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

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mRNA encoding LIGHT (homologous to *l*ymphotoxins, exhibits *i*nducible expression, competes with herpes simplex virus *g*lycoprotein D for *H*VEM, a receptor expressed by *T* lymphocytes), a member of the tumor necrosis factor superfamily of ligands, as well as mRNAs encoding LIGHT receptors [HVEM, LTβR, 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 (TNFα) and its closely related superfamily members, FasL and TNF related apoptosis inducing ligand (TRAIL).[1–3]

LIGHT [homologous to *l*ymphotoxins, exhibits *i*nducible expression, competes with herpes simplex virus (HSV) *g*lycoprotein D for *H*VEM, 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 LTβR[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] LTβR 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.

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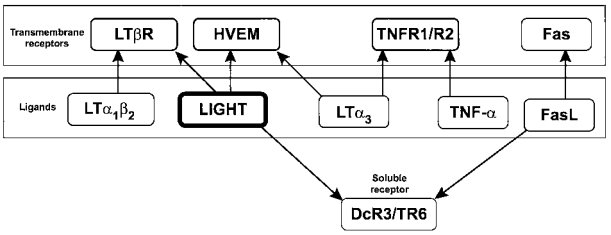

Figure 1. A schematic illustration of LIGHT and its related TNF superfamily members. **Arrows** indicate receptor-ligand interactions.

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 (Miltenyi 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-βhCG (clone CG05) (Neomarkers, Fremont, CA) to detect any contaminating syncytial fragments.[23] Less than 4% of the cytospin-prepared cells demonstrated immunoreactivity for βhCG, suggesting very few contaminating syncytial fragments. βhCG 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 μl of each 1:5 diluted cDNA sample were used in the subsequent 50 μl PCR reaction for the LIGHT primer pair. Four μl (50 μl reaction) of cDNA were used for the β-actin primer pair. Primers were derived from human LIGHT cDNA (forward: 5′-CAAGAGCGAAGGTCTCACGAGGTC-3′ and reverse 5′-TCACACCATGAAAGCCCCGAAGTAAG-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 (DNASTAR 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 μl of each reaction through 2% agarose (Amresco 3:1, Solon, OH), 0.05 μg/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 μg of total protein were separated by electrophoresis on 15% polyacrylamide SDS-PAGE gels. Proteins were electrophoretically transferred to 0.2 μm-supported nitrocellulose (Schleicher & Schuell, Keene, NH), for 75 minutes at 100 volts (27°C) in Na_2CO_3/$NaHCO_3$ 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-