Intestinal secretory cell ER stress and inflammation

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Abstract

Data from animal models and human inflammatory bowel diseases have implicated the ER (endoplasmic reticulum) stress pathway in intestinal inflammation. We have characterized the development of inflammation in Winnie mice in which ER stress arises due to a single missense mutation in the MUC2 mucin produced by intestinal goblet cells. This model has allowed us to explore the genesis of inflammation ensuing from a single gene polymorphism affecting secretory cells. In these mice, a proportion of MUC2 misfolds during biosynthesis, leading to ER stress and activation of the unfolded protein response. Winnie mice develop spontaneous complex progressive inflammation that is most severe in the distal colon. Inflammation involves Th1, Th2 and Th17 T-cells, with a progressive development of a Th17-dominated response, but also involves innate immunity, in a pattern not dissimilar to human colitis. Experimental inhibition of tolerance in this model severely exacerbates colitis, demonstrating active effective suppression of inflammation. Even though the misfolding of MUC2 is a consequence of an inherited mutation, as inflammation develops, the molecular markers of ER stress increase further and goblet cell pathology becomes worse, suggesting that inflammation itself exacerbates ER stress.

Intestinal mucosal barrier function

The intestinal mucosal surface is protected by a mucus barrier which protects the underlying epithelium from luminal commensal microbes and pathogens and influences the composition of the intestinal microbiota [1]. Loss of the mucus barrier in mice results in access of the microbiota to the epithelial surface, spontaneous colitis and increased susceptibility to infection [2–4]. The major functional components of the barrier are mucin glycoproteins and antimicrobial molecules produced by intestinal secretory cells, goblet cells and Paneth cells. These cells manufacture large amounts of protein for secretion and therefore present a major challenge for protein biosynthetic pathways. The major products of goblet cells are mucin glycoproteins, which, because of their large size, large number of disulfide bonds and homo-oligomerization into multimers, have an increased likelihood to misfold during biosynthesis. Small intestinal Paneth cells manufacture a range of secreted proteins, including defensins, lysozyme, anti-microbial lectins and collectins [5]. Defensin peptides are cysteine-rich, and disulfide bonds are important for their correct folding [6]. This, and the fact that Paneth cells also produce mucins, also renders them at substantial risk for protein misfolding and ER (endoplasmic reticulum) stress.

ER stress

The ER is responsible for synthesis, post-translational modification and folding of polypeptides to form functional proteins. Appropriate N-glycosylation and ER-resident chaperones and enzymes are essential for correct folding of proteins within the ER. The heat-shock protein GRP78 (glucose-regulated protein of 78 kDa) acts as a chaperone within the ER. GRP78 disengages from correctly folded proteins, but stays associated with misfolded proteins, triggering cellular responses, and is used to quantify ER stress because it is up-regulated in response to misfolding [7]. PDIs (protein disulfide-isomerases), such as Agr2 which is tightly co-expressed with mucin glycoproteins in the respiratory and gastrointestinal tracts, also promote correct folding by building intramolecular disulfide bonds. Despite such systems, a small proportion of proteins misfold, and misfolding increases with increasing protein complexity. The ER therefore has mechanisms to recognize misfolded proteins and remove them from the ER for degradation by the proteosome in a process called ERAD (ER-associated degradation) [8]. Factors which increase misfolding include increased protein synthesis, missense polymorphisms, inhibition of N-glycosylation, altered ER Ca2+ levels, energy depletion, reactive oxygen species, osmotic stress, microbial toxins and viral infection.

Protein misfolding triggers a network of phosphorylation, signal transduction and transcriptional events known as

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Abbreviations used: ATF6, activating transcription factor 6; Chop, cyclic AMP/CMYK/enhancer-binding protein-homologous protein; DSS, dextran sodium sulfate; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GRP78, glucose-regulated protein of 78 kDa; IBD, inflammatory bowel disease; IRE1, inositol-requiring enzyme 1; PDI, protein disulfide-isomerase; T-cell, thymic stromal lymphopoietin; UC, ulcerative colitis; UPR, unfolded protein response; XBP1, X-box binding protein 1.

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the UPR (unfolded protein response). The UPR involves enzymes and transcription factors engaged in parallel to restore ER homoeostasis. GRP78 is a key trigger for virtually all UPR pathways via removal from its association with the ER-resident UPR pathway-initiating molecules IRE1 (inositol-requiring enzyme 1), PERK [PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase] and ATF6 (activating transcription factor 6) during protein misfolding. ER stress can be controlled by the UPR; however, prolonged ER stress can induce inflammation and apoptosis. There are three main functional outcomes of the UPR: (i) decreased translation; (ii) restoration of protein folding; and (iii) degradation of misfolded proteins (ERAD). In addition to affecting events in the ER, the UPR can modulate other cellular programmes with important consequences for intestinal epithelial cells. Prolonged or severe ER stress can result in premature apoptosis, which, in the case of secretory cells, will decrease the capacity to secrete the major components of the mucosal barrier [9]. ER stress also triggers activation of the inflammatory transcription factor NF-κB (nuclear factor κB). Thus ER stress in intestinal secretory cells will lead to reduced production of key elements of the secreted barrier to microbes, in addition to direct inflammatory signalling, both of which can lead to activation of innate and adaptive immunity [10].

Because intestinal secretory cells produce the secreted mucosal barrier that protects from infection and inflammation [2,11], chronic ER stress in these cells is likely to promote inflammation in three ways: (i) by reducing the effectiveness of the mucosal barrier due to decreased secretion of antimicrobial molecules and mucins; (ii) by secretory cell apoptosis; and (iii) by UPR-initiated inflammatory signals released by the stressed secretory cells [10]. Data from murine models have now unequivocally demonstrated how a range of different defects in ER stress-related pathways can lead directly to chronic intestinal inflammation.

**Secretory cell pathology in intestinal inflammation**

Goblet cell pathology is one of the hallmark features of the IBD (inflammatory bowel disease) UC (ulcerative colitis). In UC, and, to a lesser extent, Crohn’s disease, goblet cells are reduced in number and are characterized by small goblet cell thecae, which equates to fewer stored mucin granules in the cytoplasm [12,13]. Despite goblet cell pathology being a key feature of UC, it has largely been overlooked as a contributing factor for several reasons. The first reason is based on a flawed interpretation of the morphological presentation of these goblet cells in IBD. The prevailing dogma is that the goblet cells in UC have reduced mucin granules due to enhanced secretion of the mucin. In other words, mucin secretion is said to have surpassed the production rate, leading to a lower accumulation of granules in the cytoplasm. This is a misinterpretation which overlooks the demonstrated capacity of intestinal goblet cells to continuously produce and secrete extremely large amounts of mucins under the influence of appropriate stimuli, such as that provided by Th12 cytokines in response to parasitic infection. Under these conditions, the thecae typically increase in size despite the high secretion rate.

A second contributing factor to the lack of focus on goblet cell pathology is the fact that similar goblet cell pathology is seen during infection with some enteric pathogens. Thus the goblet cell phenotype in UC has been attributed to the action of inflammatory factors on goblet cell secretion. We argue that, rather than just driving secretion, inflammatory factors are affecting goblet cell physiology, specifically inducing ER stress, thus causing the phenotype seen in disease. Supporting this thesis, in UC and Crohn’s disease, there is an accumulation of the MUC2 precursor, and there are many cells that lack goblet cell morphology (i.e. lack stored mucin) that are filled with the non-glycosylated MUC2 precursor [14–16]. Our interpretation is that the goblet cells are experiencing stress in their protein biosynthetic pathway, resulting in misfolding of the MUC2 precursor and reduced production of mature mucin for secretion. This view is well supported by electron microscopy studies showing vacuolation in goblet cells in UC [12,15,17].

Recently there has been a surge of interest in Paneth cell pathology in ileal Crohn’s disease. In ileal Crohn’s disease, Paneth cells are reduced in number, and there are data suggesting a reduction in production of defensins and other antimicrobial molecules [18–20]. The reduced antimicrobial granule phenotype in Paneth cells in ileal Crohn’s disease has also been linked with polymorphisms in autophagy genes [20]. This is demonstrated in an equivalent phenotype in mice hypomorphic for autophagy genes which has been shown to require co-infection with norovirus [21]. These observations implicate ER stress and related pathways in Paneth cell pathology in ileal Crohn’s disease.

The **Winnie mouse model of UC-like ER stress-induced colitis**

We have described two spontaneously arising independently generated ENU (N-ethyl-N-nitrosourea) mutants, Winnie and Eeyore, with spontaneous intestinal inflammation resulting from single missense mutations in two different D-domains of MUC2 causing goblet cell and Paneth cell ER stress [15]. Colitis in the Winnie strain has multiple similarities to UC, including goblet cell pathology, a distal gradient of colitis, a strong Th17 response and changes in the microbiome [15,22].

**Evidence of secretory cell ER stress**

The single amino acid substitutions caused by the Winnie and Eeyore mutations result in accumulation of the MUC2 precursor in the ER of the goblet cells and Paneth cells in the intestine. Immunohistochemistry using an anti-(MUC2 precursor) antibody shows a progressive accumulation of the precursor accompanied by a progressive decrease in the size of goblet cell theca where MUC2 granules are stored (Figure 1). Using electron microscopy, we have shown that the precursor accumulates in the ER, forming large vacuoles...
that are characteristic of severe ER stress. Vacuolization is most severe in the longer-lived goblet cell lineage found at the base of the crypts in the proximal and mid-colon of the mouse [15]. In vitro expression of the affected D-domain from wild-type and Winnie mice suggests that inappropriate N-terminal oligomerization of MUC2 is responsible for the misfolding.

Accumulation of the MUC2 precursor and vacuolization of goblet cells are accompanied by clear molecular evidence of ER stress and activation of ERAD and the UPR [15]. Markedly increased mRNA levels of ER chaperones (Grp78, Grp94), ERAD components [Edem1 (ER degradation enhancer, mannosidase α-like 1)], and elements of the UPR (spliced Xbp1 (X-box-binding protein 1), Atf4, Atf6 and Chop [C/EBP (CCAAT/enhancer-binding protein)-homologous protein 1]) are found in the intestine of Winnie mice. The morphological features of goblet cell pathology and the molecular markers of ER stress progressively deteriorate as mice age and progressive inflammation develops. This observation suggests that inflammatory factors combine with the misfolding mutations and exacerbate ER stress.

**Generation of complex inflammation in Winnie mice**

Winnie mice provide a unique opportunity to explore the generation of inflammation that results from a single gene mutation restricted to intestinal secretory cells. Therefore we have carefully dissected the inflammatory response as it emerges in the colon of these mice [22]. Interestingly, even though the goblet cell pathology is greatest in the proximal colon where the long-lived goblet cell lineage resides, inflammation is most severe in distal regions of the colon, as is the case in UC. Determination of location of inflammation in the intestine is likely to depend on the local balance between inflammatory signalling by the epithelium, recruitment and activity of effector leucocytes, and the presence and efficacy of suppressor mechanisms which can be derived from epithelial cells, antigen-presenting cells and T-regulatory lymphocytes.

Antigen-presenting cells such as dendritic cells and macrophages are the initiators of inflammatory responses. Large amounts of the cytokine IL (interleukin)-1β are produced in the Winnie mouse colon, which is likely to be derived from these antigen-presenting cells and/or epithelial cells. TSLP (thymic stromal lymphopoietin) is a regulatory cytokine produced by intestinal epithelial cells which is capable of suppressing dendritic cell activation, thus regulating immune responses to the normal microbial flora in the gut. In Winnie mice, there is decreased production of TSLP that is consistent with stressed epithelial cells suppressing TSLP production and/or release. This is accompanied by increases in the number and activation status of intestinal lamina propria dendritic cells. These dendritic
cells produce IL-6 and IL-23 together with chemokines, which is a pattern associated with stimulation of T_{H17} cells. Increased mRNA for IL-17A, IL-17F, TGFβ (transforming growth factor β) and CCR6 in the colon of Winnie mice is consistent with an active T_{H17} response, and intracellular cytokine staining shows that the major source of IL-17 is CD4⁺ T-cells. Despite the apparent dominance of the T_{H17} response in Winnie mice, substantial amounts of T_{H1} and, to a lesser extent, T_{H2}, cytokines are also produced [22]. This complex multi-cytokine pattern of inflammation is completely consistent with that observed in non-biased studies examining multiple cytokines in human IBD.

In order to determine whether innate inflammation is important in the colitis, we crossed Winnie mice on to a Rag-1-knockout background. These RaW mice have the same goblet cell defect, but lack both effector and regulatory lymphocytes. In the absence of lymphocytes, innate inflammation with relatively heavy neutrophil infiltration develops in RaW mice. In young mice, the inflammation in RaW mice is less than in Winnie mice in the distal colon and slightly greater than in Winnie mice in the proximal colon. However, as mice age, the inflammation in Winnie mice progressively worsens, whereas, with the exception of the caecum, the inflammation does not progress in RaW mice [22]. These experiments show that, in the absence of effectors and regulatory lymphocytes, innate inflammation develops with a different gradient of location from that when lymphocytes are present. This is, of course, an artificial, although informative, model, and when naive T-cells are introduced into RaW mice, very strong inflammation rapidly ensues, showing further that the intestinal microenvironment is primed to stimulate a strong T-cell response.

T-regulatory cells are not reduced in the Winnie mouse intestinal lymph nodes and mucosa [22], and our studies show that IL-10-mediated tolerance is very active in Winnie mice (M.A. McGuckin, unpublished work). In the Winnie phenotype, there is no deficiency in tolerance, but tolerance is overcome by inflammatory signalling originating from the ER-stressed epithelial cells and the diminished mucus barrier.

**Evidence from other animal models for a role for ER stress in colitis**

In addition to Winnie and Eeyore mice, there has emerged a series of murine models with defects in the ER stress and UPR pathways that collectively demonstrate the likely importance of this pathway in IBD. ER PDIs are enzymes which build and, if necessary, reduce disulfide bonds which are important in forming correct structures during protein folding. Constitutive knockout of the Agr2 ER PDI co-expressed with MUC2 in intestinal goblet cells and Paneth cells results in ER stress, abrogation of MUC2 biosynthesis and spontaneous inflammation [23]. Interestingly, inducible knockout of Agr2 causes rapid Paneth cell and goblet cell apoptosis and severe inflammation of tissues including the ileum [24]. AGR2 polymorphisms have been linked with IBD [25].

The IRE1 ribonuclease splices the XBP1 mRNA, resulting in the coding of a transcription factor which induces UPR target genes, including chaperones, PDIs and components of ERAD [26,27]. Knockout of Ire1b in mice increases sensitivity to DSS (dextran sodium sulfate)-induced colitis [28], whereas intestinal-specific inducible knockout of XBP1 results in Paneth cell and goblet cell apoptosis, spontaneous ileal inflammation and increased sensitivity to DSS-induced colitis [29]. XBP1 polymorphisms have been linked with IBD [29].

As part of the UPR, ATF6 translocates from the ER to the Golgi where it is cleaved by the S1P and S2P proteases, releasing a transcription factor which induces UPR target genes including GRP78 [30]. Mice hypomorphic for Mbp1 (membrane-bound transcription factor peptidase, site 1) (an S1P orthologue) develop spontaneous increases in inflammatory cytokines without obvious histological colitis and increased sensitivity to DSS [31]. CHOP is a UPR-triggered transcription factor which initiates ER stress-induced apoptosis. Knockout of CHOP protects mice from DSS-induced epithelial apoptosis and colitis, showing that ER stress is important in this commonly used model of UC [32].

Taken together, these models show that either increased misfolding or an inappropriate UPR lead to chronic intestinal inflammation and allude to the multiple mechanisms by which ER stress could contribute to IBD. In IBD, environmental factors such as luminal toxins and/or host inflammatory factors, including reactive oxygen species, could stress the ER and lead to protein misfolding and activation of the UPR and inflammatory signalling. Polymorphisms in major proteins, chaperones and PDIs could also lead to protein misfolding and the same phenotype. Even in the absence of environmental factors or folding polymorphisms, the large protein production load of secretory cells is likely to result in a substantial baseline of ER stress. Inappropriate responses to that ER stress caused by polymorphisms in ERAD, autophagy or UPR genes could result in stress, particularly when protein synthesis is increased during infectious or inflammatory triggers. In this scenario, the ER stress phenotype may not emerge until a strong environmental pressure, perhaps an infection, initiates pathology.

Although mouse models have shown that ER stress leads to intestinal inflammation, there is still much to learn about the importance of ER stress in IBD. Whether ER stress is primary or secondary in IBD, mucosal healing requires full restoration of secretory cell function to restore the mucus barrier to the microbes. Thus alleviating ER stress is a potentially efficacious treatment approach in IBD which requires exploration.

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