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Complement modulates the function of the ubiquitin–proteasome system and endoplasmic reticulum-associated degradation in glomerular epithelial cells

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ABSTRACT

In experimental membranous nephropathy, complement C5b-9 induces sublethal glomerular epithelial cell (GEC) injury and proteinuria. C5b-9 also activates mechanisms that restrict injury or facilitate recovery. The ubiquitin–proteasome system (UPS) selectively degrades damaged or abnormal proteins, while misfolded proteins in the endoplasmic reticulum (ER) undergo ER-associated degradation (ERAD). In this study, we investigated the effect of complement on the UPS and ERAD. We monitored UPS function by transfection of rat GECs with a UPS reporter, GFP^u (CL1 degron fused with green fluorescent protein). By analogy, CD3δ-yellow fluorescent protein (YFP) was employed as a reporter of ERAD. We demonstrated decreased GFP^u levels in GECs after incubation with antibody and complement, compared with control. Using C8-deficient serum with or without purified C8, cycloheximide (an inhibitor of protein synthesis), and the proteasome inhibitor, MG132, we confirmed that the decrease of GFP^u was mediated by C5b-9, and subsequent proteasomal degradation of the reporter. Inhibition of the c-Jun N-terminal kinase attenuated the effect of complement on GFP^u degradation. Complement, however, increased the level of CD3δ-YFP in GECs, implying an impairment of ERAD, likely due to an overabundance of misfolded proteins in the ER. The overall ubiquitination of proteins was enhanced in complement-treated GECs and in glomeruli of rats with experimental membranous nephropathy, although ubiquitin mRNA was unchanged in GECs. Proteasome inhibition with MG132 increased the cytotoxic effect of complement in GECs. Complement-stimulated UPS function, by accelerating removal of damaged proteins, may be a novel mechanism to limit complement-induced injury.

1. Introduction

The health of an organism largely depends on the exactness of signals sent among constituent cells. Much of this information is transmitted through proteins, which are either secreted from the cell, are embedded in the plasma membrane, or are part of the intracellular molecular network. Considering the high rate of translation, and that stress may result in the incorporation of incorrect amino acids or the disruption of proper protein folding, it becomes evident that protein biogenesis is an inherently error-prone process. The endoplasmic reticulum (ER) is an essential cellular compartment for protein maturation, and about one-third of all cellular proteins are translocated into the lumen of the ER, where they are modified to form their proper tertiary structure [1]. The quality of the protein folding process in the ER is vigorously controlled by molecular chaperones (e.g., BiP/GRP78, GRP94, calnexin) [2,3]. Only correctly folded proteins are permitted to leave the ER, while misfolded proteins are selected for degradation. One important mechanism for a cell to clear misfolded proteins is through targeted proteolysis by the ubiquitin–proteasome system (UPS). UPS-mediated protein degradation involves tagging of the ε-NH_2 group of an internal substrate lysine residue by covalent attachment of multiple ubiquitin molecules and degradation of the tagged protein by the 26S proteasome complex, with release of free and reusable ubiquitin. Ubiquitin is attached to its substrate in a three step process through an enzymatic cascade, comprising ubiquitin activase (E1), one of several ubiquitin conjugases (E2s), and one of many different ubiquitin ligases (E3s) [4]. Since the 76 amino acid ubiquitin molecule contains seven lysine residues, which can each be targeted for ubiquitin conjugation (i.e. ubiquitylation), consecutive rounds of ubiquitylation can result in the formation of long and diverse ubiquitin chains [4]. The current paradigm states that attachment of at least four ubiquitin moieties to the substrate is necessary for targeting to the 26S proteasome [5,6]. In a last crucial step, the ubiquitin chain is removed from

Abbreviations: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERK, extracellular-signal regulated kinase; GEC, glomerular epithelial cell; GFP, green fluorescent protein; HIS, heat-inactivated serum; HPRT, hypoxanthine ribosyltransferase; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; NS, normal serum; PHN, passive Heymann nephritis; RT-qPCR, reverse transcriptase real-time quantitative polymerase chain reaction amplification; UPS, ubiquitin–proteasome system; YFP, yellow fluorescent protein

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the substrate by associated ubiquitin isopeptidases, and this appears to permit more efficient translocation into the catalytic core for degradation [7]. UPS activity plays a role in regulating the level of proteins synthesized in the ER. The process, which removes terminally misfolded proteins from the ER, is known as ER-associated degradation (ERAD) and requires retrograde transport of substrates to the cytoplasm, the site of the 26S proteasome [8,9]. However, a broad spectrum of insults can disrupt this process, causing accumulation of misfolded proteins and failure of the ER to deal with this excessive protein load. Thus, the term ER stress is used to define any perturbation that compromises the protein folding functionality of the ER [10]. It is implicated in several diseases, ranging from ischemia–reperfusion injury to neurodegeneration (e.g. Alzheimer disease, Parkinson disease) to diabetes [11–13]. In the kidney, there is also evidence for an important role for ER stress in glomerular visceral epithelial cell (GEC; podocyte) injury [14–17].

Membranous nephropathy is an organ-specific complement-mediated disease and a frequent cause of idiopathic nephrotic syndrome in adults. Morphologically, it is characterized by subepithelial immune deposits containing IgG and complement, expansion of the glomerular basement membrane, and diffuse effacement of podocyte foot processes. In experimental membranous nephropathy, heterologous antibody binds to podocyte antigens [18,19]. Subsequently, immune complex formation leads to activation of the complement system with assembly of the C5b-9 membrane attack complex [19]. Generally, nucleated cells require multiple C5b-9 lesions for lysis, but at lower doses, C5b-9 induces sublethal (sublytic) injury and various metabolic effects [18,19]. In GECs, C5b-9 can activate cytosolic phospholipase A_2, protein kinase C, mitogen-activated protein kinase (MAPK) pathways, cell cycle proteins, transcription factors, and others [18,19]. Recent studies have demonstrated that C5b-9-induced GEC injury is associated with damage to the ER and an increased expression of ER stress proteins in GECs in culture and in the passive Heymann nephritis (PHN) model of membranous nephropathy in the rat [14,15].

In the present study, we investigated the effects of complement on UPS and ERAD function in GECs. We employed proteasome and ERAD reporters, and we analyzed changes in ubiquitination of proteins to provide a global assessment of UPS activity, which includes the interactions of E1, E2s, E3s, ubiquitin isopeptidases, together with the degradative capability of the proteasome. Complement increased UPS activity and protein ubiquitination, and proteasome inhibition exacerbated complement cytotoxicity.

2. Materials and methods

2.1. Materials

Tissue culture reagents and Lipofectamine 2000 were obtained from Invitrogen (Burlington, ON). Cycloheximide, doxorubicin, E64, N-ethylmaleimide, ionomycin, MG132, PD98059, SB203580, SP600125, tunicamycin, C8-deficient serum, C8, rabbit anti-ubiquitin antiserum, mouse anti-tubulin antibody, and rabbit anti-actin antibody were obtained from Sigma-Aldrich Canada (Mississauga, ON). 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), GE Healthcare (Baie d'Urfé, QC), and Jackson ImmunoResearch (West Grove, PA). Rabbit anti-cyclin A, mouse anti-green fluorescent protein (GFP), mouse anti-hemagglutinin epitope (HA), and mouse anti-ubiquitin antibodies, as well as U0126 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pEGFP^u was kindly provided by Dr. Ron Kopito (Stanford University, Stanford, CA) [20] and pHA-ubiquitin has previously been described by Treier et al. [21]. CD3δ-yellow fluorescent protein (YFP) was purchased from Addgene and was described previously [22]. The dominant negative c-Jun N-terminal kinase (JNK) cDNA (JNKapf) was described previously [23].

2.2. Cell culture and transfection

Primary cultures of GECs were established from explants of rat glomeruli, and have been characterized previously [24,25]. GECs were cultured in K1 medium, and experiments were done with cells between passages 25 and 70. GECs were transiently transfected using a DNA (μg) to Lipofectamine 2000 (μl) ratio of 1:2. Mouse embryonic fibroblasts were cultured in DMEM with 10% fetal calf serum [15].

2.3. Incubation of cells with antibody and complement

To activate complement, GECs in monolayer culture were incubated with rabbit anti-GEC antiserum (5% vol/vol) in modified Krebs–Henseleit buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 1 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 5 mM glucose, and 20 mM Hepes, pH 7.4, for 40 min at 22 °C. GECs were then incubated with normal human serum (NS; diluted in K1 medium), or with heat-inactivated (decomplemented) human serum (HIS; 56 °C for 1 h) in controls, at 37 °C. In some experiments, antibody-sensitized GECs were incubated with C8-deficient human serum, or C8-deficient serum reconstituted with purified C8 (80 μg/ml undiluted serum). We have generally used heterologous complement in order to facilitate studies with complement-deficient sera, and to minimize possible signaling via complement-regulatory proteins, however, in earlier studies, we confirmed that homologous (rat) complement activated analogous signaling pathways in GECs. Although there was some variability in concentrations of complement activity among batches of sera, we selected 2.5% as a "sublytic" concentration, i.e. a concentration that induced minimal cytolysis. In GECs, complement is not activated in the absence of antibody [25]. To activate complement in mouse embryonic fibroblasts, the cells were incubated with antiserum, as described above, followed by 5% NS (sublytic concentration), or HIS in controls [15].

2.4. Immunoprecipitation and immunoblotting

To prevent deubiquitination of proteins, cell lysates were prepared in buffer containing 1% Triton X-100, 125 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 20 μM pepstatin, 0.2 mM PMSF, and 10 mM N-ethylmaleimide, pH 7.4. Lysates were incubated with primary antibody (non-immune IgG in controls), followed by agarose-coupled protein A. Immune complexes or cell lysates were boiled in Laemmli sample buffer, and were subjected to SDS-PAGE under reducing conditions. Equal amounts of proteins were loaded into each lane of the gel. Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 5% bovine serum albumin, 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 NIH ImageJ software [14,15].

2.5. RNA extraction and analysis

RNA extraction and cDNA synthesis was done by use of the RNeasy Mini Kit and the QuantiTect Reverse Transcription Kit from Qiagen. Reverse transcriptase real-time quantitative polymerase chain reaction amplification (RT-qPCR) was performed with a pair of target-specific primers, and detection was with iTaq SYBR Green Supermix with ROX from Bio-Rad Laboratories (Applied Biosystems 7300 Real-Time PCR System). The primers for GFP^u were 5′-GTCCAGGAGCGCACCATCT-3′ (forward) and 5′-ATGCCCTTCAGCTCGATGC-3′ (reverse). Standardization of gene expression measurements was done against hypoxanthine ribosyltransferase (HPRT); primers 5′-TCGAAGTGTTGGATACAGGCCAGA-3′ (forward) and 5′-TACTGGCCACATCAACAGGACTCT-3′ (reverse).