MAJOR ARTICLE

Cryptosporidium Infection of Human Intestinal Epithelial Cells Increases Expression of Osteoprotegerin: A Novel Mechanism for Evasion of Host Defenses

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Cryptosporidium **parasites are pathogens of human intestinal epithelial cells. To determine which genes are regulated during early infection, human ileal mucosa cultured as explants was infected with *C. parvum* or *C. hominis,* and gene expression was analyzed by microarray. The gene for osteoprotegerin (OPG) was up-regulated by both parasites. OPG mRNA was also significantly increased in biopsy specimens obtained from a volunteer experimentally infected with *C. meleagridis,* compared with levels in a prechallenge biopsy specimen. After in vitro infection of HCT-8 cells, there was an early peak in production of OPG mRNA protein. Treatment of infected cells with the OPG ligand tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induced epithelial cell apoptosis and reduced parasite numbers, and recombinant OPG blocked these effects. These results suggest a novel TRAIL-mediated pathway for elimination of *Cryptosporidium* infection and a role for OPG in modulating this host response.**

Diarrheal diseases remain a major cause of morbidity and mortality worldwide [1]. However, relatively little is known about the host defenses in the human gut, especially in the small intestines. Cryptosporidiosis is caused by protozoan parasites that infect human intestinal epithelial cells. The host immune response is critically important in control of cryptosporidiosis [2]. In immunocompetent patients, cryptosporidiosis presents as a self-limited diarrheal illness. After recovery, normal hosts are partially resistant to reinfection. By contrast, patients with AIDS develop chronic diarrhea, which is often fatal. In the absence of an effective host response, cryptosporidiosis cannot be reliably cured with available antiparasitic drugs. AIDS-associated chronic cryptosporidiosis can resolve with immune recovery, but the efficacy of antiretroviral therapy may be compromised by malabsorption of HIV drugs in the setting of chronic diarrhea [3].

The mechanisms by which parasites are cleared and the mechanisms used by the parasites to evade the host response are poorly understood. $CD4^+$ T cells play a key role in the control of human cryptosporidiosis [4–6]. Cytokines, particularly interferon-γ, are important in the immune response controlling cryptosporidiosis in murine and human studies [7–9].

To complete their life cycle, *Cryptosporidium* organisms depend on survival of the host epithelial cell for at least 48 h. Studies of infected intestinal tissues suggest that there is increased apoptosis of both infected and adjacent cells [10, 11]. Initial invasion induces apoptosis of the infected cells [12–15]. However, within a few hours, the parasite activates host cell NF-κB, which, in turn, activates antiapoptotic mechanisms. By 48–72 h,

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Table 1. Genes that are up-regulated in normal human tissue after *Cryptosporidium* infection.

	C. hominis		*C. parvum*	
Gene name (symbol)	Fold change	*P*	Fold change	*P*
TNF receptor superfamily, member 11b (osteoprotegerin) *(TNFRSF11B)*	2.34	.019	1.58	.094
Platelet-derived growth factor D *(PDGFG)*	1.55	.049	1.56	.030
CD59 molucule, complement regulatory protein *(CD59)*	1.51	.051	1.47	.043
CD24 molecule *(CD24)*	1.67	.143	1.95	.047
Striatin, calmodulin-binding protein *(STRN)*	1.27	.191	1.52	.032

NOTE. Paired infected and uninfected tissues from each of 3 normal tissue samples were compared by microarray analysis. TNF, tumor necrosis factor.

antiapoptotic factors no longer dominate, and the host cell dies. Epithelial cell death has features of both apoptosis and necrosis [16]. Interestingly, early apoptosis of the infected cells in vitro leads to decreased parasite numbers [17, 18]. Thus, epithelial cell apoptosis may be critical for clearing parasites before the intracellular forms become infectious.

We used microarray analysis to study the effects of *Cryptosporidium* infection on normal human intestinal mucosa in vitro. We identified the gene for the tumor necrosis factor (TNF) superfamily receptor osteoprotegerin (OPG) as being the most prominently up-regulated. This led to the hypothesis that the parasite up-regulates OPG as a way of evading the response of the host. In the present article, we detail evidence for a novel OPG-mediated mechanism for evasion of host defenses.

MATERIALS AND METHODS

Parasites. *C. parvum* oocysts (Iowa isolate) were purchased from the University of Arizona and stored at 4°C in an antibiotic solution (0.01% Tween 20 containing 100 U/mL penicillin and 100 μg/mL gentamicin). *C. hominis* oocysts (TU502; isolated from gnotobiotic pigs and provided by D. Akiyoshi and S. Tzipori of Tufts University School of Veterinary Medicine) were stored in a 2.5% potassium dichromate solution at 4°C. The excystation and preparation of parasites for infections was done according to a standard protocol [19, 20]. The oocysts were washed with 0.15 mol/L PBS (pH 7.2), centrifuged (3000 *g,* 15 min, 4°C), and treated with acidic H_2O (pH 2.5) for 20 min at 37°C, with vortexing every 5 min. The oocysts were centrifuged (3000 *g,* 4 min, 20°C), resuspended in parasite maintenance medium (Dulbecco's modified Eagle medium containing 4.5 g/L D-glucose, 0.58 g/L L-glutamine, 3.7 g/L sodium bicarbonate, 0.2 g/L bovine bile, 0.004 g/L folic acid, 0.001 g/L 4-aminobenzoic acid, 0.004 g/L D-calcium pantothenate, 0.88 g/L ascorbic acid, 1% heat-inactivated fetal bovine serum [FBS], 2.4 g/L HEPES, 100,000 U/L penicillin, 100,000 μg/L streptomycin, and 250 μg/L amphotericin B, adjusted to pH 7.4), and incubated for 3 h in a 37°C water bath. The intact and empty oocysts were counted and used to determine the percentage of excystation.

Ileal tissue explants and biopsies. Ileal tissue was obtained from 3 individuals undergoing surgical procedures for unrelated noninfectious conditions (ileostomy takedown, right hemicolectomy for localized colon cancer, and trauma). Laboratory personnel collected ileal tissue in the operating room at the time of surgery. The tissue was immediately transported to the laboratory in CMRL-1066 medium. The mucosal layers were removed mechanically, and the mucosa from each patient was divided into four 3-mm^2 pieces. Each piece was placed luminal side up, onto polycarbonate-coated insert cups. The inserts were then placed into culture wells containing CMRL-1066 medium supplemented with 5 g/L D-glucose, 0.3 mmol/L L-glutamine, 0.3 g/L L-methionine, 0.3 g/L tricine, 5% heat-inactivated FBS, 100,000 U/L penicillin, 100,000 μg/L streptomycin, and 250 μg/L amphotericin B. A small amount of culture medium was also added to the apical surface of each insert cup to keep the explants hydrated. The explants were incubated (3–4 h; 95% O_2 and 5% CO_2) before in vitro infection, as described elsewhere [20].

Oocysts of *C. parvum,* oocysts of *C. hominis* (1×10^6/sample, in 5 mL), and sterile excystation solution were inoculated onto the apical surface of the explants. The explant tissues were kept in vitro for 24 h at 37°C in a chamber containing 95% O_2 and 5% CO_2 and then collected in RNase inhibitor solution (RNA*later;* Ambion) and stored frozen. Control tissue from before infection was placed immediately in RNase inhibitor solution. Explant samples were disrupted and homogenized mechanically by sonication, and total RNA was extracted with the RNeasy Mini Kit (Qiagen) per the manufacturer's protocol. To extract RNA from cultured cells or jejunal biopsy specimens, we disrupted cells by use of QIAshredder columns and the RNeasy Plus Extraction Kit (Qiagen). The quality of the RNA obtained was confirmed by capillary electrophoresis performed with an Agilent 2100 Bioanalyzer.

Gene expression profiles were probed by microarray analysis using the GeneChip Human Genome U133 Plus 2.0 Array (version 2.0; Affymetrix) (33,000 human genes) by the Baylor College of Medicine Microarray Core Facility. Data were adjusted for background and normalized and summarized by the robust multiarray average method [21–23], using the Bioconductor

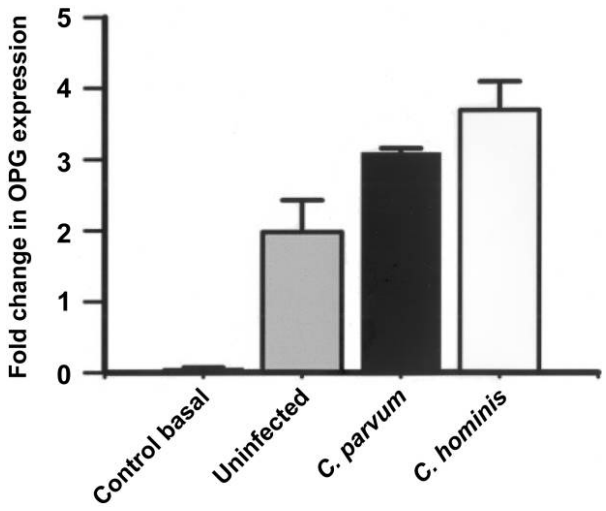

Figure 1. Real-time reverse-transcription polymerase chain reaction for osteoprotegerin (OPG) expression in intestinal tissue (explants) after 24 h. The results are shown as fold increases in OPG mRNA, indicating baseline, uninfected tissue at 24 h *(gray bar),* tissue infected with *Cryptosporidium parvum (black bar),* and tissue infected with *C. hominis (white bar).* The statistical differences between all groups were calculated using an analysis of variance test. The uninfected controls at 24 h were different from each of the other groups ($P < .05$). Compared with baseline, there were significant differences in the *Cryptosporidium*-infected groups ($P < .01$).

package (http://bioconductor.org; version 1.9). The result was a data set of expression values, with each probe set having a single expression value per chip and with the data in $\log_2$ scale. For each probe, data were filtered by whether there was a signal (present or absent calls calculated by GeneChip Operating Software (version 2.0; Affymetrix). Probe sets that had absent calls on all chips were excluded in further analysis. The data were then imported into SAS software (version 9.0; SAS Institute). The *P* values were adjusted by the Benjamini-Hochberg method to control for false-discovery rate resulting from the 2370 probe sets [24].

In addition, jejunal biopsy specimens from a volunteer were provided by P. Okhuysen. The volunteer had undergone an experimental challenge with *Cryptosporidium meleagridis* and underwent upper endoscopy with a pediatric colonoscope before and 20 days after an experimental infection with 1×10^5 *C. meleagridis* oocysts. Jejunal biopsy specimens were placed directly in RNA*later,* and RNA was extracted with the RNeasy Plus Extraction Kit.

Tissue culture and in vitro infection. HCT-8 cells (1×10^6) were plated and cultured in 6-well plates with complete medium (RPMI 1640 plus L-glutamine, 10% heat-inactivated FBS, 100,000 U/L penicillin, and 100,000 μg/L streptomycin) until the cells reached 95% of confluence. *C. parvum* oocysts (1×10^6 in 5 mL) were excysted and then added to each plate (2 h at 37°C; 95% O_2 and 5% CO_2). The cells were then washed 3 times with sterile PBS to remove remnant oocysts, and new complete medium was added to each well. Cultured supernatant fluid was aspirated at baseline and at 1, 6, 12, and 24 h after infection and then stored at 4°C until assayed by ELISA. For reverse-transcription polymerase chain reaction (PCR) experiments, supernatant was removed, and the cells were detached with 0.05% trypsin, collected at the same points, and stored at −70 °C until use.

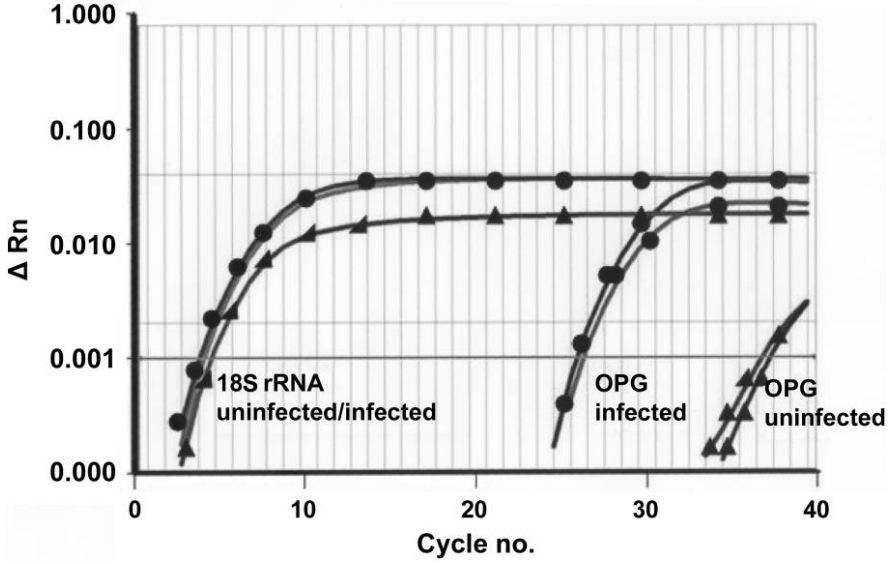

Figure 2. Expression of osteoprotegerin (OPG) in intestinal biopsy specimens after experimental *Cryptosporidium* infection. Jejunal biopsy specimens were obtained from a volunteer before *(triangles)* and 20 days after *(circles)* infection with 1×10^5 oocysts of *C. meleagridis.* OPG mRNA was analyzed by real-time polymerase chain reaction in duplicate and normalized for human 18S rRNA content. OPG mRNA calculated from the cycle threshold demonstrated a 1281-fold increased expression in postchallenge specimens compared with that in paired prechallenge specimens. Rn, fluorescence emission intensity of the normalized reporter.

ELISA. OPG secretion from HCT-8 cells was determined by ELISA using a commercial kit (R&D systems). Briefly, 2 μg/mL anti-OPG diluted in PBS was added to a 96-well plate and incubated overnight. Samples were blocked with a PBS/1% bovine serum albumin solution (3 h, room temperature). Supernatant (100 μL) or standards were added (2 h, room temperature). Detection antibody (100 ng/mL, 100 μL) was added to each well. After 2 h of incubation, streptavidin conjugated to horseradish peroxidase solution was added, and the plate was incubated in the dark for 20 min at room temperature. A 1:1 mixture of H_2O_2 and tetramethylbenzidine solution was added and incubated for another 20 min. Finally, the reaction was stopped by 50 μL of 2*N* H_2SO_4, and the samples were read at 450 nm.

Real-time PCR analysis. The samples were amplified using the SYBR Green SuperScript One-Step Kit (Invitrogen). For each reaction, we used 0.5 μg of RNA and specific primers: for OPG, 5'-ACT AGT TAT AAG CAG CTT ATT TTT ACT G-3' (forward) and 5'-GGA GGC ATT CTT CAG GTT TGC TG-3' (reverse); and for human 18S rRNA, 5'-CCG ATA ACG AAC GAG ACT CTG G-3' (forward) and 5'-TAG GGT AGG CAC ACG CTG AGC C-3' (reverse). Reactions were performed with the ABI PRISM 7300 system (Applied Biosystems), and conditions were as follows: for cDNA synthesis, 50°C for 30 min and then 94°C for 2 min; for PCR amplification, 94°C for 30 s and then 60°C for 1 min, for 40 cycles. The cycle-threshold values were determined using the 7300 system SDS software, to calculate the fold increase between control and infected samples according to the relative quantification method described elsewhere [25]. The signal for OPG mRNA was expressed as a ratio to the signal for human 18S rRNA, as described elsewhere [26].

Parasite staining. For some in vitro experiments, *C. parvum* oocysts were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) following a protocol described elsewhere [27], with modifications. The oocysts were washed with PBS and centrifuged at 300 *g* for 4 min. The pellet was resuspended in H_2O (pH 2.5) and incubated in ice for 10 min. The oocysts were centrifuged as before, resuspended in a solution of 0.8% taurocholate-PBS, and incubated at 37°C for 15 min. CFSE (4 mmol/L) was added to the sample, which was incubated for another 15 min. Oocysts were then washed 3 times with PBS and resuspended in 0.8% taurocholate–RPMI complete medium.

TNF-related apoptosis-inducing ligand (TRAIL) and OPG effect by flow cytometry. OPG functions as a soluble decoy receptor for 2 TNF superfamily ligands: TRAIL and TRANCE (TNF-related activation-induced cytokine; also known as "RANK ligand"). To determine the effect of recombinant TRAIL and OPG on parasite numbers, we plated HCT-8 cells in 12-well plates and infected them with CFSE-stained parasites (5×10^5, 2 h, 37°C). Infected cells were treated with recombinant TRAIL (Biosource) at concentrations of 0, 25, 50, and 100 ng/mL (37°C, 5% CO_2). To determine the effect of OPG on parasite numbers, recombinant OPG (100 ng/mL; R&D Systems) was added to infected cells treated with TRAIL at concentrations of 0, 25, 50, or 100 ng/mL. After incubation (6 h), cells were detached with 0.05% trypsin, washed with 1× PBS, fixed with 4% formaldehyde in PBS (20 min), and analyzed by flow cytometry performed with the Beckman-Coulter EPICS XL-MCLs flow cytometer. The results were analyzed with WinMDI software (version 2.9).

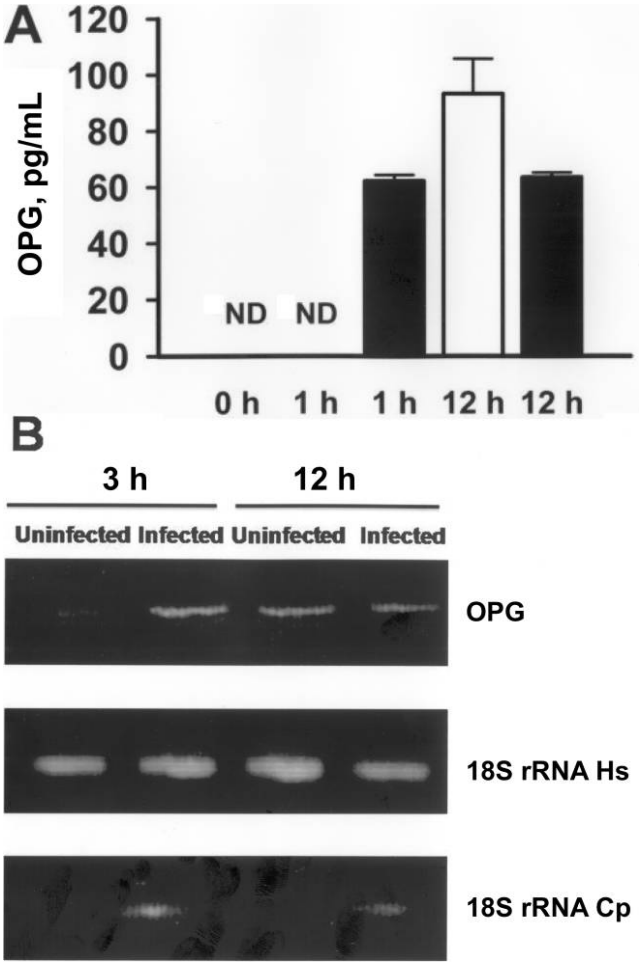

Figure 3. Production of osteoprotegerin (OPG) by HCT-8 cells after *Cryptosporidium parvum* infection. *A,* Infection of HCT-8 cells. Cells were infected with 3 *C. parvum* oocysts per HCT-8 cell. Aliquots were removed at baseline and 3 and 12 h after culturing and assayed for OPG by sandwich ELISA. Supernatants from infected *(white bar)* and uninfected *(black bars)* HCT-8 cells are shown. *B,* OPG expression. mRNA levels in uninfected and infected HCT-8 cells were determined by reverse-transcription polymerase chain reaction after 3 and 12 h of incubation. Controls included primers for human rRNA (r18S Hs) and *Cryptosporidium* rRNA (r18S Cp). ND, not detected.

RESULTS

OPG expression defined by microarray analysis. To test for host factors that might modulate the early response to infection,

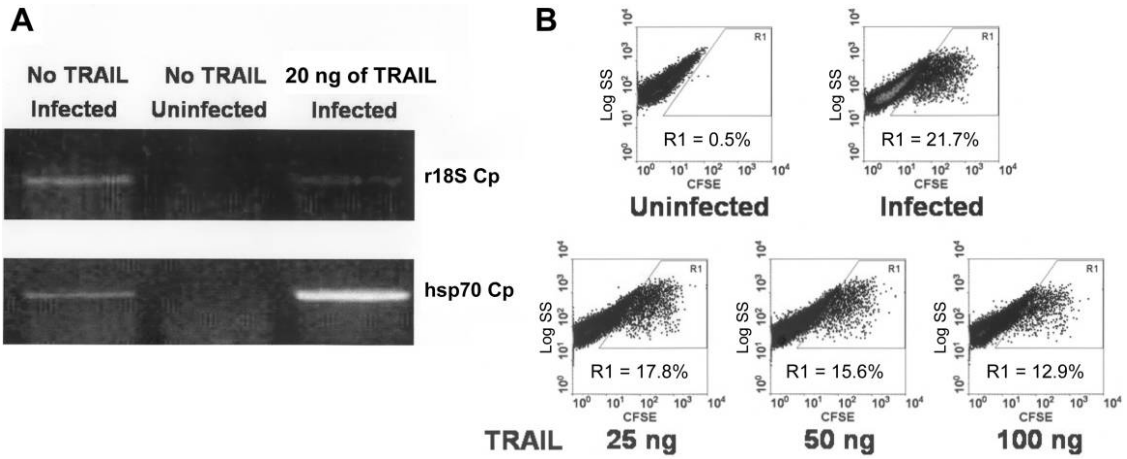

Figure 4. Effect of recombinant tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) on *Cryptosporidium* infection of epithelial cells. *A,* Reverse-transcription polymerase chain reaction of HCT-8 cells to detect *Cryptosporidium* rRNA (r18S) and hsp70. Cells were infected with 1×10^6 *Cryptosporidium parvum* and incubated for 8 h in medium with or without 20 ng of recombinant TRAIL. *B,* Flow cytometry of HCT-8 cells infected with *C. parvum* oocysts labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). The groups were treated with several concentrations of TRAIL (0, 25, 50, and 100 ng). R1, region 1 (area of infected cells); SS, side scatter.

we developed a system to infect normal human small intestinal tissues cultured as mucosal explants [20]. Overall, the gene expression profile was very similar for all specimens. This reflects the fact that we used paired specimens, that *Cryptosporidium* infects only epithelial cells, and that only a minority of the epithelial cells were infected. Several genes were significantly up- or down-regulated (table 1). Of the genes found to be significantly up-regulated in the presence of *Cryptosporidium,* the gene for OPG was up-regulated after infection with both species of *Cryptosporidium* but with a greater fold change in *C. hominis* than in *C. parvum*. We confirmed the level of expression in explant tissues by quantitative real-time PCR (figure 1). Overall, there was significantly increased expression of OPG mRNA after culture of the tissues as explants compared with baseline, even in uninfected tissues. In uninfected tissues, there was a 2-fold increase in OPG mRNA after overnight incubation. Expression was further increased after infection with either *C. parvum* (3-fold) or *C. hominis* (nearly 4-fold) (both $P < .05$ compared with uninfected tissues and $P < .01$ compared with baseline tissues) (figure 1). All of these levels are relatively modest, but given the low rate of infection and the fact that the explant tissues include lamina propria as well as epithelium, this represents a substantial up-regulation of expression.

To confirm that OPG was expressed in vivo, jejunal biopsy specimens were obtained from a volunteer before and after experimental infection with *Cryptosporidium meleagridis*. Compared with a biopsy specimen obtained before challenge, there was a 1281-fold increase in OPG mRNA in the postchallenge biopsy specimen (figure 2). Thus, we confirmed increased expression of OPG mRNA in the infected tissue.

***Secretion and expression of OPG by epithelial cells in response to* Cryptosporidium.** To determine whether OPG is produced by epithelial cells, we infected HCT-8 cells in vitro with *C. parvum*. As early as 1 h after infection, we detected an increase in OPG mRNA. Expression was markedly up-regulated in infected but not control cells at 3 h after infection (figure 3). Subsequently, the amount of OPG mRNA in control tissues increased such that by 12 h there was no longer a difference between infected and control cells. OPG protein was detected in culture supernatants as early as 1 h after infection. By 12 h, however, the levels were similar for infected and uninfected tissues. Thus, there is an early burst of OPG mRNA and protein secretion that begins near the time of infection.

Elimination of parasites from infected cells induced by TRAIL. Because TRAIL causes apoptosis, we reasoned that it might be involved in the clearance of infected cells. To test this hypothesis, HCT-8 cells were infected with *C. parvum*. Shortly thereafter, the infected cells were treated with varying concentrations of TRAIL. The number of parasites in the cells was then assessed by real-time PCR for parasite-specific rRNA and for parasite-specific hsp70 (figure 4). Overall, there was a marked decrease in parasite rRNA. By contrast, parasite-specific hsp70 actually increased. Because hsp70 is up-regulated by stress, these data suggest that parasite numbers are decreasing and that the remaining parasites are stressed. To confirm that parasite numbers were decreasing, oocysts were stained with CFSE before infection. At 6 h after treatment with TRAIL, the numbers of parasites remaining were counted by flow cytometry, with reductions of >50% (figure 4).

Induction of apoptosis in* Cryptosporidium*-infected cells by TRAIL. Under pathological conditions, TRAIL became a potent inducer of apoptosis in intestinal epithelial cells. To determine the effect of TRAIL as an inducer of apoptosis in *Cryptosporidium*-infected cells, we treated the cells with several

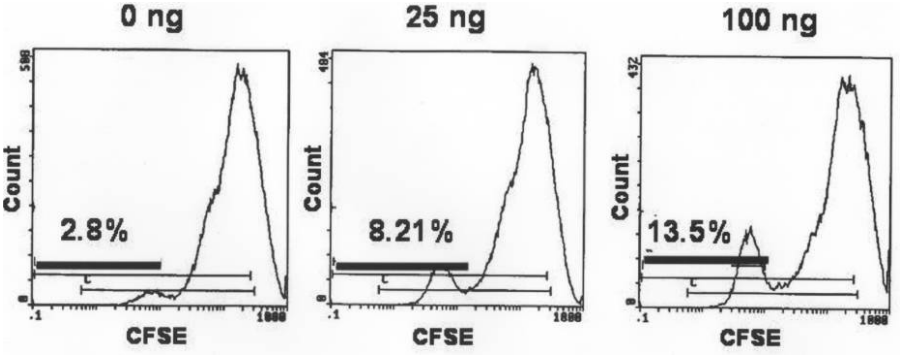

Figure 5. Apoptosis in infected HCT-8 cells. Cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated with different concentrations of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) (0, 25, and 100 ng) for 8 h. The apoptosis rate was deemed to be the reduction in CFSE signal, as determined by flow cytometry.

concentrations of recombinant TRAIL (0, 25, and 100 ng). We observed that only a small minority of infected cells underwent apoptosis in the absence of TRAIL. By contrast, the addition of TRAIL increased the level of apoptosis in a dose-dependent fashion. Thus, TRAIL induced apoptosis of infected cells (figure 5).

Protection of* Cryptosporidium *by OPG. The ability of OPG to bind to TRAIL and prevent it from associating with death-inducing receptors suggests that OPG could act as an additional soluble decoy receptor for TRAIL, preventing induction of the apoptotic signaling cascade by TRAIL and thereby providing cells with a survival advantage. To test the hypothesis that OPG protects the parasites against TRAIL-mediated apoptosis, we used *Cryptosporidium*-infected HCT-8 cells treated with 0, 25, 50, or 100 ng of TRAIL plus 100 ng of recombinant OPG. The results show that OPG blocks the effect of TRAIL on parasite numbers (figure 6).

DISCUSSION

In this study, we used in vitro infection of human explants and microarray analysis to determine gene expression in response to *Cryptosporidium* infection. The result showed that a limited number of genes were significantly overexpressed after infection. Unexpectedly, the microarray analysis demonstrated that OPG was one of the most prominently up-regulated genes in the infected explants. The results were consistent for both *C. parvum* and *C. hominis*. We also confirmed expression of OPG mRNA in jejunal tissues obtained after experimental human infection. We noted significant differences in OPG production at the RNA and protein levels, particularly during the first few hours after infection in vitro. We also demonstrated that TRAIL decreases parasite numbers in infected cells and that addition of recombinant OPG blocked the effect of TRAIL.

OPG is expressed and secreted constitutively at moderate levels in different types of mammal cells, including intestinal epithelial cells [28]. Overexpression of OPG has been reported in cells under pathological conditions, such as cancer or inflammatory bowel disease [29–31].

Previous studies done with *Cryptosporidium* showed that the infected cells displayed mildly increased apoptosis compared with uninfected cells. The apoptosis rate was considerably augmented by inhibition of NF-κB, which produced a significant reduction in the number of parasites [12]. Interestingly, OPG is regulated by NF-κB, and up-regulation of OPG inhibits epithelial cell apoptosis [32]. We observed that OPG is maximally secreted within 3 h of infection, supporting the idea that OPG might protect infected cells against early apoptosis. Induction of apoptosis by TRAIL has been reported in epithelial cells [33]. TRAIL induces apoptosis by binding to TRAIL-R1 and

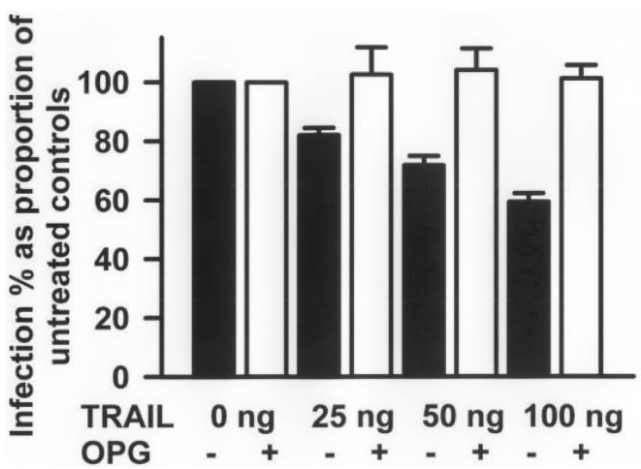

Figure 6. Effect of recombinant osteoprotegerin (OPG) on infected cells treated with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). Cells were treated with different concentrations of TRAIL *(black bars).* In addition, 100 ng of recombinant OPG was added to all of the groups of cells previously treated with TRAIL at the same concentrations *(white bars).* The decrease in infection rate was defined as the reduction in the rate of infection as a proportion of untreated controls.

TRAIL-R2. Both are classic "death receptors" characterized by an intracellular death domain, which allows direct activation of the caspase signaling, inducing apoptosis of a target cell. To test the effect of TRAIL in infection, we added TRAIL to infected HCT-8 cells and noted significant dose-dependent parasite reduction in infected cells compared with untreated cells. Thus, we hypothesized that addition of OPG might serve to antagonize TRAIL. Our results showed that addition of recombinant OPG increased the infection rate to levels comparable to that in untreated cells. Previous reports of studies using *Leishmania major* in dendritic cells described how the blockade of RANK promotes interleukin 12 production and the activation of a protective Th1 response against the infection [34]. OPG might also function to block antigen presentation by dendritic cells [35]. However, we have no clear evidence of this to date.

In the present work, we demonstrated that OPG is produced during the early stages of infection and prevents early apoptosis, allowing *Cryptosporidium* organisms to complete their life cycle. Future studies should further elucidate the role played by OPG and open the possibility that OPG can be targeted for the clearance of cryptosporidiosis.

Acknowledgments

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