation could also promote apoptosis, we stimulated RPTEC with sCD40L for time intervals from 12 to 48 h and subsequently measured apoptosis. We observed no significant change in apoptosis in the sCD40L-treated cells despite enhanced ROS formation (Figure 2C). DNase-I–treated RPTEC, which served as a positive control, showed significant increase in apoptosis/nuclear DNA fragmentation. Untreated cells served as a negative control. We interpret these observations to suggest that CD40 signals in RPTEC simultaneously induce ROS and a protective mechanism that prevents apoptosis in renal epithelial cells.

CD40 Ligation Promotes Cytoprotective HO-1 Overexpression in RPTEC

HO-1 serves as a protective gene in several inflammatory conditions, including renal injury. To analyze whether CD40 ligation may induce HO-1 protein expression in RPTEC, we stimulated the cells with sCD40L for time intervals up to 24 h. By Western blot analysis, we found that CD40–CD40L interactions promote a significant increase in HO-1 protein expression as compared with untreated controls (Figure 3A). To examine further CD40-induced HO-1 overexpression, we made use of a full-length human HO-1 promoter-luciferase construct. RPTEC first were transiently transfected with the promoter-reporter construct and were stimulated with sCD40L for 24 and 48 h, respectively. The effect of sCD40L on HO-1 transcription was assessed by measurement of luciferase activity in cell lysates. We found that CD40 ligation significantly increased HO-1 promoter activity as compared with untreated control (Figure 3B).
Thus, CD40 stimulation of RPTEC promotes HO-1 transcriptional activation as well as HO-1 protein overexpression.

To determine whether CD40-induced HO-1 overexpression is mediated through ROS, we transfected RPTEC with the HO-1 promoter-luciferase construct and then stimulated the cells with sCD40L for 24 h in presence or absence of an antioxidant (100 μM vitamin C). As observed by others (24), we found that vitamin C can significantly inhibit CD40-induced ROS generation (data not shown). As shown in Figure 3C, the CD40-induced HO-1 transcriptional activation was significantly downregulated with the antioxidant treatment. Thus, CD40-induced ROS plays an important role in HO-1 overexpression in RPTEC.

We next wished to analyze whether upregulation of HO-1 could promote cytoprotection in RPTEC. To this end, we transfected the cells with an HO-1 overexpression plasmid (pcDNA3-HO-1). Control cells were transfected with an empty expression vector. We first confirmed that HO-1 expression was significantly increased in pcDNA3-HO-1-transfected cells (data not shown). Twenty-four hours after transfection, the cells were treated with 250 μM exogenous H₂O₂ for 15 min and were incubated in normal medium for 8 h. We observed that the H₂O₂ treatment significantly increased apoptosis in pcDNA3-transfected cells as compared with untreated empty vector-transfected control (Figure 3D, left and middle); however, transfection with the HO-1 overexpression plasmid significantly inhibited H₂O₂-induced apoptosis in these cells (Figure 3D, right). Thus, HO-1 plays an important cytoprotective role in ROS-induced apoptosis of RPTEC.

CD40–CD40L Interactions Promote NF-κB–Mediated HO-1 Transcriptional Activation in RPTEC

The human HO-1 promoter has potential binding sequences for CD40-inducible transcription factors NF-κB and AP-2 adjacent to the transcription start site (Figure 4A) (44). To determine whether NF-κB and AP-2 are functional in CD40-induced HO-1 transcriptional activation in RPTEC, we used PCR-generated radiolabeled HO-1 promoter sequences that contained binding sites for either NF-κB (bp −362 to −132, relative to transcription start site) or AP-2 (bp −150 to 24, relative to transcription start site)
start site) in EMSA (Figure 4A). We first generated nuclear lysates from RPTEC that were stimulated with sCD40L for 60, 90, and 120 min, respectively. We found that ligation of cells with sCD40L significantly increased the binding of nuclear protein(s) to the NF-κB–HO-1 promoter sequence in a time-dependent manner (Figure 4B). This specific protein–DNA complex formation in both untreated and sCD40L-treated cells was competed away with 100-fold molar excess of NF-κB consensus oligonucleotide, confirming that the CD40-induced nuclear protein binding to the HO-1 promoter sequence is NF-κB (Figure 4, B and C). In contrast, there was no significant change in CD40-induced binding of nuclear proteins to the AP-2–HO-1 promoter sequence (data not shown). These data suggest that promoter binding of NF-κB is of importance in CD40-induced transcriptional activation of HO-1 in RPTEC.

**Discussion**

The expression of CD40 is prominent on many cell types during inflammation, including renal endothelial cells and tubular epithelial cells (16,17). During tubulointerstitial inflammation, infiltrating CD4+ T cells and platelets expressing CD40L are closely approximated with CD40-expressing renal tubular epithelium (6,17,20,21). In this study, we have shown that CD40 signals can mediate inflammatory as well as anti-inflammatory signals in RPTEC. We found that despite generating ROS in RPTEC, CD40 ligation can also promote the expression of the protective gene HO-1 in part through the activation of NF-κB.
ROS are important regulators of cell death and apoptosis, and the role of oxidant stress in necrosis is well established (45). ROS, such as superoxide, H$_2$O$_2$, and hydroxyl radical, have been implicated as important effectors of necrotic cell death after ischemic and toxic injury to RPTEC (46). Consistent with our observations in RPTEC, Lee et al. (47) demonstrated that CD40 engagement on B lymphocytes also generates ROS, which promotes c-Jun N-terminal kinase activation and IL-6 secretion. Urbich et al. (24) reported that CD40L inhibits EC migration by increasing production of endothelial ROS; however, the inhibitory effect of CD40L was not related to the induction of apoptosis or the blockade of cell-cycle progression.

In this study, we have shown that although CD40 ligation in RPTEC generates ROS, the cells do not undergo apoptosis. In contrast, H$_2$O$_2$-induced ROS can induce apoptosis in RPTEC. These findings suggest that CD40 signals may induce the activation of a cytoprotective pathway.

HO-1 has been shown to be an important protective gene in the kidney and is known to be expressed by tubular epithelial cells (40). Hypoxia, heat-shock proteins, and other factors are known to induce HO-1 expression (35,48); however, to our knowledge, very few studies have described immune-mediated induction of HO-1. Here we have shown that some tubules in rejecting kidneys express high levels of CD40 as well as HO-1.
as compared with normal renal tubules. Moreover, some portions of the same tubule co-express both of these molecules. We have found that induction of HO-1 in RPTEC can inhibit ROS-induced apoptosis. However, we have also observed that inhibition of HO-1 by its selective inhibitor can promote some apoptosis in RPTEC after sCD40L treatment (data not shown). This suggests that in selected tubules in vivo during inflammation, HO-1 may be functional as an antiapoptotic protein. Some reports have suggested that CD40 activation may stimulate the expression of protective genes in other cell types (28,29). Recently, Zazzeroni et al. (28) reported that Gadd45β is induced by CD40 and that this induction suppressed Fas-mediated killing in B cells. Hess et al. (49) showed that CD40 induces resistance to TNF-mediated apoptosis in a fibroblast cell line. Here,
we have demonstrated that CD40 ligation in RPTEC induces the transcriptional activation as well as the protein expression of HO-1, partly through ROS generation. We observed that although there was some increase in ROS generation by 6 h after sCD40L treatment, it takes 16 to 20 h to express HO-1. This suggests that CD40-induced and ROS-mediated HO-1 expression may involve activation of some intermediary molecule.

The analysis of human HO-1 promoter sequence revealed the presence of binding sites for the transcription factors NF-κB and AP-2 adjacent to the transcription start site (44). The finding of NF-κB and AP-2 binding sites on the human HO-1 promoter suggests that HO-1 will be induced in processes in which these transcription factors are activated (e.g., after CD40-CD40L interactions). It is also possible that differential activation of transcription factors in response to CD40 ligation may dictate differences in cell fate. Ahmed-Choudhury et al. (29) showed that CD40 signals induce a transient activation of NF-κB but that sustained AP-1 activation is associated with apoptosis in hepatocytes. In contrast, prolonged NF-κB activation and lack of AP-1 activation in intrahepatic EC result in proliferation. Here, we found that CD40 ligation induces the binding of NF-κB to the HO-1 promoter sequence; however, the binding of AP-2 remained unaltered (data not shown). If CD40 induces a transient NF-κB-mediated HO-1 expression, then these findings are suggestive that the HO-1 protective response will also be transient. Together, we suggest that the early transient expression of HO-1 is of importance in cytoprotection. We are currently evaluating whether there are other protective genes (e.g., Gadd45α, described above [28]) in RPTEC that may be functional at later times to protect against chronic inflammatory processes.

In summary, CD40 signals have bifunctional roles in RPTEC to generate both proinflammatory ROS and anti-inflammatory HO-1 expression. Regulating the balance between proinflammatory and anti-inflammatory processes via CD40-induced HO-1 may be of importance in the treatment of inflammatory renal diseases.

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