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Proteasome Inhibition of Pathologic Shedding of Enterocytes to Defend Barrier Function Requires X-Linked Inhibitor of Apoptosis Protein and Nuclear Factor κB

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BACKGROUND & AIMS: Although we are beginning to understand where, when, and how intestinal epithelial cells are shed, physiologically, less is understood about alterations in cell fate during minimally invasive epithelial infections. We used a piglet model of *Cryptosporidium parvum* infection to determine how elimination of infected enterocytes is balanced with the need to maintain barrier function. **METHODS:** We studied the effects of enterocyte shedding by *C parvum*–infected ileum on barrier function ex vivo with Ussing chambers. The locations and activities of caspase-3, nuclear factor κB (NF-κB), and inhibitor of apoptosis proteins (IAP) were assayed by enzyme-linked immunosorbent assay, immunoblot, and tissue immunoreactivity analyses and using specific pharmacologic inhibitors. The location, specificity, and magnitude of enterocyte shedding were quantified using special stains and light microscopy. **RESULTS:** Infection with *C parvum* activated apoptotic signaling pathways in enterocytes that resulted in cleavage of caspase-3. Despite caspase-3 cleavage, enterocyte shedding was confined to villus tips, coincident with apoptosis, and observed more frequently in infected cells. Epithelial expression of X-linked inhibitor of apoptosis protein (XIAP), activation of NF-κB, and proteasome activity were required for control of cell shedding and barrier function. The proteasome blocked activity of caspase-3; this process was mediated by expression of XIAP, which bound to cleaved caspase-3. **CONCLUSIONS: We have identified a pathway by which villus epithelial cells are maintained during *C parvum* infection. Loss of barrier function is reduced by active retention of infected enterocytes until they reach the villus tip. These findings might be used to promote clearance of minimally invasive enteropathogens, such as by increasing the rate of migration of epithelial cells from the crypt to the villus tip.**

Keywords: Protozoan; Intestine; Parasitic Infection; Cell Death.

The gastrointestinal tract is lined by a single layer of epithelial cells that serve as a barrier to luminal antigens and pathogens while also absorbing the water and nutrients required for life. In the small intestine, these epithelial cells arise from stem cells residing in the crypts whose progeny migrate up the villi and are individually shed into the intestinal lumen. Only recently have we begun to understand where, when, and how intestinal epithelial cells are physiologically shed from the villi. By most accounts this shedding occurs coincident with apoptosis, is confined predominantly to the villus tip, and does not impair maintenance of epithelial barrier function.[1–4] Far less is understood about how cell fate may be altered in response to a minimally invasive infection of the intestinal epithelium. For most tissues, the host will limit spread of infection by executing infected cells through apoptosis. However, in the intestinal epithelium, it is unclear whether the host balances signals compelling the elimination of infected cells with a necessity to prevent loss of barrier function. A clear understanding of host strategy in combating these infections is essential to the design of rational therapies to aid intestinal epithelial defense.

In humans, replication of *Cryptosporidium* spp (*C parvum*) within villous enterocytes of the small intestine causes an accelerated loss of epithelial cells resulting in severe villous atrophy, nutrient malabsorption, and debilitating diarrhea.[5] Although epithelial cell loss is a key feature of *C parvum* infection, the mechanisms arbitrating this cell death are unclear. This can be attributed in part to a failure of conventional models to recapitulate the clinical infection. For example, experimentally infected mice do not develop villous atrophy, crypt hyperplasia, mucosal inflammation, or diarrhea.[6,7]

A consistent response of epithelial cell cultures to *C parvum* infection is the induction of caspase-dependent apoptosis.[8,9] The clinical relevance of epithelial apoptosis in human cryptosporidiosis remains to be established. In fact, a noteworthy histologic feature of severe infection is a conspicuous “lack of apoptotic cells even in cases of florid cryptosporidiosis.”[5] It is possible that apoptotic cells are quickly shed from the small intestinal epithelium and therefore not visible in biopsy specimens. On the other hand, when faced with overwhelming infection, apoptosis of enterocytes may be actively repressed. Cell

Abbreviations used in this paper: cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; IAP, inhibitor of apoptosis protein; NF-κB, nuclear factor κB; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; XIAP, X-linked inhibitor of apoptosis protein.

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culture models lend support to the possibility that epithelial apoptosis is inhibited in *C parvum* infection. Although apoptosis of epithelial cells is unquestionably increased by *C parvum* infection in these models, most of the infected epithelial cells do not undergo apoptosis, and infected monolayers are more resistant to pro-apoptotic chemotherapeutics.[10–12] In some studies, protection from apoptosis was attributed to activation of the nuclear transcription factor nuclear factor κB (NF-κB)[12,13]; however, the mechanism by which NF-κB controls apoptosis in the infected monolayers is unknown.

Repression of apoptosis in cell culture models of *C parvum* infection is largely attributed to the actions of *C parvum*. From an in vivo perspective, however, repression of apoptosis could ostensibly benefit the host. In people and experimentally infected piglets, massive early epithelial cell losses from *C parvum* infection culminate in a highly attenuated epithelium that maintains its continuity despite an escalating burden of parasites. These observations suggest that repression of apoptosis may be driven by the host to prevent loss of barrier function at the expense of retaining infected cells on the villi.

Using a neonatal piglet model of *C parvum* infection that uniquely recapitulates human cryptosporidiosis, the present studies have identified a novel mechanism by which the intestinal epithelium attenuates apoptosis and cell shedding to preserve barrier function. *C parvum* infection in vivo precipitated widespread activation of villous epithelial apoptosis signaling culminating in the cleavage of caspase-3. Despite caspase-3 cleavage, epithelial cell shedding remained largely confined to the villous tips, was coincident with apoptosis, and was preferential to infected cells. X-linked inhibitor of apoptosis protein (XIAP) expression and NF-κB activation in the epithelium were critical for both control of cell shedding and preservation of barrier function and dependent on proteasome activity. Proteasome-dependent repression of epithelial caspase-3 activity could be specifically attributed to expression of XIAP, an inhibitor of apoptosis protein (IAP) capable of inhibiting active caspase-3 and to which binding to cleaved caspase-3 was shown by coimmunoprecipitation.

Materials and Methods

Animals

One-day-old piglets were infected by orogastric tube with 10^8 *C parvum* oocysts on day 3 of life and killed at peak infection 3–5 days later. Sections of ileum were collected for histology, histomorphometry, epithelial cell isolation, and in vitro barrier function studies (Supplementary Materials and Methods). All studies were approved by the Institutional Animal Care and Use Committee.

Immunofluorescence and Immunohistochemistry

Frozen sections of ileal mucosa were fluorescence immunolabeled using anti-active caspase-3, anti-M30, anti–*C parvum*, and isotype control antibodies. Formalin-fixed, paraffin-embedded sections of ileal mucosa were immunostained for phospho-p65, for cytokeratin, and by means of terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (Supplementary Materials and Methods).

Epithelial Cell Exfoliation, Immunoblots, and Coimmunoprecipitations

The villous epithelium was exfoliated from fresh sections of piglet ileum in an oxygenated chelation buffer containing 2.5 mmol/L glucose as previously described[14] and frozen at −80°C. Protein extraction, quantification, electrophoretic separation, transfer, and exposure were performed using standard approaches. Primary antibodies included rabbit anti–caspase-3, mouse anti-XIAP, rabbit anti-survivin, goat anti–cellular inhibitor of apoptosis protein 1 (cIAP1), and rabbit anti–cellular inhibitor of apoptosis protein 2 (cIAP2). Positive controls included HeLa and Jurkat cell lysates. Coimmunoprecipitation experiments between XIAP, survivin, and cleaved caspase-3 were performed (Supplementary Materials and Methods).

Caspase-3 and NF-κB Activity

Protein extracts from piglet ileal mucosa were assayed for caspase-3 and NF-κB activity by enzyme-linked immunosorbent assay (Supplementary Materials and Methods).

Barrier Function Studies

Transepithelial electrical resistance ($\Omega \cdot cm^2$) and mucosal-to-serosal flux of ^{3}[H]labeled mannitol were measured for piglet ileal mucosa after mounting in 1.13-cm^2 aperture Ussing chambers using standard techniques (Supplementary Materials and Methods).

Ex Vivo Inhibition of Proteasome Activity, Caspase-3, NF-κB, and XIAP

Inhibitors of proteasome activity, caspase-3, NF-κB, and XIAP were added alone and in combination to both the serosal and mucosal reservoir of the Ussing chamber for 285–300 minutes (Supplementary Materials and Methods), after which time the mucosa was removed and flash frozen in liquid nitrogen or processed for light microscopic and immunohistochemical studies.

Data Analysis

Data represent means ± SEM. For all analyses, $P \leq .05$ was considered significant. Data were tested for normal distribution and variance and analyzed using parametric or nonparametric statistics as appropriate (SigmaStat; Jandel Scientific, San Rafael, CA). Parametric data were analyzed using paired and unpaired *t* tests and one-way or repeated-measures analysis of variance. Nonparametric data were analyzed using Mann–Whitney rank sum test or Wilcoxon signed rank test. n represents number of piglets.

Results

Discordance of Executioner Caspase-3 Cleavage and Epithelial Apoptosis in C parvum Infection

To identify apoptosis of intestinal epithelial cells in *C parvum* infection in vivo, we performed Western analysis and immunohistochemistry to quantify and localize epithelial cleavage of a terminal arbiter of apoptosis, caspase-3. In uninfected piglets, the villous epithelium was characterized by the presence of only procaspase-3. In

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