Soluble Receptor (DcR3) and Cellular Inhibitor of Apoptosis-2 (cIAP-2) Protect Human Cytotrophoblast Cells Against LIGHT-Mediated Apoptosis

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LIGHT (tumor necrosis factor superfamily 14) is among the powerful apoptosis-inducing cytokines synthesized in human placentas. Here, we investigated mechanisms protecting cytotrophoblast (CTB) cells from LIGHT-mediated apoptosis. Viability assays and caspase-3 immunoblots using recombinant LIGHT were done to establish that CTB cells purified from term placentas resist LIGHT-induced apoptosis. Although the cells were also resistant to killing by another placental cytokine, interferon- γ (IFN- γ), a combination of the two induced apoptosis. Killing was prevented by DcR3-Fc fragment but not control human-Fc fragment, showing that apoptosis occurs via the LIGHT pathway and that soluble receptors provide protection. Next, two cellular inhibitors of apoptosis expressed in CTB cells, cellular inhibitor of apoptosis (cIAP)-1 and cIAP-2, were investigated for protection. Cellular IAP-1 was unchanged after stimulation with LIGHT whereas cIAP-2 mRNA and protein were elevated. The increase was abrogated by treating CTB cells with LIGHT + IFN- γ , implying a central role for cIAP-2 in preventing LIGHT-mediated apoptosis and an ability of IFN- γ to overcome cIAP-2 protection. Definitive evidence was provided in experiments that showed that cIAP-2 anti-sense morpholinos permit LIGHT to induce apoptosis in HT-29 cells. In summary, the data are consistent with the postulate that placental CTB cells are protected from LIGHT-mediated apoptosis by both soluble receptor, DcR3, and cIAP-2. (*Am J Pathol* 2004, 165:309–317)

Human placentas are sites of production of essentially all of the apoptosis-inducing tumor necrosis factor (TNF) family ligands identified to date.¹ Among these is LIGHT [homologous to lymphotoxin, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator (HVEM), a receptor expressed by T lymphocytes], which is also known as TNF superfamily 14. LIGHT is a 29-kd type II transmembrane protein that is present in both the cytosol and on cell mem-

branes, and circulates as metalloproteinase-cleaved soluble protein.^{2–4} LIGHT is not only a powerful mediator of T-cell activation^{2,3,5,6} but also induces apoptosis in some tumor cells.^{3,5,6–10}

LIGHT is biologically active as a homotrimer^{5,9} and has three receptors. Two of these are membrane-bound and transduce intracellular signals [HVEM, lymphotoxin- β receptor (LT β -R)], and one is a soluble receptor (DcR3, also known as TR6). Although it was originally suggested that coincident expression of the two membrane-bound receptors is required for apoptotic signaling,⁷ more recent studies have demonstrated that LT β -R alone is capable of transducing an apoptotic signal.^{8,9} DcR3, the soluble receptor, can prevent LIGHT from binding to either HVEM or LT β -R.^{11–13} A second cytokine, interferon (IFN)- γ has been shown to facilitate LIGHT-mediated apoptosis in tumor cells using an as yet unidentified pathway.^{7,13}

LIGHT and its three receptors are abundant in human placentas. Specific mRNAs as well as proteins have been reported.^{1,5,14,15} Immunohistochemical studies have identified LIGHT signals in both the syncytiotrophoblast layer and in the villous mesenchymal cells of term placentas, and immunoblots have detected abundant LIGHT protein in placental lysates. Although light microscopic identification of cytotrophoblast (CTB) cells is difficult in term placentas because of the rarity of this subpopulation, lysates of CTB cells purified from term placentas contain LIGHT.¹⁵ LIGHT is also found in the amnion membrane, in the fetal mesenchymal cells located between the amnion and chorion membranes, and in the decidua.¹⁵ All three LIGHT receptors have been localized to specific cells in human term placentas and the extraplacental membranes by immunohistochemistry, and isolated CTB cells have been shown to contain all three receptor mRNAs¹ and specific proteins.¹⁵ Neither LIGHT nor any of its receptors is present in the extravillous CTB cells that comprise the chorion membrane, indicating that signaling through the LIGHT/LIGHT-R sys-

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tem is not an invariable function of all trophoblast-derived trophoblast cell subpopulations.

The human placenta is also a site of production of IFN- γ and its receptors,^{16–19} which is relevant to potential LIGHT-mediated apoptosis because of its known ability to enhance LIGHT-mediated apoptosis.^{7,13} The binding of IFN- γ to its receptor activates two Janus family kinases, Jak1 and Jak2, which phosphorylate the IFN- γ receptor subunit, IFNGR-1, on specific tyrosine residues. These then provide docking sites for the STAT-1 transcriptional activator. The importance of this series of interactions to mobilization of cell death pathways is revealed in studies on cells lacking STAT-1, which show reduced cell death in response to apoptotic stimuli.²⁰

Local production of LIGHT and its receptors as well as the facilitator cytokine, IFN- γ , raises a critical question about how might placental cells be protected from LIGHT-mediated apoptosis. Although one pathway could be through interference by the soluble receptor, DcR3, other mechanisms are possible. For example, human CTB cells contain a number of proteins in the inhibitor of apoptosis (IAP) family.²¹ Two members of this family, cIAP-1 and cIAP-2 (also known as HIAP-2 and HIAP-1, respectively), interfere with TNF- α -mediated apoptosis by blocking activation of caspase-3.^{22–24} Apoptosis-inducing ligands in the TNF superfamily are highly homologous, with 27 to 34% identity, suggesting that cIAP-1 and cIAP-2 might also interfere with LIGHT-mediated apoptosis.

To address this critical biological question, we investigated LIGHT-mediated apoptosis in purified CTB cells harvested from term placentas, and explored the possibility that DcR3, cIAP-1, and/or cIAP-2 protect these cells. The results indicate that LIGHT is prevented from killing CTB cells by both soluble receptor and cIAP-2. Simultaneous exposure to IFN- γ overcomes this latter protective effect, suggesting that in cases of infection, placental cells may become more susceptible to LIGHT-mediated damage.

Materials and Methods

Reagents

Recombinant human LIGHT (rhLIGHT), rhDcR3/TR6-Fc, and rhIFN- γ were purchased from R&D systems (Minneapolis, MN). The characteristics of these proteins have been reported.^{5,11,14,25} Chrompure human IgG₁ Fc fragment (Hu-Fc) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Placentas and CTB Purification Procedures

Human placentas from cesarean section deliveries performed at term for benign conditions or to relieve fetal distress were obtained under a protocol approved by the Human Subjects Committee of the University of Kansas Medical Center. Samples were taken randomly from floating villi after dissecting and discarding the fibrous plate. Underlying pathology was not evident on either gross or

histological examination of samples. CTB cells were purified from term placenta by enzymatic digestion, gradient centrifugation, and immunomagnetic purification using a monoclonal antibody against HLA-A,B,C (W6/32, no. HB95; American Type Culture Collection, Manassas, VA), as described.^{16,26,27} To assess purity of CTBs, Cytospin (Shandon, Pittsburgh, PA) preparations of cells were analyzed by immunohistochemical staining using mouse anti-pan cytokeratin (Lu-5; Biogenex, San Ramon, CA), which detects all trophoblast cells, and mouse anti-CD14 (Zymed, San Francisco, CA), which detects contaminating macrophages. Less than 1% of cells were immunoreactive for CD14. We further qualified the purity of our samples by immunoblotting and immunohistochemical staining using mouse anti- β -hCG (clone CG05; Neomarkers, Fremont, CA) to detect any contaminating syncytial fragments.²⁶ Less than 4% of the cytopsin-prepared cells demonstrated immunoreactivity for β -hCG suggesting very few contaminating syncytial fragments. β -hCG protein was not detectable by immunoblot in these samples indicating that highly pure populations of CTBs were isolated.

Cell Culture

The human colon adenocarcinoma cell line, HT-29, was purchased from American Type Culture Collection. HT-29 cells and CTB cells were grown in their respective complete growth media.^{12,28} After a 12-hour incubation at 37°C in 5% CO₂, cells were washed once with media to remove nonadherent cells, and were cultured with or without cytokines for 2 or 4 days as indicated in the Results section and in the figure legends.

MTT Assay

Purified CTB cells (1×10^5 /well) were treated with serial dilutions of rhLIGHT alone or together with rhIFN- γ (100 U/ml), rhTR6-Fc (4 μ g/ml), Hu-Fc (4 μ g/ml), or control medium in a total volume of 100 μ l of serum-free complete Iscove's-modified Dulbecco's (IMDM) medium (Cellgro, Herndon, VA) in 96-well flat-bottom microplates. The CellTiter 96 nonradioactive assay (Promega, Madison, WI) was used to assess cell viability. In brief, the cells were incubated with modulators or control medium for 48 or 96 hours then the indicator dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] was added and cultures were continued for 6 hours at 37°C, 5% CO₂. Subsequently, 100 μ l of stop solution was added and, after incubating the microplates overnight in a humidified chamber to facilitate solubilization of formazan crystals, the A₅₇₀ was measured by spectrophotometer (Elx-808; Bio-Tek Instruments Inc., Winooski, VT). HT-29 cells (5×10^3 /well) grown in medium containing 10 U/ml rhIFN- γ served as a positive control. In some experiments these cells were incubated with serial dilutions of rhLIGHT in complete Dulbecco's modified Eagle's medium (Cellgro), as above for CTB cells.

Immunoblotting

Protein samples were prepared from lysed cells by standard methods as previously reported.¹⁵ Protein quantification was performed using the manufacturer's protocol (Bio-Rad Laboratories, Richmond, CA). Twenty-five μg of total protein were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, then were electrophoretically transferred to 0.2- μm -supported nitrocellulose (Schleicher & Schuell, Keene, NH), for 75 minutes at 100 V (27°C) in Tris/glycine buffer (Bio-Rad Laboratories). For detection of cIAP-2, caspase-3, and actin, 0.75 $\mu\text{g}/\text{ml}$ of rabbit anti-human cIAP-2 antibody (H-85; Santa Cruz Biotechnology, Santa Cruz, CA), 1 $\mu\text{g}/\text{ml}$ of rabbit anti-human caspase-3 antibody (H-277; Santa Cruz Biotechnology), or 1:5000 rabbit anti-human actin (Sigma Chemical Co., St. Louis, MO), respectively, was prepared in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T) and 2% nonfat milk (Bio-Rad Laboratories) and incubated with the membranes for ~15 hours at 4°C. Membranes were washed in TBS-T and incubated with 0.08 $\mu\text{g}/\text{ml}$ of goat anti-rabbit Ig-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories) for 1 hour at 27°C. Membranes were washed in TBS-T and subjected to chemiluminescent detection (Pierce, Rockford, IL).

cIAP-2 Knockdown

For these experiments, HT-29 cells were used as a model system because in preliminary experiments, solutions containing a morpholino delivery agent reduced the viability of normal CTB cells. The Gene Tools LLC (Philomath, OR) special delivery protocol was used after optimization of morpholino delivery. Briefly, a 25-mer, fluoresceinated morpholino oligomer with the sequence, 5'-TGCTGTTTCTACTATGTTTCATAAT-3' and a fluoresceinated standard control oligomer with the sequence, 5'-CCTCTTACCTCAGTTACAATTTATA-3' were generated by Gene Tools LLC. Oligomers were incubated with the delivery agent, ethoxylated polyethylenimine, for 20 minutes and then mixed with serum-free media. HT-29 cells (5×10^3 cells/well in 96-well plates and 4×10^5 cells/well in 6-well plates) were incubated for 12 hours in complete Dulbecco's modified Eagle's medium at 37°C, 5% CO_2 . Cells were washed with D-PBS (Sigma Chemical Co.) and the morpholino/ethoxylated polyethylenimine/Dulbecco's modified Eagle's medium mixture was added. After incubation for 3 hours at 37°C, 5% CO_2 , the delivery mixture was aspirated and fresh complete Dulbecco's modified Eagle's medium (in some cases with cytokines) was added. After 72 hours cells were photographed using a fluorescent microscope. After 96 hours the cells were lysed to acquire protein or the MTT assay was conducted on cells in wells using the protocol described above.

Statistics

All quantitative data were subjected to analysis of variance or paired *t*-test as appropriate, using statistical

Figure 1. Analysis of cell viability after cytokine treatments using the MTT assay. **A:** CTB cells treated with rhLIGHT for 48 and 96 hours. **B:** CTB cells treated with rhIFN- γ (100 U/ml) for 48 and 96 hours. **C:** CTB cells treated with rhLIGHT + rhIFN- γ (100 U/ml) for 48 and 96 hours. **D:** HT-29 cells treated with rhLIGHT + rhIFN- γ (10 U/ml) for 48 and 96 hours (positive control). Data are shown as a percentage of the control values (no cytokine added). Error bars represent the means $\pm$ SEM of six preparations of CTB cells purified from six different placentas (**A–C**) and six different preparations of HT-29 cells (**D**). Each assay was performed in triplicate. *, $P < 0.05$; **, $P < 0.01$.

analysis systems (SAS Institute, Inc., Cary, NC). Pre-planned comparisons were conducted to determine differences between controls and treatments. Data are presented as means $\pm$ SEM.

Results

rhLIGHT Fails to Kill CTB Cells

To investigate the ability of rhLIGHT to initiate cell death in CTB cells, purified preparations of these cells obtained from term placentas were treated with medium alone or with increasing concentrations of the cytokine. Viability was measured using the MTT assay. Because CTB cells do not proliferate *in vitro*, MTT values closely approximate the number of viable cells.^{26,28}

In Figure 1A test values are shown as a percentage of the negative control values to normalize the results obtained with CTB cells from six different placentas. Error bars represent the mean and SEM for each CTB cell experiment when compared with the control. At all concentrations, rhLIGHT failed to reduce the viability of CTB cells ($P > 0.05$). Increasing the time of incubation of the CTB cells with rhLIGHT from 48 hours to 96 hours had no effect (Figure 1A).

rhIFN- γ Fails to Kill CTB Cells but Facilitates rhLIGHT-Mediated Killing

Because IFN- γ is synthesized in placentas and might either induce apoptosis or augment LIGHT-mediated ap-

optosis, experiments were done to test the effects of this cytokine on CTB cells. Figure 1B shows that 100 U/ml of rhIFN- γ failed to mediate CTB cell death. None was observed at either 48 hours or 96 hours. This experiment was conducted in triplicate on six different preparations of CTB cells, and the results are shown as percentages of control values together with the mean and SEM for each CTB cell experiment when compared with the control.

Figure 1C shows the results of experiments in which rhLIGHT and rhIFN- γ were added to cultures of CTB cells. The dose of rhIFN- γ was unchanged throughout these experiments; the six different preparations of CTB cells were treated with a physiological concentration of 100 U/ml¹⁹ together with increasing concentrations of rhLIGHT. The results are shown as percentages of control values together with the mean and SEM for each CTB cell experiment when compared with the control. The combination of rhIFN- γ and rhLIGHT caused a concentration-dependent decrease in CTB cell viability at both 48 hours and 96 hours of incubation, but not at 24 hours (data not shown). At 48 hours, a statistically significant decrease from control values ($P < 0.05$) was observed using 10 ng/ml of rhLIGHT whereas at 96 hours, a statistically significant change from control ($P < 0.01$) was observed in cells treated with only 1 ng/ml of rhLIGHT.

The HT-29 cell line, which is highly sensitive to killing by rhIFN- γ + rhLIGHT,⁷ served as a positive control. Figure 1D shows that as expected, HT-29 cell viability was significantly reduced ($P < 0.01$) at both 48 hours and 96 hours in the presence of 10 U/ml of rhIFN- γ and 1 ng/ml of rhLIGHT as well as all higher concentrations. We also verified the report that IFN- γ alone marginally reduced viability of HT-29 cells,²⁹ ~16% at 96 hours in our experiments (data not shown).

Killing of CTB by rhIFN- γ + rhLIGHT Is Blocked by Soluble LIGHT Receptor, DcR3

To establish the role of LIGHT in the killing observed in CTB cells and HT-29 cells treated with rhIFN- γ + rhLIGHT, we repeated the experiment described above with the addition of either rhDcR3-Fc fragment or control-Fc fragment (human Fc, negative control). Figure 2A shows that as expected from the data shown in Figure 1C, rhIFN- γ + rhLIGHT significantly decreased the viability of CTB cells ($P < 0.05$). Viability was not restored by the addition of control-Fc fragment. By contrast, the addition of DcR3-Fc fragment fully restored viability. The same was true for HT-29 cells, as shown by Yu and colleagues¹² and in Figure 2B; the two cytokines significantly reduced viability of HT-29 cells ($P < 0.01$) and viability was restored by DcR3-Fc fragment but not control-Fc fragment. These experiments showed that killing of CTB cells occurs via interaction between LIGHT and one or another or both of its apoptosis-inducing receptor(s), HVEM and LT β -R, and that soluble receptor, DcR3, is capable of exerting a protective role.

Figure 2. Analysis of cell viability after exposure of rhLIGHT/rhIFN- γ -treated cells to control-Fc or DcR3-Fc using the MTT assay. **A:** CTB cells. **B:** HT-29 cells (positive control). Cells treated with rhLIGHT/rhIFN- γ for 96 hours as before, were also exposed to either 4 μ g/ml of control-Fc (human Fc) or the same concentration of the LIGHT-soluble receptor, DcR3-Fc. Data are shown as a percentage of the control value (no Fc fragment). Error bars represent the means $\pm$ SEM of three preparations of CTB purified from three different placentas or three different preparations of HT-29 cells. All data points represent triplicate wells. *, $P < 0.05$; **, $P < 0.01$.

Identification of Apoptosis as the Mechanism of CTB Cell Death

To determine whether the reduction in cell numbers observed in the MTT assays in which cells were treated with rhLIGHT + rhIFN- γ was because of the specific process of programmed cell death (apoptosis), CTB cells were treated for 96 hours with phosphate-buffered saline (PBS) (control), rhLIGHT (100 ng/ml), rhIFN- γ (100 U/ml), or rhLIGHT (100 ng/ml) + rhIFN- γ (100 U/ml) then tested for active caspase-3 by immunoblotting. Figure 3 demonstrates that CTB cells grown in medium alone exhibited no caspase-3 fragments and that neither rhLIGHT nor rhIFN- γ alone induced active fragments. By contrast, the combination of cytokines generated readily detectable active p20 and p17 fragments. These experiments showed

Figure 3. Induction of caspase-3 fragments in CTB cells by rhLIGHT + rhIFN- γ . Cells were treated for 96 hours with (left to right): control PBS (C), rhLIGHT (100 ng/ml) (L), rhIFN- γ (100 U/ml) (I), or rhLIGHT (100 ng/ml) + rhIFN- γ (100 U/ml) (L/I), then immunoblotted to reveal caspase-3. Arrows indicate the active heterodimer (p20 and p17) as well as the inactive p24 fragment. The immunoblot is representative of three independent experiments.

clearly that CTB cell death after exposure to rhLIGHT + rhIFN- γ was because of apoptosis.

cIAP-2 Expression Is Differentially Regulated by LIGHT and IFN- γ

Human placental CTB cells contain several IAP family proteins.²¹ Experiments to determine whether IAP proteins protected CTB cells from LIGHT-mediated apoptotic cell death were conducted on two of these proteins, cIAP-1 and cIAP-2. Analysis by immunoblotting demonstrated that cIAP-1 protein levels in CTB cells as well as HT-29 cells were unaffected by treatment of the cells for 48 hours or 96 hours with rhLIGHT, rhIFN- γ , or a combination of the two cytokines (data not shown). By contrast, as shown in Figure 4, A and B, cIAP-2 protein levels in harvests of CTB cells were increased after exposure to rhLIGHT (48 hours, $P < 0.05$; 96 hours, $P < 0.05$). Levels were not significantly altered by exposure to rhIFN- γ or the combination of rhLIGHT + rhIFN- γ .

Figure 5 shows that HT-29 cells had essentially the same cIAP-2 profile as CTB cells. After 96 hours of incubation, rhLIGHT had stimulated significantly higher levels of cIAP-2. No statistically significant change was observed when the cells were treated with either rhIFN- γ or a combination of the two cytokines.

In the Absence of cIAP-2, rhLIGHT Alone Mediates Apoptosis

To obtain unequivocal evidence that cIAP-2 protects against LIGHT-mediated apoptosis, we developed a morpholino anti-sense knockdown assay. Primary CTB cells used in the previous experiments were shown in preliminary experiments to be highly sensitive to morpholino diluent toxicity. The HT-29 cells, which were similar to CTB cells in their resistance to LIGHT-mediated killing, their expression of HVEM, and LT β -R,⁷ their sensitivity to killing by IFN- γ + LIGHT, and their production of cIAP-2, were therefore used as a substitute.

Figure 6A demonstrates that the fluorescein-tagged morpholinos, both control and cIAP-2-specific, were readily observed inside cells. Figure 6B shows that treatment of HT-29 cells with an anti-sense morpholino specifically targeted to the 5' untranslated region of cIAP-2

Figure 4. Effects of rhLIGHT, rhIFN- γ , and rhLIGHT + rhIFN- γ on the abundance of cIAP-2 protein in CTB cells at 48 hours (A) and 96 hours (B). CTB cells were incubated with (left to right) control PBS (C), rhLIGHT (100 ng/ml) (L), rhIFN- γ (100 U/ml) (I), or rhLIGHT (100 ng/ml) + rhIFN- γ (100 U/ml) (L/I), then immunoblotted to reveal cIAP-2 protein. The blots were then incubated to reveal actin. Arrows indicate cIAP-2 and actin protein, respectively. Data gathered by scanning densitometer representing the mean fold change over control (normalized to actin) $\pm$ SEM of three preparations of CTB cells, purified from three different placentas, are shown below representative blots.

message diminished their production of cIAP-2 protein. Approximately 50% reduction was demonstrated by using scanning densitometer for analyses. Although LIGHT alone was unable to kill HT-29 cells transfected with control morpholinos (Figure 7), the data shown in Figure

7 demonstrate a dose-dependent effect, in which cIAP-2 knockdown permitted rhLIGHT, at levels as low as 1 ng/ml, to trigger HT-29 cell apoptosis in the absence of rhIFN- γ . Thus, these experiments demonstrated that cIAP-2 has a major role in protecting HT-29 cells from killing by LIGHT.

Discussion

In this study we report for the first time that CTB cells from term placentas are entirely resistant to killing by the potent, apoptosis-inducing, endogenous placental cytokine, LIGHT, and show that two mechanisms are used for protection. The first pathway is via inhibition by the soluble LIGHT receptor, DcR3. The second is via induction of an inhibitor of apoptosis, cIAP-2. Figure 8 illustrates potential intracellular and extracellular pathways by which interference with apoptosis may be established in CTB cells. Figure 8 also shows a potential pathway for facilitation of LIGHT-mediated apoptosis through blocking of cIAP-2 by IFN- γ .

LIGHT alone was unable to mediate apoptosis of CTB cells despite their expression of both HVEM and LT β -R. In cancer cells, expression of both receptors is associated with LIGHT-mediated apoptosis.^{7,14,30} Thus, pathways in normal CTB cells are clearly different from those in many cancer cells. However, as is the case for other types of cells, including the HT-29 cells tested in this study, IFN- γ increased the sensitivity of CTB cells to LIGHT-mediated apoptosis. Our experiments using soluble DcR3 fragments showed definitively that IFN- γ potentiates LIGHT-mediated apoptosis although IFN- γ itself is

not an inducer of apoptosis in CTB cells. Further, we identified cIAP-2 as a major target for the combination of IFN- γ + LIGHT; CTB cell death accompanied the reduction of cIAP-2, strongly implicating this apoptosis inhibitor in protection of CTB cells. The cIAP-2 promoter region contains a binding site for IFN regulatory factor-1 (IRF-1), which might comprise a regulatory element³⁰ leading to apoptosis, a postulate worthy of experimental evaluation. As with CTB cells,¹⁵ hepatocytes express both HVEM and LT β -R. The report of Matsui and colleagues³¹

Figure 7. MTT analysis of HT-29 cells treated first with cIAP-2 or control morpholinos and then with PBS (control) or rhLIGHT (1 to 1000 ng/ml) for 96 hours. Data are shown as a percentage of the control values (no cytokine added). Error bars represent the means $\pm$ SEM of three preparations of CTB cells purified from three different placentas. Each assay was performed in triplicate. *, $P < 0.05$.

demonstrating that LIGHT pretreatment protects these cells from TNF- α -mediated apoptosis suggests that mechanisms stimulated by encounter with LIGHT that result in protection may be found in other types of cells and organs.

Interference with apoptosis of placental cells by soluble receptors in the TNF receptor (TNF-R) superfamily is not unknown. The first of the potent, Th1-type cytokines to be reported in placentas was TNF- α ,^{32,33} and the first of the death-dealing receptors demonstrated in human placenta was TNF-R1.³² Abundant soluble TNF-R1 is found in amniotic fluid and maternal blood and urine, and, as with DcR3 blocking of LIGHT-mediated apoptosis, interferes with killing by TNF- α .³⁴ It does not seem unreasonable to suggest that *in vivo*, DcR3 interferes with LIGHT-mediated signaling and cell death just as it does *in vitro* in the experiments reported here. Nevertheless, more extensive studies, possibly involving DcR3 knockdown, will

be necessary to resolve any true biological significance. Interestingly, immunohistochemical experiments show that DcR3 is localized to exactly the same cells as LIGHT and immunoblot analyses document high levels of DcR3 protein in the amniochorion. It will therefore be of interest to test amniotic fluid for this effective inhibitor of LIGHT-mediated cell death.

Interference in apoptosis mediated through TNF death receptor pathways has been documented for two of the six IAP cytosolic proteins, cIAP-1 and cIAP-2.^{24,35} Because of this observation and the recent report of cIAP mRNA and proteins in first trimester and term placentas as well as in purified CTB cells,²¹ we focused on these two inhibitors. Treatment with LIGHT enhanced cIAP-2, but not cIAP-1 expression. Thus, LIGHT is now one of the group of powerful cytokines, which include TNF- α and insulin-like growth factor-1 (IGF-1), known to be capable of increasing this apoptosis inhibitor.^{36,37} Although it was not possible to perform the definitive anti-sense cIAP-2 morpholino experiments in primary human placental CTB cells because of toxicity, HT-29 cells serving as a substitute were clearly protected from LIGHT-mediated apoptosis by cIAP-2. The question of whether or not cIAP-2 was protective was answered but raised the further question of which pathway for protection was activated. Investigators have recently focused on the ability of other apoptosis mediators to increase nuclear factor- κ B in resistant cells.^{38,39} It is therefore of considerable interest that transcription factor binding sites in the cIAP-2 gene include not only sites for binding nuclear factor of activated T cells (NFAT) and AP-1 but also sites for binding nuclear factor- κ B.³⁰

Although the experiments presented here show clearly that both inhibition by soluble receptor and inhibition by endogenous cIAP-2 are effective protective mechanisms, this does not preclude the possibility that CTB cells are protected by additional measures, as would be suggested by the lesser reduction in viability when compared to HT-29 cells. For example, CTB cells appear to be protected against the equally powerful apoptosis-inducing ligand, FasL, another member of the TNF family of ligands present in placentas,^{40,41} by exhibiting dysfunctional Fas receptor signaling.⁴² However, at least one of the apoptosis-signaling receptors, HVEM and/or LT β -R, is functional in CTB cells because rhLIGHT + IFN- γ killed the cells, and killing was abrogated by DcR3-Fc fragments. Interestingly, the DcR3 soluble receptor for LIGHT is shared by FasL,¹² suggesting the possibility that this molecule might be an additional protector of CTB cells against FasL-mediated apoptosis. Also, XIAP and NAIP, two other members of the IAP family, are present in CTB cells.²¹ Neither, however, has been shown to have any effect in preventing apoptosis through TNF superfamily death receptors. Finally, decoy receptor strategies might be used, as appears to be the case with TRAIL, a third apoptosis-inducing TNF superfamily ligand abundantly produced in human placentas.^{1,43} The DcR1 decoy receptor is particularly prominent in CTB cells⁴³ and may play a role in their protection against this killer molecule, but this remains to be demonstrated experimentally.

Figure 8. A schematic illustration summarizing regulation of LIGHT/IFN- γ -mediated apoptosis in human villous CTB cells.

An entirely unexpected outcome of the experiments that we and others have conducted on the TNF superfamily ligands and receptors expressed in human placenta is the acquisition of evidence that in this organ, the ligand/receptor pairs may serve entirely different functions than in immunity. For example, TNF- α appears to promote invasion of maternal decidua by trophoblast cells,⁴⁴ to regulate β -hCG production,^{45,46} and to participate in CTB cell fusion to the syncytiotrophoblast layer.^{47,48} Yui and colleagues²⁸ proposed in 1994 that TNF- α is central to placental development and differentiation, killing CTB cells to facilitate the close association of fetal vessels with syncytiotrophoblast that is required for transport of nutrients into the fetus. TRAIL may protect placenta against killing by anti-paternal cytotoxic cells⁴³ and FasL seems to prevent transfer of maternal cells into the fetus.⁴⁹

Having determined in this study that LIGHT alone does not kill CTB cells, it is now imperative to search for other critical functions that may be performed in placenta by this newly discovered member of the TNF superfamily of ligands. It is hoped that further investigation into regulation of CTB cell death may be useful in understanding the growing number of placental pathologies linked to dysregulated apoptosis.

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