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Toll-like receptor profiling of seven trophoblast cell lines warrants caution for translation to primary trophoblasts

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ABSTRACT

Introduction: Excessive placental inflammation is associated with pregnancy complications. Toll-like receptors (TLRs) are sensors for danger signals from infections and damaged tissue and initiate inflammation. Trophoblasts in the placenta broadly express TLRs. Trophoblast cell lines are used as surrogates for primary trophoblasts for *in vitro* studies, but the inflammatory translatability of trophoblast cell lines warrants examination. We aimed to assess TLR1-10 gene expression and activation in seven trophoblast cell lines and compare this to primary trophoblasts.

Methods: The five choriocarcinoma trophoblast cell lines BeWo, JAR, JEG-3, AC1M-32 and ACH-3P, and the two SV40 transfected trophoblast cell lines HTR-8/SVneo and SGHPL-5 were included and compared to primary first trimester trophoblasts (n = 6). TLR1-10 gene expression was analyzed by RT-qPCR. Cells were stimulated by specific TLR1-9 ligands for 24 h and cytokine release was measured by a 10-plex immunoassay.

Results: All choriocarcinoma cell lines demonstrated broad TLR gene expression, but lacked functional cytokine response to TLR ligand activation. In contrast, SV40 transfected cell lines showed restricted TLR gene expression, but SGHPL-5 cells displayed significantly increased levels of interleukin (IL)-6, IL-8, IL-12 and vascular endothelial growth factor A after TLR3 and/or TLR4 activation ($P < 0.01$), while TLR2 activation increased IL-6 and IL-8 levels ($P < 0.05$). HTR8/SVneo cells responded to TLR3 activation by increased IL-6 and interferon (IFN)-γ ($P < 0.05$). The SGHPL-5 TLR profile most closely resembled primary trophoblast.

Discussion: The characterized trophoblast cell line TLR profiles serve as a reference and warrant caution when selecting trophoblast cell lines as *in vitro* models for immune responses in primary trophoblasts.

1. Introduction

Normal pregnancy is characterized by natural mild inflammation, while disturbances leading to excessive placental inflammation may have harmful consequences for mother and fetus [1,2]. Fetal trophoblasts are the main placental cell type and directly interact with maternal cells. In early pregnancy, trophoblasts invade the spiral arteries in the uterine wall to facilitate vessel adaptions required for optimal placental development. At later gestation, trophoblasts cover the fetal villous structures forming a

Abbreviations: CT, threshold cycle; FBS, fetal bovine serum; FC, fold change; IFN, interferon; IL, interleukin; IP, interferon-γ-inducible protein; P3CSK4, Pam3CysSerLys4; FSL-1, Pam2CGDPKHPKSF; poly(I:C), polyinosinic:polycytidylic acid; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; PCA, principal component analysis; TBP, TATA box binding protein; TLR, Toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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placental barrier that interacts with maternal blood [3]. The delicate interplay between trophoblasts and maternal cells is sensitive to danger signals from infections and tissue damage.

Toll-like receptors (TLRs), which belong to the family of pattern recognition receptors, serve as the body's immediate sensors of danger and are essential for initiating inflammation [4]. TLR activation induces an inflammatory repair process involving production of inflammatory cytokines and recruitment of immune cells to the site of injury. Ten different human TLRs, each responding to a specific set of ligands, have been identified [4]. TLR expression and activation in primary trophoblasts [5–10] may contribute substantially to development of inflammatory pregnancy complications such as preeclampsia [11–13]. In Tangerås/Stødle et al. [7] we demonstrated a broad functional TLR profile in primary first trimester trophoblasts, while the trophoblast cell line BeWo showed no TLR mediated cytokine response.

Trophoblast properties have been widely studied in normal and complicated pregnancies [14]. Primary trophoblasts are the ideal choice for such studies, but the availability of placental tissue is often restricted, isolation of trophoblasts is labor intensive, and the isolated cells have a restricted life span in culture. To overcome these limitations a variety of trophoblast cell lines are commonly used [15]. Among these are naturally immortalized cell lines obtained from choriocarcinoma tissue, such as BeWo [16], JAR [17] and JEG-3 [18]. The cell lines AC1M-32 [19] and ACH-3P [20] have been generated by fusion of the AC1-1 cell line (a JEG-3 mutant [21]) with primary term or first trimester trophoblasts, respectively. In addition, trophoblast cell lines such as HTR-8/SVneo [22] and SGHPL-5 [23] have been generated by SV40 large T antigen transfection. Trophoblast cell lines represent a valuable tool for studying placental function and it is essential that these models are thoroughly characterized and compared to primary trophoblasts. This study aimed to assess TLR1-10 gene expression and activation in seven trophoblast cell lines and compare this to primary first trimester trophoblasts.

2. Materials and methods

2.1. Cell lines

The choriocarcinoma trophoblast cell lines BeWo, JAR (#HTB-44, ATCC, Manassas, Virginia), JEG-3 (#HTB-36, ATCC), AC1M-32 and ACH-3P, and the SV40 transfected trophoblast cell lines HTR-8/SVneo and SGHPL-5 were included (Table 1). The BeWo, ACH-3P and AC1M-32 cell lines were generously provided by Professor Berthold Huppertz (Medizinische Universität, Graz, Austria), the HTR-8/SVneo cell line by Professor Charles H. Graham (Queens University, Kingston, Canada), and the SGHPL-5 cell line by Professor Guy Whitley (Saint George's Hospital, University of London, UK). All cell lines were cultured in specific medium (Table 1) with 100 mg/ml penicillin–streptomycin (Sigma–Aldrich, St. Louis, Missouri) at 37 °C and 5% CO_2 and tested negative for mycoplasma (Lonza, Basel, Switzerland).

2.2. TLR gene expression

TLR1-10 gene expression was analyzed by RT-qPCR as previously described [7]. In short, 1.5 μl cDNA (iScript/qScript cDNA synthesis kit, Bio-Rad, Hercules, CA/Quanta, Gaithersburg, Maryland) was added to SYBR Green Supermix/FastMix (Bio-Rad/Quanta) together with 400 nM/300 nM of forward and reverse primers for TLR1-10 or the reference gene TATA box binding protein (TBP) (Bio-Rad/Quanta) [7]. The samples were analyzed in triplicates on a Chromo4 detector using MJ Opticon Monitor software version 3.1 (Bio-Rad) at 95 °C for 5 min, 40 cycles of 95 °C for 5 s, 60 °C–66 °C for 10 s, and 72 °C for 8 s.

2.3. TLR ligand activation and quantitation of cytokine response

Cells were seeded in 96-well plates and stimulated at 80% confluence with or without specific TLR ligands in 100 μl culture medium (triplicates); Pam3CysSerLys4 (P3CSK4; TLR2/1 ligand, 100 ng/ml, #L2000, EMCmicrocollection GmbH, Tübingen, Germany), Pam2CGDPKHPKSF (FSL-1; TLR2/6 ligand, 50 ng/ml, #L7000, EMCmicrocollection GmbH), polyinosinic:polycytidylic acid (poly (I:C); TLR3 ligand, 50 μg/ml, #27-4729-01, Amersham Pharmacia Biotech, Uppsala, Sweden), *Escherichia coli* lipopolysaccharide (LPS) (TLR4 ligand, 100 ng/ml, #tlrl-pelps, InvivoGen, San Diego, CA), flagellin (TLR5 ligand, 1 μg/ml, #tlrl-stfla, InvivoGen), R848 (TLR7/TLR8 ligand, 1 μg/ml, #tlr-r848-5, InvivoGen), and CpG oligodeoxynucleotide (ODN) 2006 (TLR9 ligand, 20 μM, TIBMolBiol, Berlin, Germany). LPS was sonicated for 5 min prior to use. After 24 h supernatants were collected, centrifuged, and stored at −80 °C.

For quantification of cytokine responses, supernatants were thawed on ice and analyzed with a human 10-plex cytokine immunoassay (Bio-Rad) on a Bio-Plex 200 system (Bio-Rad) powered by Luminex xMAP Technology. The levels of IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12 (p70), interferon-(IFN)-γ inducible protein (IP)-10, tumor necrosis factor (TNF)-α, IFN-γ, and vascular

Table 1
Characteristics and culture conditions of the seven trophoblast cell lines.

Cell line	Origin	Culture life span	Medium[b]	Supplement[b]	FBS[b]	Ref.
Choriocarcinoma cell lines						
BeWo	Choriocarcinoma	Unlimited	DMEM/Ham's F12	20 μM L-glutamine	10%	[16]
JAR	Choriocarcinoma	Unlimited	RPMI 1640	1 mM sodium pyruvate 1 μM HEPES D-glucose	10%	[17]
JEG-3	Choriocarcinoma	Unlimited	MEM	20 μM L-glutamine 1 mM sodium pyruvate 0.1 mM NEAA	10%	[18]
AC1M-32	AC1-1[a] fused with primary term trophoblast cells	Unlimited	Ham's F12		10%	[19]
ACH-3P	AC1-1[a] fused with first trimester cytotrophoblasts	Unlimited	Ham's F12		10%	[20]
SV40 transfected cell lines						
HTR-8/SVneo	Cells from tissue pieces of first trimester placental villi	Unlimited	RPMI 1640		5%	[22]
SGHPL-5	Primary first trimester extravillous trophoblasts	Passage 25	Ham's F10	20 μM L-glutamine	10%	[23]

[a] Hypoxanthine guanine phosphoribosyltransferase (HGPRT)-defective mutant of JEG-3.
[b] Fetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium), DMEM (BioWhittaker), Ham's nutrient mixture F12 (SAFC Biosciences, Hampshire, UK), L-glutamine (Sigma–Aldrich, St. Louis, Missouri), RPMI 1640 (Sigma–Aldrich), sodium pyruvate (PAA Laboratories GmbH, Pasching, Austria), HEPES (Gibco), D-glucose (Sigma–Aldrich), MEM medium (Gibco, Carlsbad, CA), non-essential amino acid (NEAA) cell culture supplement (Lonza, Basel, Switzerland), Ham's nutrient mixture F10 (Gibco).