Differential Expression of LIGHT and Its Receptors in Human Placental Villi and Amniochorion Membranes

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mRNA encoding LIGHT (homologous to lymphotoxins, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes), a member of the tumor necrosis factor superfamily of ligands, as well as mRNAs encoding LIGHT receptors [HVEM, LT β R, and TR6 (DcR3)] are present in placentas and cytotrophoblast cells at term. To establish translation of these messages and determine directions for functional studies, term placentas, amniochorion membranes, and purified cytotrophoblast cells were evaluated by immunoblotting and immunohistochemistry. Ligand and receptor proteins were identified in lysates from all three sources although the soluble receptor, TR6, was scarce in placentas and all receptors were in low abundance in cytotrophoblast cells. These results were confirmed and cell type-specific expression was documented by immunohistochemistry. Ligand and receptor proteins were differentially expressed according to cell type. For example, HVEM was identified on syncytiotrophoblast but not in villous mesenchymal cells; amnion epithelial cells were positive for all proteins whereas chorion membrane cytotrophoblasts exhibited none. Because LIGHT is a powerful cytokine that can alter gene expression and promote apoptosis, these experiments suggest that ligand-receptor interactions may critically influence structural and functional aspects of human placentas through as yet undefined autocrine/paracrine pathways. (*Am J Pathol* 2002, 161:2011–2017)

The placenta and extraplacental membranes, which are derived from the implanted blastocyst, are tolerated by the mother throughout pregnancy. Mechanisms underlying maternal acceptance of these semi-allogeneic tissues are as yet unclear but are believed to include immune modulation by tumor necrosis factor α (TNF α) and its closely related superfamily members, FasL and TNF related apoptosis inducing ligand (TRAIL).^{1–3}

LIGHT [homologous to lymphotoxins, exhibits inducible expression, competes with herpes simplex virus

(HSV) glycoprotein D for HVEM, a receptor expressed by T lymphocytes], a newly identified member of this growing family of molecules, is reportedly transcribed in human placentas.^{4–6} It is not known whether these messages are translated and no potential functions have been suggested. Much is known of the involvement of LIGHT in the human immune system. LIGHT, as with other members in this superfamily, forms a homotrimer.^{6,7} LIGHT has both cytosolic and membrane-bound forms.⁸ The membrane-bound form may be cleaved by matrix metalloproteinases to act as a soluble protein.^{8–10} Soluble LIGHT enhances mixed lymphocyte reactions (MLR)⁶ and is a major mediator of graft-versus-host disease.¹¹ LIGHT is reportedly required for dendritic cell-mediated primary allogeneic T cell responses,⁹ is known to induce T lymphocyte proliferation and secretion of Th-1 cytokines,^{9,11} and participates in the induction of cell-mediated immunity.^{9,11,12} LIGHT, as with most members of the TNF superfamily, has the ability to trigger apoptosis in some tumor cells in culture and *in vivo*.^{11,13}

The increasingly varied roles postulated for LIGHT indicate that function will depend on receptor expression and cytokine environment. LIGHT binds to three receptors, TR6 (DcR3), HVEM, and LT β R^{5–7,14,15} (Figure 1). TR6 is a soluble receptor that competes with HVEM for LIGHT binding,^{15,16} abrogates LIGHT-mediated apoptosis,^{15–17} prolongs the survival time of mice receiving heart allografts,¹⁶ and favors Th-2 lymphokine production in a MLR.¹⁶ HVEM is present on T cells and is important in LIGHT-mediated T cell costimulation.^{5,6,9,11,18,19} LT β R is not found on T or B cells but is found on stromal cells and in some tumor cells, where it transduces apoptotic signals.^{5–7,20} The messages for all three receptors have been detected in the human placenta, but translation and cellular localization remain unknown.^{4,14,21,22}

In this study, our goal was to identify translated proteins in the LIGHT ligand/receptor system and establish their cellular localization in term placentas, amniochorion, and purified cytotrophoblast cells. We have uncovered potential autocrine, juxtacrine, and paracrine signaling pathways that may be important in placental structure and function.

Supported by National Institutes of Health grants HD24212 and HD33994 (to J.S.H.) and from the Lawson-Mann fellowship (to R.M.G.).

Accepted for publication August 15, 2002.

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Figure 1. A schematic illustration of LIGHT and its related TNF superfamily members. **Arrows** indicate receptor-ligand interactions.

Materials and Methods

Reagents

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Cell Lines and Tissues

The human trophoblast-derived choriocarcinoma cell line, JAR, was purchased from the American Type Culture Collection (ATCC no. HTB-144) (Manassas, VA). Human placentas were obtained from normal cesarean section delivery at term, in accordance with a protocol approved by the Human Subjects Committee of the University of Kansas Medical Center. Samples were taken randomly from the floating villi and reflected amniochorion for further analysis. Underlying pathology was not evident on histological examination of the samples.

Isolation of Term Cytotrophoblasts

Cytotrophoblasts were isolated from term placenta by enzymatic digestion and gradient centrifugation as described.^{23,24} Cytotrophoblasts were further purified from this cell suspension by removal of HLA-A,B,C-positive cells using the monoclonal antibody W6/32 (ATCC no. HB95) and goat anti-mouse Ig-conjugated magnetic microbeads (Milenyi Biotec Inc., Auburn, CA) according to the protocol recommended by the manufacturer. To assess purity of cytotrophoblasts, Cytospin (Shandon, Pittsburgh, PA) preparations of cells were analyzed by immunohistochemical staining using mouse anti-pan cytokeratin (Lu-5) (Bio Genex, San Ramon, CA), which detects all trophoblast cells, and mouse anti-CD14 (Zymed, San Francisco, CA), which detects contaminating macrophages. Less than one percent of the cells was 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.²³ 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 cytotrophoblasts were isolated. Purified cells were immediately lysed for protein and RNA preparations.

Analysis by RT-PCR

Whole placenta ($n = 1$), cytotrophoblasts purified from a different placenta ($n = 1$), and JAR cells were analyzed. RNA was isolated from 8×10^6 cells, or 100 mg tissue, using 1 ml TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Total RNA was treated with DNase I (AMP-D1) according to the manufacturer's instructions. First-strand cDNA synthesis was then performed using this treated total RNA and Moloney murine leukemia virus reverse transcriptase (Life Technologies) according to the enzyme manufacturer's protocol. Ten μ l of each 1:5 diluted cDNA sample were used in the subsequent 50 μ l PCR reaction for the LIGHT primer pair. Four μ l (50 μ l reaction) of cDNA were used for the β -actin primer pair. Primers were derived from human LIGHT cDNA (forward: 5'-CAAGAGCGAAGGTCTCA-CGAGGTC-3' and reverse 5'-TCACACCATGAAAGC-CCCGAAGTAAG-3') and human β -actin cDNA (forward 5'-CACCCCGTGCTGCTGACCGAGGCC-3' and reverse 5'-CCACACGGAGTACTTGCGCTCAGG-3') sequences, respectively (National Center for Biotechnology Information databases), using the PrimerSelect program (DNA-STAR Inc., Madison, WI), and were synthesized by Gemini Biotech (Alachua, FL). The amplification schedule for LIGHT was: 94°C for 5 minutes; 38 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 2 minutes; and 72°C for 7 minutes. The amplification schedule for β -actin was: 94°C for 45 seconds; 30 cycles of 94°C for 45 seconds; 60°C for 30 seconds; 72°C for 2 minutes; and 72°C for 7 minutes. All reactions were conducted in a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA). Products were analyzed by electrophoresis of 10 μ l of each reaction through 2% agarose (Amresco 3:1, Solon, OH), 0.05 μ g/ml ethidium bromide, followed by UV transillumination. The correct sequence of product derived from LIGHT primers was confirmed by dRhodamine terminator cycle sequencing using the ABI 310 DNA sequencer (Applied Biosystems) in the Center for Reproductive Sciences at the University of Kansas Medical Center.

Analysis by Immunoblotting

Protein samples were prepared from homogenized placental tissue ($n = 3$) and lysed primary cells purified from the same placentas ($n = 3$). Protein quantification was performed as per the manufacturer's protein assay protocol (Bio-Rad Laboratories, Richmond, CA). Fifty μ g of total protein were separated by electrophoresis on 15% polyacrylamide SDS-PAGE gels. Proteins were electrophoretically transferred to 0.2 μ m-supported nitrocellulose (Schleicher & Schuell, Keene, NH), for 75 minutes at 100 volts (27°C) in $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH 9.9). For detection of specific proteins, primary antibodies were prepared in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T) and 3% nonfat milk (Bio-Rad Laboratories) and incubated on the membranes for approximately 15 hours at 4°C (Table 1). Membranes were washed in TBS-T and incubated with the respective sec-

Table 1. Summary of Antibodies Utilized

Human antigen	Species	Isotype	Conjugation	Immunoblot concentration	Immunohistochemistry concentration	Source*
LIGHT	Mouse	IgG ₁ (monoclonal)	—	2.00 μ g/ml	—	J. Ni
LIGHT (C-20)	Goat	IgG (polyclonal)	—	—	10.0 μ g/ml	SCB
TR6/DcR3	Rabbit	IgG (polyclonal)	—	0.10 μ g/ml	10.0 μ g/ml	J. Ni
HVEM (N-19)	Goat	IgG (polyclonal)	—	0.40 μ g/ml	10.0 μ g/ml	SCB
LT β R (N-15)	Goat	IgG (polyclonal)	—	0.20 μ g/ml	10.0 μ g/ml	SCB
Control	Mouse	IgG ₁ (monoclonal)	—	2.00 μ g/ml	10.0 μ g/ml	BDP
Control	Goat	IgG (polyclonal)	—	0.40 μ g/ml	10.0 μ g/ml	Vector
Control	Rabbit	IgG (polyclonal)	—	0.10 μ g/ml	10.0 μ g/ml	Vector
Mouse IgG	Goat	IgG (polyclonal)	HRP [†]	0.08 μ g/ml	—	BDP
Goat IgG	Rabbit	IgG (polyclonal)	HRP	0.08 μ g/ml	—	JL
Rabbit IgG	Goat	IgG (polyclonal)	HRP	0.08 μ g/ml	—	JL
Mouse IgG	Horse	IgG (polyclonal)	Biotin	—	5.0 μ g/ml	Vector
Goat IgG	Horse	IgG (polyclonal)	Biotin	—	5.0 μ g/ml	Vector
Rabbit IgG	Goat	IgG (polyclonal)	Biotin	—	5.0 μ g/ml	Vector

*J. Ni: Dr. J. Ni, Human-Human Hybridoma, Inc., Bethesda, MD; SCB: Santa Cruz Biotechnology, Santa Cruz CA; BDP: BD Pharmingen, San Diego CA; Vector: Vector, Burlingame CA; JL: Jackson ImmunoResearch Laboratories, West Grove, PA.

[†]Horse radish peroxidase.

ondary antibody-enzyme conjugates for 1 hour at 27°C (Table 1). Membranes were washed in TBS-T and subjected to chemiluminescent detection (Pierce, Rockford, IL).

Analysis by Immunohistochemistry

One cm³ samples were surgically excised, at random from 4 placentas, from both cotyledons embedded in basal plate and amniochorion membranes, fixed in 4% paraformaldehyde, and then embedded in paraffin blocks. Paraffin sections were deparaffinized in Histo-clear (National Diagnostics, Atlanta, GA) and rehydrated in an ethanol gradient. Tissue sections were blocked with 10% normal horse or goat serum for 1 hour at 27°C. The blocker was removed and primary antibody was added (Table 1). The tissue sections were incubated for 15 hours at 4°C. Washes were performed in phosphate-buffered saline (PBS) with 0.3% Tween-20 (PBS-T) and the peroxidase block was performed with 0.5% H₂O₂ in methanol for 30 minutes. The respective biotinylated secondary antibody was added and incubated for 1 hour at 27°C (Table 1). Following washes with PBS-T, the streptavidin peroxidase label (Zymed) was incubated for 10 minutes at 27°C. The sections were washed with PBS and the 3-amino-9-ethylcarbazole in N,N-dimethylformamide (AEC) color development substrate (Zymed) was incu-

bated for 10 minutes at 27°C. Sections were washed in water and counterstained with Mayer's hematoxylin.

Results

Characterization of LIGHT in the Human Placenta at Term

We used RT-PCR to test for LIGHT message in placenta, purified cytotrophoblasts, and JAR cells (a choriocarcinoma-derived cell line). We detected LIGHT message in all three samples (Figure 2). Our findings agree with previous reports that placenta and JAR cells contain message for LIGHT^{4,5,7} and we demonstrate, for the first time, LIGHT message in purified cytotrophoblast cells.

Immunoblotting was used to establish the presence of LIGHT protein in human term placentas. LIGHT protein was detected in lysates from three placentas as well as in their matching amniochorion membranes (Figure 3). LIGHT protein was also detected in lysates of purified cytotrophoblast cells (Figure 3).

Figure 2. RT-PCR of whole placenta, purified cytotrophoblasts, and JAR cells. β -actin was used as a loading control. The sequence of the 470-bp product matches that of human LIGHT.

Figure 3. Immunoblot of whole placenta, amniochorion and purified cytotrophoblast protein for LIGHT [predicted molecular weight (M.W.), 26.4 kD], TR6 (predicted M.W., 32.7 kD), HVEM (predicted M.W., 30.4 kD) and LT β R (predicted M.W., 46.7 kD). LIGHT migrates at approximately 33 kD, TR6 at 35 kD, HVEM at 50 kD and LT β R at 75 kD. Actual migration significantly differs from the predicted molecular weight for HVEM and LT β R, suggesting post-translational modifications.

Figure 4. Immunolocalization of LIGHT, TR6, HVEM, and LTβR in term human placenta and amniochorion. **A, C, E, and G** show placenta and **B, D, F, and H** show amniochorion immunolocalized for LIGHT, TR6, HVEM, and LTβR and their controls, respectively. Annotations: a, amnion; m, mesenchymal layer; c, chorion; d, decidua. **A:** Arrow (vertical), syncytiotrophoblast layer; arrow (horizontal), mesenchymal cell. **B:** Arrow (vertical), amnion; arrow (horizontal), mesenchymal cell; arrow (blue), decidua capsularis. **C:** Arrow (vertical), mesenchymal cell; arrow (horizontal), syncytiotrophoblast layer. **D:** Arrow (horizontal), amnion; arrow (vertical), mesenchymal cell; arrow (blue), decidua capsularis. **E:** Arrow, syncytiotrophoblast layer. **F:** Arrow (horizontal), amnion; arrow (vertical), decidua capsularis. **G:** Arrow (vertical), mesenchymal cell; arrow (horizontal), syncytiotrophoblast layer. **H:** Arrow (vertical), amnion; arrow (horizontal), mesenchymal cell; arrow (blue), decidua capsularis. Table 1 has antibody specifications. Images captured at magnification, ×200.

LIGHT protein was localized in sections of placentas and fetal membranes using immunohistochemistry. LIGHT protein was observed in placental mesenchymal cells (Figure 4A) and in the syncytiotrophoblast layer (Figure 4A). We noted that a small proportion of placental syncytiotrophoblast demonstrated patchy immunoreactivity with anti-

LIGHT (< 1 to 2%, data not shown). In the amniochorion, LIGHT protein was strongly expressed by the amnion and fetal mesenchymal cells and was detectable in the decidua capsularis (Figure 4B). LIGHT protein was not detected in the chorion (Figure 4B). The negative controls were not immunoreactive for goat IgG (Figure 4, A and B).

Table 2. Summary of Immunoblot Results

	Ligands	Receptors		
	LIGHT	TR6	HVEM	LT β R
Term tissue (<i>n</i> = 3)				
Placenta	+	0	+	+
Fetal membranes	+	+	+	+
Primary cells (<i>n</i> = 3)				
Cytotrophoblasts (purified)	+	+	+	+

*+, positive; 0, negative.

Localization of LIGHT's Soluble Receptor in the Human Placenta at Term

Immunoblot experiments showed an intense signal for TR6 protein in amniochorion lysates (Figure 3). Although TR6 protein was not detected in lysates of placental villi unless the blots were overexposed (data not shown) it was readily detected in purified cytotrophoblasts (Figure 3).

Immunohistochemical studies demonstrated weak signals for TR6 protein in the syncytiotrophoblast layer and in the villous mesenchymal cells of term placentas (Figure 4C). By contrast, TR6 protein was abundantly expressed in the amnion and in the decidua capsularis (Figure 4D). TR6 protein was detected in most fetal mesenchymal cells adjacent to the amnion but was not observed in the chorion (Figure 4D). The negative controls were not immunoreactive for rabbit IgG (Figure 4, C and D).

Localization of LIGHT's Membrane-Bound Receptors in the Human Placenta at Term

HVEM and LT β R protein were detected in placenta, amniochorion, and cytotrophoblast lysates by immunoblotting (Figure 3). HVEM and LT β R migrate as larger molecular weight proteins than their amino acid content would suggest, which is not uncommon among TNF receptor (TNFR) superfamily members given their potential for post-translational modification.²⁵

Immunohistochemical studies detected HVEM protein in the syncytiotrophoblast layer (Figure 4E). HVEM protein was also localized to the amnion and decidua capsularis (Figure 4F). HVEM protein was not detected in villous (Figure 4E) or membrane (Figure 4F) fetal mesenchymal cells. LT β R protein was localized throughout the placenta (Figure 4G). The amnion, fetal mesenchymal cells, and decidua capsularis all contained immunoreactive LT β R (Figure 4H). HVEM (Figure 4F) and LT β R (Figure 4H) protein were not detected in chorion membranes. The negative controls were not immunoreactive for goat IgG (Figure 4, E–H).

The immunoblot and immunohistochemistry data for LIGHT, TR6, HVEM, and LT β R are summarized in Tables 2 and 3.

Discussion

This study provides evidence that LIGHT, TR6, HVEM, and LT β R proteins are expressed in term human pla-

Table 3. Summary of Immunohistochemical Results

	Ligand	Receptors		
	LIGHT	TR6	HVEM	LT β R
Term placenta (<i>n</i> = 4)				
Syncytiotrophoblast layer	+	+	+	+
Fetal mesenchymal cells	+	+	0	+
Term fetal membranes (<i>n</i> = 4)				
Amnion	+	+	+	+
Fetal mesenchymal cells	+	+	0	+
Cytotrophoblasts (chorion)	0	0	0	0
Decidua capsularis	+	+	+	+

*+, positive; 0, negative.

centa and amniochorion. By examining these tissues we established that the syncytiotrophoblast layer expressed LIGHT, HVEM, LT β R and, to a lesser extent, TR6 protein; villous mesenchymal cells expressed only LIGHT, LT β R, and TR6 protein; amnion epithelial cells and the decidua capsularis expressed LIGHT and all of its receptors whereas cells in the chorion membrane did not exhibit either ligand or receptor proteins; and purified cytotrophoblasts expressed all four proteins.

The biological implications of the expression patterns are as yet unclear. The interaction of the placenta with active maternal immune cells could initiate a cascade of biochemical events that culminate in placental demise but this clearly does not happen. There is increasing evidence that LIGHT promotes inflammation, yet the syncytiotrophoblast layer, which is exposed to immune cells in circulating maternal blood, expresses this cytokine. This suggests an alternative function for LIGHT at the maternal fetal interface. HSV can gain entry into T cells through HVEM¹⁴ and LIGHT can interfere with this process.⁵ Evolutionary pressure to prevent entry of virus might have driven the expression of LIGHT on T cells,^{5,8} and this might also account for its presence on syncytiotrophoblast. The fetal mesenchymal cells, which are not exposed to maternal immune cells under normal conditions, also express LIGHT. In this environment, LIGHT may be able to promote inflammation^{6,9,11,12,26} and contribute to placental defense from circulating pathogens, as is the case with LIGHT in other contexts.

LIGHT shares significant homology with TNF α and seems to possess the same capacity to activate or suppress the proliferation of distinct cellular populations. Both TNF α and LIGHT receptors can recruit specific intracellular factors [TNF receptor-associated factor-1 (TRAF-1), and TRAF-2], initiating a cascade of events culminating in NF κ B activation.^{7,27–30} TNF α has been detected in the uterus, embryo, and placenta and has been postulated to play a positive role in female reproduction.^{31–38} Given the sequence homology, shared signal transduction pathway, and mutual localization in the placenta, it is reasonable to suggest that LIGHT and TNF α might act similarly to promote homeostasis in maternal and fetal tissues during pregnancy.

A notable finding in this study was that TR6 was weakly expressed in the term placenta but easily detected in the amniochorion. The high level of TR6 expression in the amnion suggests that this protein is secreted into the amniotic fluid where it may modulate the actions of LIGHT and FasL. Infection and preterm labor have been associated with high levels of TNF α in human amniotic fluid.³⁹ The level of soluble TNFR in amniotic fluid varies with gestational age and has been postulated to protect the fetus from excess TNF α .⁴⁰ Similarly, TR6 may be important in neutralizing excess LIGHT in the amniotic fluid.

The co-expression of LT β R and HVEM on the cytotrophoblasts and in cells located in the decidua capsularis recapitulates the dual expression observed in some tumor cell lines in which LIGHT causes apoptosis.⁴¹ LIGHT, as with TNF α ,⁴² may therefore be able to promote apoptosis in human cytotrophoblasts and this interaction could be modulated by autocrine or paracrine TR6 expression. It would be interesting to explore the relative expression level of LT β R and HVEM proteins in pathological specimens as these proteins exhibit some variable expression in our samples of normal placenta.

The results of the present study locate LIGHT, TR6, HVEM, and LT β R protein in specific maternal and fetal cells important in the maintenance of human pregnancy. Studies on the function and regulation of placental LIGHT have yet to be done but sequence homology and cellular localization data suggest that this cytokine will mirror TNF α as a powerful and potentially pleiotropic mediator of successful pregnancy.

Acknowledgments

We thank Dr. Judith Pace and the P30/U54 Reproductive Sciences Center for providing cultured cell lines and purified W6/32 monoclonal antibody.

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