Endoplasmic reticulum stress in glomerular epithelial cell injury

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Cybulsky AV, Takano T, Papillon J, Kitzler TM, Bijian K. Endoplasmic reticulum stress in glomerular epithelial cell injury. Am J Physiol Renal Physiol 301: F496–F508, 2011.—Focal segmental glomerulosclerosis (FSGS) may be associated with glomerular epithelial cell (GEC; podocyte) apoptosis due to acquired injury or mutations in specific podocyte proteins. This study addresses mediation of GEC injury, focusing on endoplasmic reticulum (ER) stress.

We studied signaling in cultured GECs in the presence or absence of the extracellular matrix (ECM). Adhesion to collagen supports cell survival, but adhesion to plastic (loss of contact with ECM) leads to apoptosis. Compared with collagen-adherent cells, GECs on plastic showed increased protein misfolding in the ER, and an adaptive-protective ER stress response, including increased expression of ER chaperones, increased phosphorylation of eukaryotic translation initiation factor-2α (eIF2α), and a reduction in protein synthesis. Activation of these ER stress pathways counteracted apoptosis. However, tunicamycin (a potent stimulator of ER stress) changed the ER stress response from protective to cytotoxic, as tunicamycin induced the proapoptotic ER stress gene, C/EBP homologous protein-10, and exacerbated apoptosis in GECs adherent to plastic, but not collagen. In GECs adherent to plastic, adaptive ER stress was associated with an increase in polyubiquitinated proteins and “choking” of the proteasome. Furthermore, pharmacological inhibition of the proteasome induced ER stress in GECs. Finally, we show that ER stress (induction of ER chaperones and eIF2α phosphorylation) was evident in experimental FSGS in vivo. Thus interactions of GECs with ECM may regulate protein folding and induction of the ER stress response. FSGS is associated with induction of ER stress. Enhancing protective aspects of the ER stress response may reduce apoptosis and possibly glomerulosclerosis.

Apoptosis; extracellular matrix; glomerulonephritis; podocyte; ubiquitin-proteasome system

Adhesion of cells to the extracellular matrix (ECM) can facilitate cell survival, modulate proliferative responses to polypeptide growth factors, and promote differentiation. Thus cell homeostasis reflects a balance of proliferation, apoptosis, and differentiation (16, 17, 23). Apoptosis may be triggered by inadequate ECM-cell contact, insufficient exposure to growth factors, disruption of the cytoskeleton, and other factors (16).

The pathways that regulate cell survival may involve adhesion receptors, including integrins and protein kinases, such as focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), as well as multiple downstream effectors, including Bcl-2 family proteins, caspases, and others (6, 16–18, 20, 21, 23, 28). For example, FAK plays a major role in mediating signals emanating from ECM via β1-integrins, and FAK may link integrin signals to the Ras-ERK pathway (6, 20).

Exposure of cells to environmental stress increases expression of stress proteins in cellular compartments, such as the endoplasmic reticulum (ER). Normally, secretory, luminal, and membrane proteins attain their correctly folded conformation in the ER via ER-resident enzymes and chaperones. To rescue misfolded proteins, the ER has in place quality control machinery, including the unfolded protein response (UPR) (24, 40, 41, 52, 53). Upon accumulation of misfolded proteins in the ER, activating transcription factor-6 (ATF6) moves to the Golgi and is cleaved by proteases. The cleaved cytosolic fragment migrates to the nucleus to activate transcription of ER stress proteins, e.g., the glucose-regulated proteins (grp), grp94 and bip (grp78), and others. In parallel, inositol requiring-1 activates its endoribonuclease activity, cleaving X-box binding protein-1 (Xbp1) mRNA and changing the reading frame to yield a potent transcriptional activator. Normally, ER stress proteins serve as protein chaperones for exocytosis from the ER and may complex with defective proteins to target them for degradation. During stress, the induction of ER chaperones may limit accumulation of abnormal proteins. Another aspect of the UPR involves PERK (PKR-like ER kinase), which is activated to phosphorylate the eukaryotic translation initiation factor-2α subunit (eIF2α). This process reduces initiation AUG codon recognition; thus the general rate of translation is reduced (which decreases the protein load on a damaged ER). The UPR may allow cells to recover from stress and may be protective toward additional insults, but substantial/prolonged ER stress may lead to apoptosis via induction of specific genes, e.g., C/EBP homologous protein-10 (CHOP; also known as GADD153), and/or activation of caspase-12 or protein kinases (24, 33, 40, 50–52).

Glomerular visceral epithelial cells (GECs) or podocytes are intrinsic components of the kidney glomerulus and play a key role in the maintenance of glomerular permselectivity (29, 32, 37, 43). Under normal conditions, podocytes are in contact with ECM (i.e., glomerular basement membrane) and are exposed to trivial concentrations of growth factors, and there appears to be little turnover of podocytes. Glomerular permselectivity is dependent on the maintenance of the appropriate structure of podocytes and the filtration slit diaphragms, including nephrin, a key component of the slit diaphragm. Certain forms of glomerulonephritis are associated with podocyte injury, which may lead to apoptosis or proliferation, detachment from the underlying glomerular basement membrane, impaired glomerular function or permselectivity (proteinuria), and, in association with ECM, expansion to glomerulosclerosis. Podocyte injury occurs in human focal segmental glomerulosclerosis (FSGS), in the context of ECM expansion (49). Familial forms of FSGS, associated with mutations in podocyte structural proteins further support the view that FSGS is a disease of the podocyte (29, 37, 43, 46). FSGS may feature podocyte apoptosis, or detachment, as well as proliferation; the latter is observed in the collapsing variant of FSGS, where the proliferating cells appear to be podocytes with a dysregulated phenotype and loss of the p27 cyclin-dependent kinase inhib-
itor (38, 49). Based on studies in experimental animal models, there is evidence that in FSGS, podocyte apoptosis may lead to “podocyteptenia” and consequently glomerulosclerosis (26, 30, 38, 47, 49). Detachment of podocytes from the glomerular basement membrane also seems to be an important event underlying formation of the sclerotic lesions seen in FSGS (38, 48). Alterations in expression of podocyte structural proteins and filtration slit diaphragm components have also been reported (29, 37, 43).

To address the mechanisms of apoptosis/cell survival in FSGS, we have previously used an experimental model where signaling was studied in cultured rat GECs in the presence or absence of ECM and integrin engagement (10, 11). We have shown that adhesion of GECs to collagen activated FAK, c-Src, and the Ras-ERK pathway and facilitated survival (prevented apoptosis), but in the absence of ECM (i.e., in GECs adherent to plastic substratum), GECs underwent apoptosis (2). GECs adherent to plastic exhibited increased caspase-8, -9, and -3 activities, increased expression of the proapoptotic protein Bax, and decreased antiapoptotic protein Bcl-X, compared with collagen. Stable expression of constitutively active mutants of Ras, FAK (CD2-FAK), or mitogen-activated protein kinase kinase (R4F-MEK) activated the ERK pathway and supplanted the requirement of collagen for survival. In contrast, expression of a Ras mutant that activates phosphatidylinositol 3-kinase, but blocks ERK activation, or pharmacological inhibition of the ERK pathway decreased survival on collagen (2). Moreover, expression of a mutant form of α-actinin-4 (which is associated with FSGS in humans) increased apoptosis of GECs (3). In vivo, glomeruli isolated from rats with experimental FSGS (puromycin aminonucleoside nephrosis) revealed activation of signaling analogous to our cell culture model, i.e., increased β1-integrin expression, along with increased activation of FAK, c-Src, and ERK (2). Together, these changes suggested that in experimental FSGS, there may be altered signaling from the ECM to podocytes, but precisely how such signals regulate podocyte apoptosis requires further study.

The aim of the present study was to further address the signals that regulate GEC injury in vivo and in culture, focusing on the role of the UPR. We demonstrate that in a GEC culture model, loss of contact with ECM induced an adaptive-protective UPR, and in addition, these GECs became responsive to exogenous enhancement of ER stress, which changed the ER stress response from protective to cytotoxic. Glomerular ER stress was evident in an experimental glomerulonephritis model of FSGS. Enhancement of the protective aspects of the UPR may reduce apoptosis.

**MATERIALS AND METHODS**

**Materials.** Tissue culture reagents and Lipofectamine 2000 were obtained from Invitrogen (Burlington, ON). Pepsin-solubilized bovine dermal collagen I (Vitrogen) was from Cohesion (Palo Alto, CA). Tunicamycin, MG132, 4-[2-aminoethyl]benzenesulfonylfluoride (AEBSF), and rabbit anti-ubiquitin antisera were obtained from Sigma-Aldrich Canada (Mississauga, ON). Salubrinal was purchased from Chembridge (San Diego, CA). Bisbenzimide H33342 fluorochrome and propidium iodide were obtained from Calbiochem (La Jolla, CA). N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) was from Biomol (Plymouth Meeting, PA). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, ON) and Amersham Biosciences (Baie d’Urfé, QC). [35S]methionine/cysteine (1,000 Ci/mmol) was purchased from Biosource International (Camarillo, CA). Rabbit anti-β-actin, anti-grp78, anti-Cox-4, and anti-β-actin antibodies were from Abcam (Cambridge, MA). Rabbit anti-caspase-3 antibody was from Cell Signaling (Beverly, MA). Rabbit anti-CHOP, rabbit anti-caspase-12, and rabbit anti-eIF2α antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cytochrome c antibody was from BD Biosciences (Mississauga, ON). Mouse anti-green fluorescent protein (GFP) antibody, rabbit anti-calnexin, rabbit anti-calreticulin, and rabbit anti-bim antibodies were from Assay Designs (Ann Arbor, MI). Mouse anti-green fluorescent protein (GFP) antibody, rabbit anti-CHOP, rabbit anti-caspase-12, and rabbit anti-eIF2α antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-cytochrome c antibody was from BD Biosciences (Mississauga, ON). pEGFP-C1 was kindly provided by Dr. Ron Kopito (Stanford University, Stanford, CA) (1).

**Extracellular matrix, cell culture, and transfection.** Type I collagen gel matrices were prepared as described previously (10). For practical reasons, collagen I was used in all experiments in this study, but in previous studies several key observations were confirmed using collagen IV substrata (2, 10). Primary cultures of rat GECs (“parental” GECs) were established from explants of rat glomeruli and have been characterized previously (8, 10). Unlike some other GEC culture lines, the cells used in the present study do not contain the SV40 large T antigen and thus, in our view, are better suited for studies of cell signaling. The cells used in the present study do not contain the SV40 large T antigen and thus, in our view, are better suited for studies of cell signaling. 

**Induction of glomerulonephritis in mice.** The mouse glomerulonephritis model with podocyte injury and features of FSGS was characterized previously (25). Glomerulonephritis was induced in male CD mice (~35 g; Charles River, St. Constant, QC) by two intraperitoneal injections of sheep anti-rabbit glomerular basement membrane (0.5 ml/20 g body wt on days 1 and 2), which was kindly provided by Dr. Stuart Shankland (University of Washington, Seattle, WA). Urine was collected on day 14, the mice were then euthanized, and glomeruli were isolated by differential sieving (13, 14). Other mice were injected with tunicamycin (1 mg/kg ip), and glomeruli were isolated after 24 h. In addition, some mice were injected with tunicamycin, followed by sheep anti-rabbit glomerular antiserum after 24 and 48 h. The purity of glomerular isolation was ~65%. The studies were approved by the McGill University Animal Care Committee. For histological analysis, mouse kidney tissue was fixed in formalin and embedded in paraffin. Sections were subjected to periodic acid-Schiff staining and were then examined by light microscopy.

**Immunoblotting.** Cells lysates were boiled in Laemmli sample buffer and subjected to SDS-PAGE under reducing conditions, as detailed previously (2). Equal amounts of proteins were loaded into each lane of the gel. Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 5% milk, and incubated with primary antibody, and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminescence technique (ECL). Protein content was quantified by scanning densitometry, using National Institutes of Health ImageJ software (2, 12, 14). Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

**Assays of cell viability.** Staining with Hoechst H33342 dye was used to quantify apoptosis, as described previously (2). Briefly, adherent cells were stained with H33342 (1 μg/ml) for 10 min at 37°C.
without fixation. After washing with PBS, cells were stained with propidium iodide (5 μg/ml) to identify necrotic or late apoptotic cells. Cells were examined using a Nikon Diaphot fluorescence microscope, and the number of H33342- and propidium iodide-positive cells was quantified by visual counting. H33342-positive cells (i.e., with condensed and/or fragmented nuclei) without propidium iodide staining were defined as apoptotic. Propidium iodide-positive cells were defined as late apoptotic (2). Lactate dehydrogenase (LDH) release was measured as described previously (14).

[35S]methionine/cysteine incorporation and immunoprecipitation of calnexin-associated proteins. To monitor protein synthesis, cells were washed with methionine/cysteine-free DMEM and were then incubated with methionine/cysteine-free DMEM containing [35S]methionine/cysteine (50 μCi/ml) for 20 min. After washing, cells were scraped from culture plates, centrifuged, and suspended in 0.5 ml of a solution of BSA, 1.0 mg/ml. After addition of 20% trichloroacetic acid, cells were vortexed and incubated for 30 min on ice. The mixture was filtered through a glass microfiber disk. The disk was dried, and the radioactivity was measured in a β-scintillation counter.

For immunoprecipitation of calnexin-associated proteins, cells were radiolabeled with [35S]methionine/cysteine (50 μCi/ml) for 24 h. Cells were then replated onto collagen or plastic and were chased with “cold” methionine and cysteine for 3 or 6 h. Lysates were immunoprecipitated with anti-calnexin antibody. Immune complexes were incubated with agarose-coupled protein A. Complexes were boiled in Laemml sample buffer and subjected to SDS-PAGE and fluorography (36).

Proteasome activity assays. Cells were scraped from culture dishes into buffer containing 50 mM Tris-HCl (pH 7.50), 250 mM sucrose, 5 mM MgCl₂, 0.5 mM EDTA, 2 mM ATP, and 1 mM DTT (4°C). After sonication, cells were centrifuged for 10 min at 10,000 g. Supernatants (30–50 μg protein in 50 μl) were mixed with 50 μl of 50 mM Tris-HCl (pH 7.50), 40 mM KCl, 5 mM MgCl₂, 0.5 mM ATP, 1 mM DTT, 0.5 mg/ml BSA, and 0.2 mM Suc-LLVY-AMC (chymotrypsin-like activity) (27). Proteasomal activity was measured in a spectrofluorometer after 30-min incubation at 37°C. The excitation and emission wavelengths were 360 and 460 nm, respectively. Preliminary studies demonstrated that in this assay, pretreatment of cells with the proteasome inhibitor MG132 inhibited chymotrypsin-like activity by ~80%, indicating that the large majority of the activity is associated with the proteasome.

Statistics. Data are presented as means ± SE. The t-statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t-statistic and adjusting the critical value according to the Bonferroni method. Albuminuria data were analyzed using the Wilcoxon rank sum test.

RESULTS

Loss of contact with ECM induces adaptive-protective ER stress in cultured GECs. We have previously employed an experimental model where signaling has been studied in cultured rat GECs in the presence or absence of ECM and integrin engagement (10, 11). Loss of contact with ECM (i.e., in GECs adherent to plastic substratum) leads to an increase in activities of caspases and apoptosis (indicators of GEC injury), which may be analogous to GEC detachment or alterations in adhesion signaling in vivo, features observed in experimental FSGS (48). Using this culture model, we have shown that adhesion of GECs to collagen activates FAK and the Ras-ERK pathway and supports survival (prevents apoptosis), but in the absence of ECM these kinase pathways are inactive (2). In the present study, we examined whether ECM influences the activation of stress pathways in GECs, focusing on the UPR. Resting para-

rental GECs adherent to collagen express the ER chaperone proteins bip (grp78) and grp94 at relatively low levels (Fig. 1A). In contrast, the endogenous levels of bip and grp94 were increased significantly in parental GECs adherent to plastic (Fig. 1, A and B), indicating that loss of contact with ECM induces the UPR. The increases in bip and grp94 expression in GECs adherent to plastic substratum (Fig. 1, A and B) are presented at 18 h after plating of cells. Changes in bip expression were also studied at 3 and 6 h after plating, but the changes were not significant at these time points (data not shown). By analogy to bip and grp94, expression of the ER lectin chaperone calreticulin (9) was also increased in GECs adherent to plastic compared with collagen (Fig. 1, A and B).

Another aspect of the UPR involves phosphorylation of eIF2α on serine51. This process reduces initiation AUG codon recognition, thereby reducing the general rate of translation (which aims at decreasing the protein load on a damaged ER). Compared with collagen, GECs adherent to plastic showed higher levels of phosphorylated eIF2α, although there were no significant changes in total eIF2α (Fig. 1, C and D). In keeping with increased eIF2α phosphorylation, there was a reduction in [35S]methionine/cysteine incorporation in GECs adherent to plastic, reflecting reduced protein synthesis (Fig. 1F).

The UPR is known to be induced by an accumulation of misfolded proteins in the ER (24, 40, 52). Thus we examined whether protein misfolding was increased in cells adherent to plastic by monitoring the association of proteins with the lectin chaperone calnexin. Calnexin mediates protein folding in the ER, and misfolded proteins are bound to calnexin to undergo proper folding (9, 36). Proteins were metabolically labeled with [35S]methionine/cysteine. Cells were plated onto collagen or plastic and were then chased with “cold” methionine and cysteine. After 3 or 6 h, lysates were immunoprecipitated with anti-calnexin antibody and were subjected to SDS-PAGE and fluorography. The bands on the fluorogram (Fig. 1F) represent calnexin-associated proteins, i.e., misfolded proteins which bind to calnexin while attempting to fold properly. As these misfolded proteins continue to fold, the radioactivity of calnexin-associated proteins declines over time (during the chase). At 3 and 6 h, there was enhanced association of proteins with calnexin in GECs adherent to plastic (Fig. 1F), reflecting protein misfolding, which likely caused the UPR.

FAK is an important mediator of signals elicited by adhesion of cells to ECM. Previously, we demonstrated that GECs stably transfected with a constitutively active FAK (CD2-FAK) were able to survive when adherent to plastic substrata; i.e., CD2-FAK supplanted the requirement of ECM for survival (2). In the present study, we demonstrate that basal expression of bip was low in CD2-FAK-transfected GECs (on plastic) and was comparable to parental GECs adherent to collagen (Fig. 2A). The levels of grp94 paralleled those of bip in parental GECs adherent to collagen or plastic and in CD2-FAK GECs (Fig. 2B). Similarly, phosphorylation of eIF2α in CD2-FAK GECs was low and did not significantly differ from parental GECs on collagen (Fig. 2C). Thus, unlike the parental GECs adherent to plastic, the UPR was not enhanced in the CD2-FAK GECs. In contrast to the pattern of ER stress induction, expression of the cytosolic stress protein heat shock protein (Hsp) 70 was not detectable in parental GECs adherent to collagen or plastic, and Hsp70 was not expressed in CD2-FAK GECs. Thus adhesion
to ECM modulated ER stress, but not cytosolic stress pathways.

Although phosphorylation of eIF2α is associated with a global decrease in translation, certain mRNAs may be translated preferentially after eIF2α is phosphorylated. Among these is activating transcription factor-4 (ATF4), which in-...
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In the next set of experiments, we tested the functional role of the UPR in apoptosis. Induction of ER chaperones (e.g., bip, grp94) is dependent on the cleavage of ATF6 by the site-1 and site-2 proteases. AEBSF is a specific inhibitor of the site-1 protease (35), and our preliminary studies in GECs demonstrated that AEBSF attenuated induction of bip by an exogenous stimulator of ER stress, tunicamycin (results not shown). Incubation of GECs on plastic with AEBSF increased apoptosis significantly (Fig. 4B), implying that enhanced expression of ER chaperones in these cells was antiapoptotic or protective. AEBSF also produced a small additional increase in apoptosis in GECs adherent to plastic (Fig. 4C), suggesting that in collagen-adherent cells, expression of ER chaperones also imparted cytoprotection, even though the basal expression of ER chaperones was relatively low, compared with GECs on plastic (Figs. 1 and 2).

We then examined the role of eIF2α in apoptosis. Incubation of parental GECs with salubrinal, a selective inhibitor of eIF2α dephosphorylation (4), produced a small additional increase in eIF2α phosphorylation in GECs adherent to plastic (Fig. 4C) and D). Interestingly, when GECs adherent to plastic were incubated with salubrinal, apoptosis was reduced to levels observed in GECs adherent to collagen (Fig. 4A). This result implies that apoptosis associated with loss of contact with ECM could be attenuated after enhancement of a protective aspect of the UPR, i.e., enhanced signaling via the eIF2α pathway.

Tunicamycin-induced ER stress and apoptosis. Results of the above studies suggested that enhanced expression of ER chaperones and phosphorylation of eIF2α are protective against apoptosis induced by loss of contact with ECM. However, the functional outcome of ER stress is complex and may depend on the amount of stress that cells are exposed to. In these experiments, we examined the effect of potent ER stress using tunicamycin. [Tunicamycin, which blocks N-linked glycosylation and causes an accumulation of misfolded proteins in the ER, is one of the most potent inducers of ER stress (52).] Incubation of collagen-adherent parental GECs with tunicamycin enhanced the expression of bip (Fig. 1, A and B) to approximately the same levels as seen in the resting GECs adherent to plastic. In addition, tunicamycin further increased bip expression in the GECs adherent to plastic (Fig. 1, A and B). The tunicamycin-induced increases in bip expression are presented at 18–24 h after plating of cells (Fig. 1, A and B), but they were also evident at 12 h (data not shown). Tunicamycin also induced comparable changes in the expression of grp94 (Figs. 1A and 2B).

Phosphorylation of eIF2α may be associated with induction of certain genes, including the proapoptotic gene CHOP. Tunicamycin induced significant expression of CHOP only in the parental GECs adherent to plastic (Fig. 3, A and B). In GEC adherent to collagen, induction of CHOP by tunicamycin was trivial and inconsistent and was not statistically significant (Fig. 3, A and B). This finding was distinct, compared with the effect of tunicamycin on ER chaperones. Thus significant induction of CHOP occurred only if there was loss of contact of GECs with ECM.

Caspase-12 is an ER-associated caspase, which may play a role in ER stress-induced apoptosis. Parental GECs adherent to plastic demonstrated a slight increase in caspase-12 cleavage (which reflects activation), compared with parental GECs adherent to collagen, and CD2-FAK GECs (Fig. 3, C and D). This level of activation was, however, trivial, compared with the stimulatory effect of tunicamycin, which induced a robust increase in the cleaved product (Fig. 3D).
In the next set of experiments, we addressed the effect of tunicamycin on apoptosis. Incubation of GECs with tunicamycin markedly exacerbated apoptosis and induced late apoptosis primarily in GECs adherent to plastic, as monitored by propidium iodide staining and LDH release (Fig. 4E). These effects correlated with the induction of CHOP (Fig. 3, A and B). Furthermore, release of cytochrome c paralleled the effect of ECM and tunicamycin on apoptosis (Fig. 4, F and G), implying that the mitochondrial apoptotic pathway was activated in these cells secondary to ER stress. Together, these results suggest that in resting GEC, apoptosis is triggered by lack of contact with ECM, but, simultaneously, there is enhanced expression of ER chaperones, phosphorylation of eIF2α, and reduction in protein synthesis, which attenuate apoptosis. In contrast, more sustained and potent ER stress (i.e., tunicamycin in the absence of ECM) leads to CHOP induction and greatly exacerbates apoptosis.

Ubiquitination of proteins, proteasome function, and ER stress. In these experiments, we investigated whether induction of the UPR in GECs was associated with changes in ubiquitination of proteins and proteasome function (15, 44). Adhesion of parental GECs to plastic resulted in an increase in the overall polyubiquitination of proteins (Fig. 5, A and B), suggesting that these cells are predisposed to an accumulation of misfolded or abnormal proteins. Next, we hypothesized that the misfolded proteins are degraded via the ubiquitin-proteasome pathway, but due to the abundance of misfolded proteins, the pathway may become overwhelmed and functionally impaired. Such “choking” of the ubiquitin-proteasome system is reported to increase ER stress (24, 51). The function of the ubiquitin-proteasome system was monitored by a reporter consisting of a short degron, CL1, fused to the C terminus of GFP (GFPU) (13). GFPU expression is the product of its ongoing translation and degradation; however, GFPU is rapidly degraded when ubiquitin-proteasome function is normal, and expression of GFPU is low. Impairment of the ubiquitin-proteasome system, e.g., via enhanced flux of misfolded protein(s), will reduce degradation of GFPU, resulting in an increased level of GFPU expression (1). GECs were transiently transfected with GFPU. After cells were plated on collagen, GFPU declined at 6 h, with a further substantial decline at 24 h (Fig. 5, A and B). Compared with GECs adherent to collagen, adhesion to plastic tended to enhance GFPU expression at 6 h and significantly enhanced GFPU expression at 24 h, indicating that degradation of GFPU by the ubiquitin-proteasome system was impaired (Fig. 5, A and B). In an earlier study (13), we showed that GFPU degradation was blocked by the proteasome inhibitor MG132, indicating that the reduction in the GFPU level is actually due to proteasomal degradation.

Accumulation of polyubquitinated proteins could reflect a defect in 20S proteasomal subunit peptidase activity. Thus we monitored the chymotrypsin-like activity of the 20S protea-
some using an in vitro assay. Chymotrypsin-like activity was actually enhanced in GECs adherent to plastic compared with collagen (Fig. 5C), indicating that reduced degradation of GFP was not due to a defect in 20S proteasomal peptidase activity. Finally, we demonstrated that incubation of GECs with the proteasome inhibitors MG132 and lactacystin can actually lead to upregulation of bip and CHOP expression (Fig. 5D). These results confirm that proteasome inhibition can enhance ER stress in GECs.

Effect of ECM on ER stress in other kidney cell lines. In the above experiments, it was shown that ER chaperones, including grp94, were increased in GECs adherent to plastic compared with collagen, whereas CHOP was not detectable in GECs adherent to either collagen or plastic substrata, but could...
be induced significantly with tunicamycin in the GECs adherent to plastic (Figs. 1, A and B, and 3, A and B). To determine whether this pattern of ER stress induction is unique to GECs, we examined expression of grp94 and CHOP in COS-1 and HEK-293T kidney cell lines. These cell lines do not undergo apoptosis on plastic substratum, and in this respect are distinct from GECs. Unlike GECs, in unstimulated COS-1 and 293T cells, there were no significant differences in grp94 expression between cells adherent to collagen and plastic substrata (Fig. 6). CHOP expression was trivial in both cell lines under resting conditions. Tunicamycin stimulated grp94 and CHOP in COS-1 and 293T cells, and the effect of tunicamycin was of similar magnitude on both substrata (Fig. 6). In summary, the pattern of ER stress induction in COS-1 and 293T cells appears to be distinct from GECs.

**ER stress is evident in experimental glomerulonephritis.** FSGS may feature podocyte apoptosis, detachment, or proliferation. To determine whether GEC injury in vivo is associated with induction of the UPR, we examined ER stress in a model of experimental glomerulonephritis, produced by injecting mice with sheep antibody to rabbit glomeruli (25). This model was originally described in normal and in cyclin-dependent kinase inhibitor p21 knockout mice, and it showed several features of collapsing FSGS, including podocyte injury, increased ECM in glomeruli, segmental or global glomerulosclerosis, capillary collapse, and GEC proliferation (25). These morphological abnormalities were evident in normal and p21 null mice, but were more severe in the latter. In addition, this model of FSGS was associated with an increase in glomerular cell apoptosis (visualized by terminal transferase dUTP-nick-end labeling staining) (25). We induced glomerulonephritis in normal mice, and for comparison we also treated another group of normal mice with tunicamycin. Expression of the ER chaperones bip and grp94 as well as eIF2α phosphorylation were increased significantly in glomeruli isolated from mice with glomerulonephritis compared with control (Fig. 7, A and B). Treatment of normal mice with tunicamycin resulted in significant increases in ER chaperones and eIF2α phosphorylation (Fig. 7A). In contrast to the pattern of ER stress induction, expression of the cytosolic stress protein Hsp70 was not detected in neither nephritic nor normal mouse glomeruli (Fig. 7C). Induction of CHOP was detected only in normal mice...
treated with tunicamycin, but not in mice with glomerulonephritis, or controls (Fig. 7A). Although caspase-12 was detectable in cultured GECs, we were not able to detect expression of caspase-12 in mouse glomeruli by immunoblotting.

Most of the nephritic mice showed modest to moderate albuminuria, including one mouse with severe albuminuria (Fig. 8, A and B). There was no apparent correlation between the amount of change in the expression of ER chaperones or eIF2α phosphorylation and the degree of albuminuria. Although treatment of normal mice with tunicamycin induced ER stress, tunicamycin did not induce albuminuria (Fig. 8B). Moreover, treatment with tunicamycin before the administration of an anti-glomerular antibody resulted in the appearance of severe albuminuria in three of seven mice (Fig. 8B), but due
to the large variability in albuminuria in this model of glomerulonephritis, we cannot conclude unequivocally that tunicamycin changed the amount of albuminuria significantly. Histological examination of kidneys from mice with glomerulonephritis confirmed the presence of occasional FSGS lesions (Fig. 8C). In summary, in this mouse model of FSGS, ER chaperones and eIF2α phosphorylation were increased. Induction of CHOP required more potent ER stress, induced by tunicamycin in normal mice. These results are similar to those observed in cultured GECs, which were not in contact with ECM.

**DISCUSSION**

The development of glomerular scarring in glomerular disease is linked with podocyte injury. The causes of podocyte injury may include apoptosis of podocytes, detachment of podocytes from the glomerular basement membrane, and aberrant podocyte proliferation. These potential causes of podocyte injury are present in varying degrees in FSGS (29, 37, 43, 46, 49). After parietal epithelium meets denuded areas of the glomerular basement membrane created by the loss of podocytes, a sequence of events begins, which leads to formation of an adhesion and eventually a scar. Podocytopenia is triggered by podocyte injury. Acquired forms of podocyte injury may be associated with immunological factors (e.g., T cell factors affecting permeability), oxidants, human immunodeficiency virus, toxins, and other substances. FSGS may also be associated with heritable mutations in several distinct proteins that play key roles in maintaining ultrastructure of the slit diaphragm or podocyte cytoskeleton. To better understand the mechanisms of ER stress induction in podocyte injury, we employed an experimental culture model where signaling is studied in GECs in the presence or absence of ECM and integrin engagement. This model recapitulates aspects of the podocyte in FSGS (48), including the presence of apoptosis and detachment from ECM, and thus facilitates study of signaling mechanisms associated with adhesion, which may be relevant to disease pathogenesis (10, 11). In the present study, we show that loss of contact with ECM activated the UPR in parental GECs. In resting GECs adherent to plastic, there was increased protein misfolding in the ER, and there were increases in the expression of ER chaperones, greater eIF2α phosphorylation, as well as a reduction in [35S]methionine/cysteine incorporation, reflecting reduced protein synthesis (Fig. 1). Moreover, activation of FAK (CD2-FAK-expressing cells), which supplants adhesion of cells to collagen (2), abolished the induction of ER chaperones (Fig. 2).

Bip and grp94 are chaperones involved in normal posttranslational processing of proteins in the ER. Under conditions of stress, where the amount of misfolded protein in the ER increases, induction of the UPR, including the increased production of bip and grp94, may enhance protein-folding capacity. In parallel, phosphorylation of eIF2α by PERK reduces initiation AUG codon recognition, thereby reducing the general rate of translation, which aims at decreasing the protein load on a damaged ER (24, 40, 52). These aspects of the UPR are typically cytoprotective (24, 40, 52), and based on these results it is reasonable to propose that induction of the UPR in GECs limits apoptosis. Indeed, inhibition of ATF6 processing, which attenuates increases in ER chaperones, was shown to enhance apoptosis, implying that induction of ER chaperones in GECs was antiapoptotic (Fig. 4).

During ER stress, the total amount of eIF2α phosphorylation is dependent on both PERK-mediated phosphorylation and dephosphorylation. eIF2α is dephosphorylated by a complex containing the serine/threonine phosphatase PP1 and its nonenzymatic cofactor GADD34 or Crep (a GADD34 homolog) (4). Salubrinal is a specific inhibitor of the protein complex that dephosphorylates eIF2α, and therefore, addition of salubrinal to cells would enhance signaling via the eIF2α pathway (4). We confirmed that in GECs adherent to plastic, salubrinal further enhanced eIF2α phosphorylation, and we showed that salubrinal reduced apoptosis in these GECs (Fig. 4). Most likely, apoptosis was diminished via reduction of translation, which decreases the protein load on a damaged ER. Alternatively, the ER stress-induced increase in the expression of chaperones (e.g., bip) has also been reported to depend on eIF2α phosphorylation.
activated downstream of inositol requiring-1 (vaccination) and caspase-12; UPR does have some limitations. For example, other potential apoptotic mediators of ER stress, including CHOP mRNA stability determines the outcome of the UPR. The GECs adherent to plastic also demonstrated responsiveness to induction of ER stress-associated proapoptotic pathways by tunicamycin. Thus induction of CHOP, apoptosis, and age-related apoptosis were markedly exacerbated by tunicamycin only in GECs adherent to plastic (Figs. 3 and 4), indicating that tunicamycin most likely converted a protective ER stress response in the resting GECs to a proapoptotic response. Tunicamycin also induced release of cytochrome c only in GECs on plastic (Fig. 4), suggesting that the mitochondrial apoptotic pathway had been secondarily activated by ER stress in these cells (18, 19, 28). CHOP may facilitate cell death by promoting protein synthesis and oxidation in a stressed ER, by altering gene transcription, or by other mechanisms that remain to be defined (40, 50, 52).

Why some types of ER stress are associated with cytoprotection, whereas others lead to apoptosis is not well established. One study showed that after stimulation of cells with low doses of compounds that induce ER stress, mRNAs for Bip and CHOP were induced, but while the Bip mRNA was stable and persisted, the CHOP mRNA was unstable and decayed (42). In this context, the UPR was cytoprotective. After stimulation with the same compounds at high doses, CHOP mRNA became more stable, and consequently, the UPR may become proapoptotic. Our results in GECs are consistent with this model. Thus loss of contact with ECM resulted in upregulation of Bip and grp94, but not CHOP, in resting GECs, whereas introduction of an additional potent ER stress to these cells (i.e., tunicamycin) resulted in the induction of CHOP. It should also be noted that in GECs, the effect of salubrinal on Bip mRNA phosphorylation was modest (Fig. 4). Under these conditions, salubrinal did not enhance the expression of CHOP (data not shown), and salubrinal was cytoprotective (Fig. 4). By analogy to GECs, salubrinal was reported to be cytoprotective in PC12 cells and neurons (4, 45). In contrast, salubrinal induced apoptosis in β-pancreatic cells (7), but in these β cells, salubrinal enhanced Bip mRNA stability potently, thereby upregulating CHOP. The model whereby the extent of CHOP mRNA stability determines the outcome of the UPR does have some limitations. For example, other potential apoptotic mediators of ER stress, including caspase-12 and c-Jun N-terminal kinase (50, 52), are believed to be activated downstream of inositol requiring-1α, and their activation is posttranslational and unlikely to involve CHOP mRNA stability.

The ubiquitin-proteasome system plays a key role in selective degradation of misfolded or damaged proteins and is of major importance to fundamental cellular processes and disease (15, 44). The proteasome may degrade abnormal cytoplasmic or cytoskeletal proteins, and misfolded ER proteins, which are retrotranslocated selectively to the cytoplasm, a process known as ER-associated degradation (ERAD) (5). Proteasome substrates are tagged by covalent attachment of ubiquitin molecules via a three-step reaction and are then degraded by the 26S proteasome complex. The 26S proteasome is made up of a 20S core particle, which contains peptidase activities (including chymotrypsin-like activity), and a 19S regulatory complex. For a protein to be degraded, it must associate with 19S ATPases, undergo unfolding, and translocate into the 20S core (15, 44). A large amount of misfolded proteins (and/or formation of protein aggregates) may functionally impair or choke the proteasome (1), which may lead to the activation or exacerbation of ER stress (15, 44). The mechanism of this effect is poorly understood (51), and it may involve proteasome subunits that are associated with the ER, or interference with ERAD (24, 34, 51). Thus the UPR and ERAD are intimately linked; i.e., UPR induction may increase ERAD capacity, and interference with ERAD may lead to UPR induction. In the present study, absence of contact with ECM resulted in increased protein misfolding in the ER (Fig. 1), and an increase in polyubiquitinated proteins (Fig. 5), suggesting that these cells accumulated misfolded or abnormal proteins. Second, the GECs adherent to plastic showed impaired degradation of a ubiquitin-proteasome reporter (Fig. 5), in keeping with a choking of the ubiquitin-proteasome system, although the cells did not show a defect in the chymotrypsin-like activity of the 20S proteasome when assayed in vitro (Fig. 5). Further studies will be required to determine whether proteasomal impairment in the absence of ECM was due to excessive misfolded protein, and/or altered 19S regulatory activity, e.g., reduction in ATPase activity. Finally, incubation of GECs with proteasome inhibitors led to upregulation of the UPR in GECs (Fig. 5). Together, these results support the view that ER stress induction in resting GECs on plastic may be related to a functional impairment of the ubiquitin-proteasome pathway and ERAD (24, 51).

To extend the findings in cultured cells to disease mechanisms, we investigated ER stress in an experimental glomerulonephritis model in mice that is characterized by podocyte injury, increased glomerular cell apoptosis, and features of FSGS (25). In keeping with podocyte injury, many of the nephritic mice showed at least modest to moderate levels of albuminuria, including a mouse with severe albuminuria (Fig. 8). We demonstrated that expression of the ER chaperones Bip and grp94 as well as Bip phosphorylation were increased significantly in glomeruli from mice with nephritis compared with control (Fig. 7). In contrast, induction of CHOP was detectable only in tunicamycin-treated mice, but not in the mice with glomerulonephritis, or controls (Fig. 7). The presence of glomerular ER stress in nephritic mice was not dependent on albuminuria, and treatment of mice with tunicamycin did not induce albuminuria (Fig. 8). By analogy to the results in cultured GECs (i.e., induction of ER chaperones and phosphorylation of Bip, in the absence of CHOP expression), we propose that induction of the UPR in this model of experimental glomerulonephritis may limit apoptosis of podocytes. Although we did not examine ubiquitination of proteins in the mouse model of FSGS, it should be noted that increased ubiquitin has been reported in podocytes in human FSGS (31).

Other forms of glomerulopathies associated with podocyte injury have also shown an association with upregulation of the...
proteasome. The pattern of ER stress induction in this (13) and was associated with ubiquitination and choking of the DISCLOSURES

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