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Inhibition of HTRA3 stimulates trophoblast invasion during human placental development

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ABSTRACT

Controlled invasion of extravillous trophoblast (EVT) is necessary for implantation and placentation. The serine protease HTRA3 is highly expressed in decidual cells in the late secretory phase of the menstrual cycle and throughout pregnancy. During the first trimester it is expressed in most trophoblast cell types, but not in the invading interstitial trophoblast. HTRA3 and its family members are down-regulated in a number of cancers and are proposed as tumour-suppressors. The current study investigated whether inhibiting HTRA3 in a first trimester trophoblast cell line expressing high levels of HTRA3 would alter invasion.

HTR-8/SVneo (HTR-8, derived from first trimester placenta) and a number of choriocarcinoma cells (JEG-3, AC-1M88 and AC-1M32) were screened for HTRA3 expression. Only HTR-8 cells expressed high levels of HTRA3 mRNA, consistent with HTRA3 being down-regulated in cancer. Western blotting and immunofluorescence confirmed HTRA3 protein expression and localisation in HTR-8 cells. HTRA3 was detected in conditioned medium of HTR-8 cells, confirming its secretory nature. For functional studies, both long and short forms of recombinant human HTRA3, wild type and protease-inactive mutant ($S^{305}A$) were produced using wheat-germ cell-free technology. Both have a similar molecular size, but the mutants have negligible protease activity. In addition, the mutants significantly inhibited the wild type protease activity, supporting their dominant-negative inhibition and utility as specific inhibitors of the wild type protein. Inhibition of HTRA3 by exogenous addition of HTRA3 mutant resulted in a significant increase in HTR-8 cell invasion. These results strongly support the hypothesis that HTRA3 is an inhibitor of trophoblast invasion during placental development.

1. Introduction

Adequate trophoblast invasion into the decidual stroma and spiral arteries is one of the crucial steps in human placental development. During normal pregnancy, a complex molecular dialogue takes place among the maternal endometrium (decidua), the conceptus, and the placenta. With the conceptus anchored in the maternal uterine wall, trophoblast cells cross the placental–maternal interface and invade the maternal decidua. Remodelling of maternal spiral arteries takes place to establish a vascular supply sufficient to enable optimal growth and development of the conceptus [1]. Various subsets of trophoblast originate from the trophectoderm of the blastocyst, including the villous trophoblast (cytotrophoblast, syncytiotrophoblast) which covers the placental villi, and the invasive extravillous cytotrophoblast which invade the maternal decidua [2].

Invasive trophoblast cells share several phenotypic properties with tumour cells, including some of the molecular mechanisms regulating invasion through the extracellular matrix [3,4]. Unlike tumour invasion, trophoblast invasion is under tight control that confines it to the inner one-third of the myometrium during pregnancy in women [5,6]. Thus while the invasive nature of extravillous trophoblast (EVT) is essential for proper placental development, the extent of invasion must be tightly controlled. Over-invasion and shallow invasion can lead to pathologies of pregnancy such as choriocarcinoma and preeclampsia, respectively [7–9].

Simultaneous activities of a number of proteins are known to regulate cell invasion, especially during the first trimester [10,11]. The stepwise progression commencing from the villous cytotrophoblast to the invasive extravillous cytotrophoblast is

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characterized by significant change in expression of cell adhesion molecules and proteinases that degrade the extracellular matrix [12].

The high temperature requirement factor A3 (HTRA3) belongs to a family of stress-related serine proteases that is conserved from bacteria to humans [13]. Two alternatively spliced mRNA variants (long (HTRA3-L) and short (HTRA3-S)) have been identified in human placenta [14]. Four distinct domains were found for the long form: an insulin-like growth factor binding domain (IGFBP), a kazal-type protease inhibitor domain, a trypsin-like serine protease domain and a postsynaptic density protein 95-Discs large-Zona occuldens 1 (PDZ) domain [13,14]. The short form is identical to the long form except it lacks the (PDZ) domain [14]. In addition to HTRA3, other mammalian members of HTRA family identified are HTRA1, HTRA2 and HTRA4 [13,15–18]. Each member contains a highly conserved trypsin-like catalytic domain followed by one or more C-terminal PDZ domains. In humans, the HTRA are involved in several important functions, such as cell growth, apoptosis, invasion and inflammatory reactions, and they control cell fate via regulating protein metabolism [13]. HTRA1 and HTRA3 share identical domain organization including an N-terminal signal peptide, an insulin growth factor binding domain and a kazal-type S protease inhibitor domain suggesting that they may have similar functions, but they display different expression patterns among adult human tissues [14,18]. Over-expression of both HTRA1 and HTRA3 inhibits cell growth and proliferation in vitro and in vivo, and also plays a protective role in various malignancies because of their tumour suppressive properties [19–23].

Expression of HTRA3 is significantly upregulated during mouse placental development [24]. In addition, HTRA3 is developmentally regulated and localised to human endometrium and placenta during the first trimester of pregnancy [25]. During the menstrual cycle, HTRA3 is expressed primarily in the endometrial glands, being significantly upregulated towards the mid to late secretory phases and prominent expression in stroma is detected only in the decidual cells [25]. Thus, overall endometrial HTRA3 expression is highest in the late secretory phase, when the endometrium is prepared for maternal–trophoblast interaction. The expression pattern of HTRA3 in the peri- and post-implantation uterus and also in non-villous trophoblast is much higher than HTRA1, suggesting that HTRA3 is more relevant to placentation [25,26].

HTRA3 is also expressed in certain trophoblast subtypes in the first trimester placenta. It is expressed strongly in the villous syncytiotrophoblast, trophoblast shell, and endovascular cytotrophoblast; and weakly in the distal portion of the trophoblast cell columns. However, HTRA3 is not expressed in the invasive interstitial extravillous trophoblast [25]. This distinct HTRA3 distribution at the maternal–trophoblast interface suggests that it may affect the invasive potential of trophoblast during placental development. In particular, its down-regulation in the interstitial trophoblast suggests that HTRA3 may be an inhibitor of trophoblast invasion. A recent study demonstrated that higher expression of HTRA1 was negatively correlated with trophoblast cell migration and invasion [27], but nothing is known of the actions of HTRA3.

The objective of this study was to develop a specific inhibitor of HTRA3 and to apply this to determine whether HTRA3 has a role in controlling trophoblast invasion.

2. Materials and methods

2.1. Cell culture

The HTR-8 cell line was derived from primary explant cultures of human first trimester placenta (8–10 wk gestation) and immortalised with SV40 virus [28]. HTR-8 cells, kindly provided by Dr. Charles Graham (Queen's University, Kingston, ON, Canada) were maintained in RPMI-1640 medium (Sigma) containing 20 mM HEPES, 7.5% (w/v) sodium bicarbonate (Sigma) and supplemented with 10% fetal calf serum (FCS, SAFC Biosciences, Kansas, USA), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco, Mulgrave, Australia). Cells were incubated at 37 °C in 5% CO_2 and passaged at 80–90% confluence. Cells were grown in 75-cm^2 and 25-cm^2 culture flasks (Corning, Castle Hill, NSW, Australia) for cell line maintenance and collecting cell lysates. The choriocarcinoma cell lines (JEG-3, AC-1M88 and AC-1M32) were cultured as previously reported [29] in RPMI-1640 containing 10% FCS.

2.2. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Cells were lysed and processed for total RNA isolation using an RNeasy Minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA was treated with ribonuclease-free deoxyribonuclease-1 (DNA-free kit, Ambion, Texas, USA) to remove any contaminating genomic DNA. RNA concentration was determined at 260 nm using an ND1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Total RNA (0.5 μg) was preheated at 65 °C for 5 min, then reverse transcribed at 46 °C for 2 h in 20 μl reaction mixture using 100 ng random hexanucleotide primers, 5 IU avian myeloblastosis virus reverse transcriptase, 10 mM dNTPs, 100 mM dithiothreitol, and 20 IU ribonuclease inhibitor in the presence of cDNA synthesis buffer (all from Roche, Mannheim, Germany). The resultant cDNA mixtures were heated at 95 °C for 3 min before storage at −20 °C. Negative controls were performed by omission of reverse transcriptase.

The cDNA product (1 μl) was amplified in a total volume of 26 μl using 13 μl GoTag green master mix (Promega, Hawthorn, Vic, Australia) and 10 pmols of forward and reverse primers for HTRA3 isoforms and 18 S (Table 1) [25]. All PCRs were run in duplicate with essential parameters shown in Table 1. A no template control in which DEPC water was substituted for RNA was included as a negative control. PCR products were analysed by electrophoresis on 1% agarose gel (Roche, Castle Hill, Australia) and stained with Gel Red (Biotium, Stepney, SA, Australia). As a loading control, 18 S was amplified for each RT.

2.3. Protein extraction and western blotting

HTR-8 cells were lysed with Ripa buffer and total protein (45 μg) extracted from cells, separated on 10% SDS-PAGE under reducing conditions and transferred to Hybond-P membranes (Amersham Life Science, Sydney, Australia). After overnight blocking at 4 °C in a blocking buffer (5% [w/v] skimmed milk in TBS and 0.1% [v/v] Tween 20), the membrane was incubated with a well-characterized sheep anti-human HTRA3 antibody at 1:20 dilution [25] in the blocking buffer for 2 h at room temperature, then with an HRP-conjugated donkey-anti sheep IgG (1:5000; Chemicon, Australia) for 1 h at room temperature and developed by chemiluminescence (ECL Plus system, Amersham).

2.4. Immunofluorescence

HTR-8 cells were grown on 14 mm glass coverslips till 70–80% confluent, then washed twice in phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (BDH) for 30 min at room temperature. The fixed cells were washed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 15 min at room temperature. All incubations and washes were carried out at room temperature. The cells were then blocked with 1:20 sheep serum for 30 min, incubated with HTRA3 antibody (1:20 dilution) for 2 h, washed in PBS for 3 × 5 min, and finally incubated with 1:50 polyclonal goat-anti sheep Alexafluor 568 (Invitrogen) for 1 h in the dark. The nuclei were stained with 2 μm 4′,6-diamidino-2-phenyindole dilactate (DAPI, Sigma) for 10 min and the coverslips were mounted with fluorosave reagent (Calbiochem, Kilsyth, Australia). Staining was examined with an Olympus BX60 fluorescent microscope and images were taken using an Olympus DP70 camera and DP controller imaging Leica software.

2.5. Production of recombinant human HTRA3 and mutant

Long and short form recombinant human HTRA3 proteins (HTRA3-L and HTRA3-S respectively) were synthesized using wheat-germ cell-free technology [30–32]. In

Table 1
The primer sequences and reaction conditions for RT-PCR.

Gene	Primer Sequences (5′ – 3′) F – Forward, R – Reverse	Annealing temperature (°C) and cycles (n) – Block PCR
HTRA3-L (long)	F – ATG CGG ACG ATC ACA CCA AG R – CGC TGC CCT CCG TTG TCT G	58 (30 cycles)
HTRA3-S (short)	F – GAG GGC TGG TCA CAT GAA GA R – GCT CCG CTA ATT TCC AGT	53 (35 cycles)
18 S	F – CGGCTACCACATCCAAGGAA R – GCTGGAATTACCGCGGCT	64 (26 cycles)