Cryptosporidium parvum induces apoptosis in biliary epithelia by a Fas/Fas ligand-dependent mechanism

XIAN-MING CHEN,1 GREGORY J. GORES,1 CARLOS V. PAYA,2 AND NICHOLAS F. LARUSSO3

1Center for Basic Research in Digestive Diseases, Division of Gastroenterology and Hepatology, and 2Division of Experimental Pathology, Mayo Medical School, Clinic and Foundation, Rochester, Minnesota 55905

Chen, Xian-Ming, Gregory J. Gores, Carlos V. Paya, and Nicholas F. LaRusso. Cryptosporidium parvum induces apoptosis in biliary epithelia by a Fas/Fas ligand-dependent mechanism. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G599–G608, 1999.—Although the clinical features of biliary cryptosporidiosis from opportunistic infections of the biliary tree in patients with acquired immunodeficiency syndrome (AIDS) are well known, the mechanisms by which associated pathogens, such as Cryptosporidium parvum, cause disease are obscure. Using an in vitro model of biliary cryptosporidiosis, we observed that C. parvum induces apoptosis in cultured human biliary epithelia. Both caspase protease inhibitors and neutralizing antibodies to either Fas receptor (Fas) and Fas ligand (FasL) inhibited this process; neutralizing antibodies to other apoptotic cytokines [interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β)] had no effect. C. parvum stimulated FasL membrane surface translocation, increased both FasL and Fas protein expression in infected biliary epithelia, and induced a marked increase of soluble FasL (but not IL-1β, TNF-α, and TGF-β) in supernatants from infected cells. When a coculture model is used in which infected and uninfected cell populations were physically separated by a semipermeable membrane, both uninfected biliary epithelia and uninfected Fas-sensitive Jurkat cells (but not a Fas-resistant Jurkat cell line) underwent apoptosis when cocultured with infected biliary epithelia. Moreover, both a neutralizing antibody to FasL and a metalloprotease inhibitor blocked the apoptosis in uninfected cocultured cells. Activation of caspase activity was also observed in uninfected cocultured biliary epithelia. The data suggest that C. parvum induces apoptosis in biliary epithelia by a Fas/FasL-dependent mechanism involving both autocrine and paracrine pathways. These observations may be relevant to both the pathogenesis and therapy of the cholangitis seen in AIDS patients with biliary cryptosporidiosis.

acquired immunodeficiency syndrome; cholangiopathies; opportunistic infections; parasitic diseases; caspase

Although a common cause of diarrhea in humans and animals, Cryptosporidium parvum is usually self-limited in immunocompetent individuals (37). However, in immunosuppressed patients, particularly those with the acquired immunodeficiency syndrome (AIDS), C. parvum may be life threatening (10). Currently, there is no effective medical treatment for cryptosporidiosis (10). AIDS patients infected with C. parvum also develop extraintestinal disease, most frequently of the biliary tract, resulting in sclerosing cholangitis and cholecystitis in some patients (10). Indeed, C. parvum may be found in the bile of 20–65% of patients with this so-called “AIDS-associated cholangiopathy” (2, 5, 11, 56, 59). The presence of biliary cryptosporidiosis in AIDS patients is associated with chronicity of infection as well as a poor prognosis (28).

Although the clinical and radiological features of biliary cryptosporidiosis have been well documented (59), the pathophysiological mechanisms underlying C. parvum infection of biliary epithelia are not well understood. In a previous study, we reported the development of an in vitro model of biliary cryptosporidiosis in which cultured human biliary epithelia were infected with C. parvum sporozoites (6). Using this model, we observed that C. parvum was directly cytopathic for biliary epithelia, with widespread apoptosis of biliary cells within hours after exposure (6). The data reported here show that C. parvum induces apoptosis in cultured human biliary epithelia by a mechanism involving the Fas receptor (Fas)/FasL ligand (FasL) system.

MATERIALS AND METHODS

C. parvum. C. parvum oocysts harvested from calves inoculated with a strain originally obtained from Dr. Harley Moon at the National Animal Disease Center (Ames, IA) were purchased from a commercial source (Pleasant Hill Farms, Troy, ID). Oocysts were purified using a modified ether extraction technique and then suspended in PBS and stored at 4°C. Before infecting biliary epithelial cells, oocysts were treated with 1% sodium hypochlorite on ice for 20 min and subjected to an excystation solution consisting of 0.75% taurodeoxycholate and 0.25% trypsin for 30 min at 37°C. The excystation rate was calculated as previously described by others (4, 30) and was determined for each new batch of oocysts.

Cells. H69 cells (a gift of Dr. D. J. Jefferson, Tufts University, Boston, MA) are SV40 transformed human bile duct epithelial cells originally derived from normal liver harvested for transplant. The cells continued to express biliary epithelial cell markers, including cytokeratin 19, γ-glutamyl transpeptidase, and ion transporters, consistent with biliary function and have been extensively characterized (15). Stock cultures of these nonmalignant but immortalized cells were maintained in coculture with irradiated NIH3T3 mouse fibroblasts and were grown in a hormonally supplemented medium with 10% fetal bovine serum. For experiments, cells were maintained for three passages without coculture cells to ensure that the culture was free of fibroblasts. All experiments were performed with cells between passage 23 and 26. JurKat E6–1, a human leukemia T cell that naturally expresses Fas, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). JurKat JM-3A5, a human leukemia T
cell that does not express Fas (a gift from Dr. Paul Leibson, Rochester, MN) was used as negative control for Fas/Fasl-based cytotoxicity induced by C. parvum-infected H69 monolayers. Jurkat cell lines were cultured in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Infection models. Infections were performed as described previously (6). Briefly, H69 cells were seeded into four-well chamber slides or six-well Costar tissue culture plates (Becton Dickinson Labware) and grown to 70–80% confluence. Infection with C. parvum was accomplished in a culture medium consisting of DMEM-F12, 100 µM penicillin, and 100 µg/ml streptomycin (referred to hereafter as assay medium) and freshly excysted C. parvum sporozoites. Infective organisms (excysted sporozoites treated at 65°C for 2 h) were used for sham infection controls. The viability of each oocyte preparation was determined prior to each set of experiments, and an optimal dose of oocysts and sporozoites was determined. In most experiments, a concentration of 1–5 × 10⁶ sporozoites was used.

Apoptotic cytotoxicity of C. parvum to H69 cells. Subconfluent (70–80% confluence) H69 cells in four-well chamber slides were used. Before C. parvum infection, cells were washed with DMEM-F12 and then incubated in 0.3-ml assay medium containing freshly excysted C. parvum sporozoites. Cells were either incubated with 1 × 10⁶ C. parvum sporozoites per well for different periods of time or incubated with C. parvum at concentrations from 1 × 10⁵ to 1 × 10⁶ sporozoites per well for 24 h. After incubation, slides were fixed in absolute methanol for 5 min, and the degree of apoptosis was evaluated by 4,6-diamidino-2-phenylindole (DAPI) staining and fluorescein-labeled annexin V (Pharmingen, San Diego, CA) binding.

Inhibition of apoptotic cytotoxicity by antibodies. To assess potential inhibition of apoptotic cytotoxicity of candidate apoptotic factors, neutralizing antibodies against interleukin-1β (IL-1β; 50 µg/ml; R&D Systems, Minneapolis, MN), transforming growth factor-β (TGF-β; 50 µg/ml; R&D Systems), tumor necrosis factor-α (TNF-α; 50 µg/ml, R&D Systems) and FasL, NOK-1 (17, 23) (10 µg/ml, Pharmingen), as well as Fas antagonist antibody M3 (31, 33, 34) (10 µg/ml, Immunex, Seattle, WA), were added to H69 cells grown on four-well chamber slides. M33 (Immunex), an isotype-matched irrelevant monoclonal antibody to M3, and normal goat and chicken IgG were used as control antibodies. After removal of the culture media, H69 cells were washed with DMEM-F12 and resuspended in 0.3-ml assay medium containing those antibodies. Thirty minutes after the addition of antibodies, freshly excysted C. parvum sporozoites were added. After incubation for 24 and 48 h, cells were fixed and apoptosis was assayed. In some experiments, cells were incubated with antibodies and C. parvum sporozoites for 2 h and then washed three times with PBS and fixed in absolute methanol for 5 min. Parasites that had attached to or that had invaded H69 cells were quantitated by immunofluorescence using a monoclonal antibody against a sporozoite protein (2H2; ImmuCell, Portland, ME) (46).

Inhibition of apoptotic cytotoxicity by YYAD-CHO and DEVD-CHO. H69 cells were grown on four-well chamber slides to 70–80% confluence. Prior to infection of C. parvum sporozoites, the medium was replaced with the assay medium, and the cells were incubated for 30 min with the cell permeable caspase inhibitors DEVD-CHO (2 µM; Biomol, Plymouth Meeting, PA) and YYAD-CHO (2 µM; Biomol). Infection of C. parvum sporozoites was performed as previously described. After 24 and 48 h of incubation, cells were fixed and apoptosis was determined. In some experiments, cells were only incubated with C. parvum sporozoites for 2 h and then fixed with methanol, and infection rates were determined by immunofluorescence as previously described.

Expression of FasL and Fas in infected H69 monolayers. H69 cells were grown to 70–80% confluency in T25 flasks and then exposed to C. parvum in the assay medium. After different incubation times, cells were lysed and quantitative immunoblots were performed as previously described (26, 29, 43). Briefly, samples were analyzed by SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. Membranes were sequentially incubated with primary antibodies and then with 0.2 µg/ml of horseradish peroxidase-conjugated secondary antibody and revealed by an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, England). G247–4 (Pharmingen) antibody against FasL, which recognizes both the membrane bound and soluble forms of FasL (38, 39), and clone 13 (Transduction Laboratories, Lexington, KY) against Fas were used for the immunoblotting. For immunocytochemistry of surface membrane expression of Fas and Fasl, H69 cells were grown to 70–80% confluency on four-well chamber slides and then exposed to C. parvum. In some slides, 0.2 mM 1,10-phenanthroline (Sigma, St. Louis, MO), a metalloprotease inhibitor (23) that at a dose of 0.2 mM showed no toxicity to H69 cells, was added to the assay medium at the same time as sporozoites. After 24 h of incubation, cells were fixed with 0.1 M Pipes (pH 6.95), 1 mM [ethylene-bis(oxyethylenenitri- lo)]tetraacetic acid, 3 mM MgSO₄, and 2% paraformaldehyde. Without membrane permeabilization, cells were incubated with primary monoclonal antibodies against Fas (clone 13) or Fasl (G247–4) followed by fluorescein-labeled anti-mouse antibodies. Slides were mounted with mounting medium (H-1000, Vector Laboratories) and assessed by confocal laser scanning microscopy. Contrast and intensity for each image were manipulated uniformly using Adobe (Mountain View, CA) Photoshop software.

Detection of soluble Fasl, IL-1β, TGF-β, and TNF-α. To analyze for the production of potential soluble apoptotic factors released by H69 cells in response to C. parvum infection, H69 cells were grown to subconfluence in T75 flasks. After different periods of incubation with assay medium containing freshly excysted sporozoites, supernatants were collected for assays. To detect soluble Fasl (sfasl), 15 ml of supernatants were concentrated to 0.15 ml using an ultrafree concentrator with 10,000 molecular weight limits after 1 h of spinning at 3,000 rpm at 4°C. The concentrated supernatants were then mixed 1:1 with lysine buffer [50 mM Tris, containing 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml pepstatin, and 0.5 µg/ml leupeptin adjusted to pH 7.4]. The concentrated supernatants were analyzed by SDS-PAGE and immunoblotting as previously described. G247–4 antibody, which recognizes both the membrane bound and soluble forms of FasL (38, 39), was used. IL-1β, TNF-α, and TGF-β concentrations in the supernatants were determined by ELISA using commercial kits (R&D Systems).

Apoptotic cytotoxicity of recombinant human sfasl to H69 cells. H69 cells were grown to 70–80% confluency on four-well chamber slides and then exposed to recombinant human sfasl (Calbiochem, Cambridge, MA) at concentrations from 100 to 1,000 ng/ml in the assay medium. After 10 h of incubation, cells were fixed and apoptosis was analyzed.

Apoptotic cytotoxicity of C. parvum-infected H69 monolayers for uninfected cocultured cells. Cytotoxicity of C. parvum-infected H69 monolayers for noninfected cells was evaluated by using a coculture system. For the H69/H69 coculture system, H69 cells were grown to 70–80% confluency in six-well Costar tissue culture inserts (Becton Dickinson Lab-
ware) with cells both on the inserts (upper chamber) and on the plates below the inserts (lower chamber). The two cell populations were physically separated by a polycarbonate membrane with a high density of 0.4-µm pore size, which allows free exchanges of molecules (but not sporozoites) between the upper and lower media reservoirs. After removal of H69 culture medium, cells were washed with DMEM-F12 and resuspended in assay medium. Sporozoites were then added to the assay medium of the upper chamber. In some experiments, 0.2 mM 1,10-phenanthroline was added to the medium in the upper chamber at the same time as sporozoites. After 24 h of incubation, cells in the upper and lower chambers were fixed and apoptosis was evaluated. In control experiments, sporozoites were added to the upper chamber of the inserts without H69 cells and then cocultured with H69 cells grown on the plates in the lower chamber.

For the coculture of H69 with Jurkat cell lines, the same coculture system was applied, but the upper chambers were seeded with uninfected Jurkat cells instead of H69 cells. After removal of cell culture medium, both cell populations were resuspended in the assay medium and freshly excysted C. parvum sporozoites were added to the H69 cells in the lower chamber. In some experiments, the FasL neutralizing antibody NOK-1 (10 µg/ml, Pharmingen) was added in the lower chamber. In some experiments, the FasL neutralizing antibody NOK-1 (10 µg/ml, Pharmingen) was added in the lower chamber before the addition of C. parvum sporozoites. After 24 h of incubation, H69 cells were fixed and apoptosis was determined by DAPI staining. J urkat cells were resuspended in the media and applied to slides using a Cytospin (Shandon, 1,000 rpm, 6 min). Cells on the slides were then fixed and stained with DAPI.

Caspase activity in cocultured uninfected H69 cells. H69 cells were grown to 70–80% confluency in six-well Costar tissue culture inserts with cells both on the inserts (upper chamber) and on the plates below the inserts (lower chamber) as previously described. Sporozoites were then added to the assay medium of the upper chamber. After different periods of incubation, cocultured H69 cells on the plates were washed with PBS, and the cytosolic extracts were prepared by using a lysis buffer [25 mM HEPES (pH 7.5), 5 mM magnesium chloride, 1 mM EGTA, 0.5 mM PMSF, 2 µg/ml leupeptin, and 2 µg/ml pepstatin]. Cell lysis was incubated at 37°C for 30 min in assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 10 mM dithiothreitol, and 0.5 mM EDTA) with 20 µM Ac-DEVD-aminoethylfluoromethyl coumarin (AFC) as a caspase fluorescent substrate (Enzyme Systems Products, Livermore, CA). Fluorescence at 390–475 nm was measured with a PerkinElmer fluorescence spectrophotometer (Buckinghamshire, UK). Measurements were calibrated against a standard curve of AFC (Enzyme Systems Products), and data were expressed in picomoles of released AFC per milligram of lystate proteins. Protein concentration was measured by Bradford method (Bradford reagent, Sigma).

Apoptosis measurement. Apoptosis was quantitated by DAPI staining and annexin V binding. Cells were stained with the nuclear staining dye DAPI (2.5 µM, 5 min) or fluoroscein-labeled annexin V (Pharmingen) and viewed with a fluorescence microscope. For each well on the slide, over 2,000 cells were counted, and the number of cells positive to annexin V binding or DAPI staining with nuclear changes characteristic of apoptosis (i.e., condensation, margination, and/or fragmentation) was recorded (9, 21, 42). Apoptosis was also assessed by DNA extraction and agarose electrophoresis. DNA was extracted from the cultured cells using a phenol-chloroform technique, with ethidium bromide added and run on an agarose gel. Bands were visualized and photographed under ultraviolet light (42).

Statistical analysis. All values are given as means ± SE. Means of groups were compared with Student’s (unpaired) t-test or ANOVA test where appropriate. P < 0.05 was considered statistically significant.

RESULTS

Apoptosis of C. parvum-infected human cholangiocytes. When stained with the nuclear binding dye DAPI, H69 cells in infected monolayers grown either on chamber slides or insert membranes exhibited the characteristic nuclear changes associated with apoptosis of epithelial cells to a significantly greater extent than did cells in the sham infection and normal controls (Fig. 1). Evidence of apoptosis in infected H69 cells was further confirmed by annexin V binding, which showed a similar dose-dependent increase of apoptotic cells after incubation with various concentrations of C. parvum for 24 h (Fig. 1A); an identical staining pattern of apoptotic cells with DAPI staining (Fig. 1C) was found. The number of apoptotic cells in infected H69 cell monolayers grown on four-well chamber slides increased steadily after 24 h of incubation when up to 1 × 10⁶ C. parvum sporozoites per well were added and increased consistently up to 22% when incubated with 5 × 10⁶ C. parvum sporozoites per well. When cells were incubated with 1 × 10⁶ C. parvum sporozoites per well, cytopathic effects became apparent by 12 h after exposure to the organism and increased to 12% apoptotic cells at 24 h, compared with <1% apoptosis in uninfected or sham-infected cells over the same period (Fig. 1B). Therefore, in most of the subsequent experiments, a concentration of 1 × 10⁶ C. parvum sporozoites per four-chamber slide well was used.

Inhibition of apoptosis by antibodies and caspase inhibitors. To explore possible mechanisms of C. parvum-induced apoptosis in infected H69 cells, parallel experiments were performed in the presence of various antibodies that could block Fas/FasL interactions or neutralize Fasl, IL-1b, TNF-α, and TGF-β activities. As shown in Fig. 2A, exposure of H69 cells to a Fas antagonistic antibody (M3) or a FasL neutralizing antibody (NOK-1) significantly (P < 0.01) reduced apoptosis induced by C. parvum infection; in contrast, antibodies neutralizing IL-1b, TNF-α, and TGF-β showed no effect on C. parvum-induced apoptosis. All antibodies used in the experiments had no effect on the rates of infection of H69 cells by C. parvum sporozoites (data not shown). Pretreatment of cells with the caspase inhibitors DEVD-CHO and YVAD-CHO also blocked C. parvum-induced apoptosis up to 80% (Fig. 2B). These observations suggested that Fas/FasL interactions are the major factors involved in the mechanisms of C. parvum-induced apoptosis.

Upregulation of Fas/FasL in human cholangiocytes by C. parvum. The presence of performed FasL and Fas in H69 cells was assessed by quantitative Western blotting using monoclonal antibodies against human FasL and Fas. Cell lysates from H69 cells showed a band of 37-kDa molecular mass (Fig. 3A), consistent with other reports of FasL (23, 38, 39). The intensity of FasL band decreased at 6 h postinfection but increased
significantly (P < 0.001) at 12 and 24 h postinfection (Fig. 3, A and B). Cell lysates from noninfected H69 cells contained detectable amounts of Fas, with a molecular mass of 47 kDa, and the intensity of this band also increased significantly at 24 h postinfection (Fig. 3, C and D). The membrane surface expression of Fas and FasL in infected H69 monolayers were assessed by immunocytochemistry and laser confocal microscopy without cell membrane permeabilization. H69 cells showed a distinct surface staining for Fas in sham-infected controls, but no staining for FasL was observed (Fig. 4). After incubation with C. parvum for 24 h, intensive surface staining for both Fas and FasL was detected. FasL surface expression on infected cells was further augmented by the presence of metalloprotease inhibitor 1,10-phenanthroline (Fig. 4). These observations suggest that both FasL and Fas are normally expressed in H69 cells, but whereas Fas is on the surface of uninfected cells, FasL is not normally found on the cell surface of cholangiocytes. In contrast, C. parvum infection not only increases Fas and FasL expression but also induces the translocation of FasL to the membrane and stimulates the cleavage of membrane FasL to form sFasL.

Release of sFasL from C. parvum-infected human cholangiocytes. To determine if sFasL was present in the supernatants of C. parvum-infected H69 monolayers, supernatants from C. parvum-infected H69 cells
were collected and analyzed by quantitative Western blotting. The immunogen of G247–4 is the COOH terminal of the FasL molecule, and this antibody recognizes both the membrane bound (FasL) and sFasL forms (38, 39). Although not detectable in the supernatants from sham-infection controls, sFasL increased steadily in the 24-h supernatants from infected H69 monolayers (Fig. 5, A and B). No significant increase of IL-1β, TNF-α, and TGF-β in the supernatants of C. parvum-infected H69 monolayers was found compared with noninfected or sham-infected controls using ELISA assays (Fig. 5C).

Apoptotic cytotoxicity of C. parvum-infected human cholangiocytes to cocultured H69 cells. To test the potential importance of sFasL in regulation of apoptosis in C. parvum-infected H69 cultures, cytotoxicity of C. parvum-infected H69 monolayers was further evaluated using a coculture system. H69 cells grown in the lower chamber in the coculture system, which were not directly infected with C. parvum, also showed characteristic changes of apoptosis (Fig. 6A). Apoptosis of uninfected H69 cells cocultured with infected H69 cells was further confirmed by DNA extraction and agarose gel electrophoresis, which produced a ladder-like pattern from the DNA of those cells (Fig. 6B). H69 cells cocultured with C. parvum sporozoites alone in the upper chamber (no H69 cells grown on the insert) showed no cells undergoing apoptosis (data not shown).

The role of sFasL in C. parvum-induced apoptosis in H69 cells was further confirmed by using a metalloprotease inhibitor in the H69/H69 coculture system. The metalloprotease inhibitor, 1,10-phenanthroline, which prevents the cleavage of sFasL from cell membranes, completely blocked the apoptosis in cocultured uninfected H69 cells (Fig. 6A). In contrast, an increase of apoptosis in H69 cells directly infected with C. parvum was found in the presence of this inhibitor. Furthermore, incubation of H69 cells with recombinant human sFasL for 10 h resulted in significant apoptosis in cells in a dose-dependent manner (Fig. 6C).

Activation of caspase family in cocultured biliary epithelia. To further confirm the role of Fas/FasL pathway in the mechanisms of apoptosis induced by C. parvum, cytosolic extracts were prepared from H69 cells cocultured with C. parvum-infected biliary epithelia, and caspase activity in the lysates was determined using a fluorescent substrate Ac-DEVD-AFC. A time-dependent increase of caspase activity was detected in those cocultured-uninfected H69 cells (Fig. 7).

Apoptotic cytotoxicity of C. parvum-infected human cholangiocytes to cocultured Fas-sensitive Jurkat cells. To further confirm that Fas/FasL interactions are involved in C. parvum-induced apoptosis, we designed cytotoxicity tests using our coculture system in which C. parvum-infected H69 monolayers were used as effectors and noninfected Jurkat cells as targets, in the presence or absence of the FasL neutralizing antibody NOK-1. As shown in Fig. 8, about 14% of FasL sensitive Jurkat E6–1 cells underwent apoptosis after 24 h of coculture with C. parvum-infected H69 monolayers, and the FasL neutralizing antibody NOK-1 completely blocked the apoptosis (Fig. 8). In contrast, no increase of apoptosis above control was found for the Fas-resistant Jurkat JM-3A5 cells cocultured with C. parvum-infected H69 monolayers (Fig. 8). These observations suggested that sFasL was being released from C. parvum-infected H69 cells and was inducing apoptosis of Fas-sensitive cocultured cells.

DISCUSSION
The results of our studies provide the first evidence that the Fas/FasL system is involved in the cytotoxicity of C. parvum for any epithelia. Our data show that
C. parvum infection of cultured human biliary epithelia results in apoptosis of the infected monolayers, a process that is blocked both by caspase protease inhibitors and by Fas or FasL neutralizing antibodies; 2) C. parvum infection induces membrane surface translocation of FasL and stimulates Fas and FasL protein expression in infected human biliary epithelial cells; 3) C. parvum infection releases sFasL from infected monolayers; and 4) uninfected biliary epithelia or Fas-sensitive Jurkat cells undergo apoptosis via a Fas/FasL dependent pathway when cocultured with infected biliary epithelia. The data suggest that C. parvum is cytotoxic for biliary epithelial cells via a mechanism involving Fas/FasL activation and provide insight into the potential pathogenic mechanisms whereby C. parvum causes biliary tract disease.

Apoptosis plays a critical role in the regulation of inflammation and in the host immune response. Recent data in other tissues infected with either parasites (such as Entamoeba histolytica, Schistosoma mansoni, Trypanosoma cruzi, and Toxoplasma gondii) or bacteria are consistent with the concept that microbial pathogens can kill cells by an apoptotic mechanism (8, 24, 27, 45, 57, 58, 61). However, the cellular mechanisms by which individual pathogens induce apoptosis in specific host cells, especially epithelial cells, remain obscure. For some pathogens (e.g., Shigella, Salmonella, E. histolytica) (45, 61), the cells undergoing apoptosis are limited to those directly infected by the pathogen. For other pathogens, e.g., human immunodeficiency virus (HIV) and herpes virus 6 (18, 22), the cells undergoing apoptosis are distinct from those that are infected; in this later instance, the Fas/FasL system has been mechanistically implicated (1, 7, 18, 22). Based on our data, it appears that C. parvum, like HIV and herpes virus 6, can initiate apoptosis in cells like biliary epithelia remote from those cells actually infected by the organism employing Fas/FasL.

The Fas (APO-1 and CD95)/Fas-L system has emerged as an important cellular pathway regulating the induction of apoptosis in a variety of tissues (1, 11, 14, 60). Fas is a widely expressed, 45-kDa type I membrane protein of the TNF-nerve growth factor...
family of cell surface receptors. In cells expressing Fas, apoptosis occurs after Fas interaction with 1) its natural ligand FasL, a 37-kDa type II protein; 2) agonistic anti-Fas antibody; or 3) sFasL, a biologically active form of FasL released from cell membranes by a metalloprotease (20, 23, 35, 50, 54). By activating a variety of downstream effector cellular proteases, including members of the caspase family, Fas induces apopto-

Fig. 6. Apoptosis in cocultured H69 cells and cytotoxicity of recombinant human sFasL to H69 cells. A: both infected (upper chamber) and uninfected (lower chamber) H69 showed apoptosis after 24 h of coculture. Addition of 0.2 mM 1,10-phenanthroline to upper chamber at same time as C. parvum was added completely blocked apoptosis in cocultured uninfected H69 cells. In contrast, a significant increase of apoptosis in infected cells in presence of inhibitor was found. B: ladder pattern indicative of internucleosomal cleavage characteristic of apoptosis was seen when DNA was extracted from C. parvum-infected monolayer (lane 2) or cocultured uninfected cells (lane 3) and subjected to agarose gel electrophoresis; lane 1 contains molecular weight markers (100 bp DNA ladder). This laddering was not seen in the sham-infection control cells when equal amounts of DNA was loaded (sham-infected cells, lane 4; cocultured cells, lane 5). C: recombinant human sFasL induces apoptosis in H69 cells. Incubation of cells with recombinant human sFasL for 10 h at a dose of 100–1,000 ng/ml resulted in significant apoptosis in H69 cells in a dose-dependent manner. *P < 0.01 compared with normal control. **P < 0.05 compared with infection.

sis (35, 36). The biological importance of the Fas/FasL system has been extensively studied in T cells, where it plays a critical role in the clonal deletion of autoreactive T cells and in the activation-induced suicide of T cells (20, 35, 36). Fas/FasL is also extensively expressed in epithelial cells and mediates apoptosis in epithelia in a variety of organs (3, 13, 32, 41, 44, 48). In the digestive tract, the Fas/FasL pathway has been reported to play a role in the apoptosis of colonic epithelial cells in ulcerative colitis (52), in gastric epithelial cells infected by Helicobacter pylori (47), and in hepatocytes in acute Wilson's disease (51). Biliary epithelial cells have been shown to express Fas by immunohistochemistry in primary biliary cirrhosis, primary sclerosing cholangitis (25), and in noncancerous lesions of hepatocellular carcinoma patients with chronic hepatitis or liver cirrhosis (16, 19).

To test the potential importance of the Fas/FasL pathway in regulation of apoptosis in C. parvum-infected biliary epithelia, we examined the effects of

Fig. 7. Activation of caspase activity in H69 cells cocultured with C. parvum-infected H69 cells. After different periods of coculture, uninfected H69 cells on the plates were harvested, and lysates were prepared and assayed. Caspase activity was estimated with caspase substrate Ac-DEVD-AFC. Activation of caspase activity was found in cells cocultured with C. parvum-infected H69 cells. Each point represents mean values of triplicates of 2 separate experiments. *P < 0.01 compared with controls at time 0.

Fig. 8. Apoptosis in Jurkat cells cocultured with C. parvum-infected H69 monolayers. Jurkat cells were cocultured with C. parvum-infected H69 cells. After 24 h of coculture, Jurkat cells were fixed and apoptosis was assayed by DAPI staining. Fas-sensitive Jurkat cells (Jurkat E6–1), but not Fas-resistant Jurkat cells (Jurkat JM-3A5), cocultured with C. parvum-infected H69 cells showed characteristic changes of apoptosis. FasL neutralizing antibody completely blocked the apoptosis. *P < 0.01 compared with normal control.
antagonistic anti-Fas, neutralizing anti-FasL antibodies, and caspase inhibitors on C. parvum-induced apoptosis in cultured biliary epithelia. Both types of antibodies as well as caspase inhibitors significantly reduced apoptosis in C. parvum-infected H69 monolayers by up to 80%. Our data also showed that C. parvum infection can stimulate cytoplasmic FasL membrane surface translocation and induce the expression of Fas and FasL in infected biliary monolayers. Upregulation of Fas and FasL proteins, coupled with the C. parvum-induced FasL membrane translocation and release of sFasL (an event blocked by a metalloprotease inhibitor) should adequately explain the accelerated apoptosis of both the biliary epithelia directly infected with C. parvum and those cocultured with the infected cells.

Thus it appears that FasL may function in either autocrine or paracrine pathways to produce apoptosis in this model (Fig. 9). FasL, translated to the membrane from the cytoplasm can interact with Fas on adjacent cells, activate the pathway and therefore induce apoptosis. Indeed, whereas the metalloprotease inhibitor completely blocked the apoptosis in cocultured uninfected biliary epithelia, an increase of apoptotic cytokines, such as IL-1β, TNF-α, and TGF-β, showed no significant change. Activation of caspase activity in uninfected biliary epithelial cells was also found when cocultured with C. parvum-infected biliary epithelia. These results further support the notion that C. parvum infection of human biliary epithelium induces uninfected cells to undergo apoptosis via a Fas/FasL dependent paracrine mechanism.

Reports about the cytotoxicity of sFasL are conflicting. Our present observations do not agree with those of Schneider et al. (49). In that study, investigators found that sFasL released by metalloproteases had very low Fas-mediated cytotoxicity (1,000 times less than membrane FasL). Instead, the Fas-mediated cytotoxicity found in the media was attributed to a large (>500 kDa) product that probably represented fragments of membrane-bound FasL or vesicles containing membrane-bound FasL. However, our results are in agreement with other reports that observed cytotoxicity of sFasL. For example, sFasL released from stimulated T cells produces apoptosis of Fas-sensitive Jurkat T cells; also, recombinant sFasL has been reported to induce apoptosis both in vitro and in vivo (26, 29, 54, 55). Although immortalization of cells with SV40 may decrease p53 expression and thus increase cellular resistance to apoptosis, and although sFasL is not apoptotic in all cell lines in vitro (40, 53), our data show that H69 cells are relatively sensitive to sFasL-induced apoptosis.

Although uninfected cultured human biliary epithelial cells express both Fas and FasL, our results with the cocultured biliary epithelial cells and Fas-sensitive Jurkat T cell line indicate that biliary epithelial cells mediate apoptosis of target cells only when they are infected with C. parvum. One possible explanation for this observation is that FasL may not be localized at the membrane in noninfected cells and thus is not self-toxic, as confirmed by our immunocytochemical staining showing the absence of FasL on the cell membrane surface under unstimulated conditions. This also appears to be the situation in Jurkat cells, which also express both Fas and FasL under unstimulated conditions (29). Soluble factors elaborated directly from C. parvum are not likely to play a role in the apoptotic cytotoxicity because biliary epithelial cells cocultured with C. parvum alone (separated by the insert membrane) do not undergo accelerated apoptosis.

In summary, using an in vitro model of biliary cryptosporidiosis, we found that C. parvum induced apoptosis in infected human biliary epithelial cultures, and C. parvum infection of biliary epithelial cells can further induce uninfected cells to undergo apoptosis via a Fas/FasL-dependent mechanism likely involving both autocrine and paracrine pathways (Fig. 9). Future studies should address the mechanism(s) by which C. parvum infection results in cleavage of sFasL from cell membranes and how this organism increases the expression of Fas and FasL.

We thank Drs. F. Que and A. Celli for helpful advice and Dawn Lubinski and Deb Hintz for excellent secretarial assistance. This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-24031 (N. F. LaRusso) and a grant from the Mayo Foundation.

Address for reprint requests and other correspondence: N. F. LaRusso, Center for Basic Research in Digestive Diseases, Mayo Clinic, 200 First St., S.W., Rochester, MN 55905 (E-mail: larusso.nicholas@mayo.edu).

Received 14 January 1999; accepted in final form 9 June 1999.
REFERENCES


