The Effect of Trophoblasts on T Lymphocytes: Possible Regulatory Effector Molecules - A Proteomic Analysis

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Key Words

Trophoblast • T lymphocyte • Immunesuppression • Proteomics

Abstract

Background/Aims: Tolerance of T lymphocytes at the feto-maternal interface is necessary to sustain normal pregnancy. The present investigation aimed to observe the regulatory effects on T lymphocytes by human trophoblasts and to explore possible effector molecules. **Methods:** Conditioned media was made by trophoblast culture or villous explant culture for T lymphocyte proliferation and proteomic analysis. Lymphocyte proliferation was tested by thymidine incorporation. Messenger RNA for indoleamine 2,3-dioxygenase (IDO) was detected by RT-PCR and tryptophan was assayed. The protein profile of conditioned media was assessed with shotgun mass-spectrometry and the identified proteins were bioinformatically analyzed. Human chorionic gonadotropin (HCG), human chorionic somatomammotropin (HCS), interleukin (IL)-2, 4, 10 and tumor necrosis factor (TNF)- α were assayed with radioimmunoassay (RIA). **Results:** T Lymphocyte

proliferation was inhibited by conditioned medium in a dose-dependant manner. Inhibition of IDO during previous conditioning or addition of tryptophan to the conditioned medium partly restored T lymphocyte proliferation. mRNA for IDO was expressed in trophoblasts and chorionic villi. The concentrations of tryptophan were 19.01 and 3.79 $\mu\text{mol/L}$ in unconditioned and conditioned media respectively. By proteomic procedures, 548 proteins were found in placenta-conditioned medium. Among these proteins were some proteins inhibiting T lymphocytes including HCG, HCS, AFP, pregnancy-specific beta 1-glycoprotein (SP1), glycodeilin, transforming growth factor β 2, thrombospondin-1, pigment epithelium-derived factor (PEDF), galectin-1, and macrophage migration inhibitory factor. HCG and HCS were also detected with RIA, however, no interleukins were detected in conditioned media with RIA or proteomic analysis. **Conclusions:** Trophoblasts inhibit T lymphocyte through IDO-mediated tryptophan depletion and placenta-derived immunoregulatory factors. Immunological tolerance at maternal-fetal interface represents a synergistic effect of these substances and a complex mechanism involving endocrine and immune networks.

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Introduction

The conceptus genome is half-paternal and half-maternal. Because the conceptus is a semi-allograft to its mother, it would be supposed to be attacked by maternal immune system [1, 2]. However, it is a paradox that the immunologically foreign conceptus can survive in the uterus for as long as 40 weeks without provoking maternal immune responses [1, 2]. Several mechanisms have been proposed to underlie this immunological enigma. Trophoblasts, cells of fetal origin, have been thought to be the initiator of this immunological tolerance [1, 2]. On the other hand, tolerance of T lymphocytes, the main effector cells mediating tissue allograft rejection and inflammatory processes, has been proposed to be the major mechanism of maternal tolerance to her fetus [1, 2]. However, it is still not clarified how the tolerance of T-cell is achieved.

Previous studies showed that placenta had immunoregulatory properties. Some factors derived from placenta have been proposed to have potential roles in suppressing T lymphocyte proliferation, activation and cytokine production for long time [3-11]. Although much effort has been made in searching for the substances that suppress the T lymphocytes, little was achieved in this field.

Indoleamine 2,3-dioxygenase (IDO), the initial and rate-limiting enzyme that catalyzes the catabolism of tryptophan along the kynurenine pathway, is expressed in placental trophoblasts and has been postulated to contribute to the establishment of local immuno-tolerance at maternal-fetal interface by inhibiting T lymphocyte responsiveness because inhibition of IDO led to the absorption of allogeneic concepti in mouse [12]. In humans, the findings regarding the role of IDO in inhibiting T lymphocyte responsiveness by placental trophoblasts were controversial. IDO was reported involved in mediating inhibitory effects of human placental trophoblasts on T lymphocytes by the degradation of tryptophan [8, 9]. On the other hand, Gupta et al provided different findings and proposed that some currently unknown soluble factors are responsible for the reduction of T lymphocyte responsiveness induced by trophoblasts [7]. Seeking the effector molecules by which placental trophoblasts inhibit T lymphocytes is of much interest to researchers in different fields including immunology and reproduction.

The aims of the current study were: 1) to test the hypothesis that tryptophan depletion via IDO might be the mechanism, or one of the mechanisms, by which trophoblastic cells inhibit T cell and 2) to explore potential

effector molecules that might suppress T lymphocytes.

Materials and Methods

Trophoblast isolation and culture

Placentas were obtained from women undergoing elective pregnancy termination at 8-11 gestational weeks or women undergoing elective cesarean section with normal pregnancy at term. Since trophoblasts isolated from chorionic villi of first trimester pregnancy or term placenta had similar effect on T lymphocyte proliferation in our primary experiments and trophoblasts were more abundant in first trimester villi than in term placenta, trophoblasts isolated from early pregnancy villi were used in the present study. Trophoblast isolation was performed using a modified method described by Kliman et al [13]. Briefly, walnut-sized villous tissue samples were disaggregated in Hank's balanced salt solution without calcium and magnesium. Membranes, coagulates and vessels were removed. The tissue was coarsely minced, transferred to 100 ml warmed calcium and magnesium-free Hanks' solution containing 0.125% trypsin (Sigma, St Louis, MO, USA) and 0.2mg/ml DNase I (Sigma; 1,500 Kunitz units/mg), pH7.4 and then incubated at 37°C with shaking for 20 min. The flask was set at an angle, and the tissue fragments were allowed to settle for 1 min. Then, the aliquots were collected and equal volume of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS; Sijiqing Biotech, Hangzhou, China) was added to stop trypsinization. The cell suspension was centrifuged at 1000 g for 5 min at room temperature. The resultant pellets were re-suspended in 6 ml DMEM containing 25 mM HEPES and 25 mM glucose. The remaining tissue fragment was subject to the digestion procedure two more times with addition of 50 ml, respectively, warmed fresh trypsin-DNase solution. The three resultant cell suspensions were pooled, filtered using 100-mesh screen sieve, and centrifuged at 1000 g for 10 min. The resultant pellets were re-suspended in ice-cold NH_4Cl (0.155 M in water) and left for 10 min at 0°C to lyse the erythrocytes, after which an equal volume of MDEM was added. The cells were washed and resuspended in DMEM containing 10% FCS. The trophoblast cells were adjusted to the concentration of $1 \times 10^6 \text{ ml}^{-1}$ and plated in tissue culture plates (Falcon, USA) and incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. After 72 hours of culture, supernatants were collected and used as conditioned medium for the proliferation of T lymphocyte and the assay of tryptophan. Pharmacologic IDO inhibitor 1-methyl-tryptophan might be supplemented to the concentration of 2 mM according to the purpose of the experiment.

Villous explant culture

To avoid protein contamination from FCS and to obtain enough amount of protein for proteomic analysis, chorionic villous tissues were dissected under sterile condition in serum-free DMEM. Small clusters of placental villi (40-50 mg wet weight) were placed in 24-well plates in serum-free DMEM

and were cultured at 37°C in an atmosphere containing 5% CO₂ for 72 hours. When the culture was finished, the supernatants were collected after centrifuged at 3000 g for 20 min.

Assay of the Proliferation of T lymphocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy female volunteer donors by Ficoll-Paque gradient centrifugation and T lymphocytes were purified with human T cell enrichment column (R&D Systems, USA). T lymphocytes were plated in 96-well flat bottom plates at a concentration of $1 \times 10^6 \text{ ml}^{-1}$ and cultured in a humidified atmosphere of 5% CO₂ and 95% air for 72 hours. Cells were stimulated with 5 µg/ml phytohaemagglutinin (PHA, Sigma, USA) and pulsed with 1.0 µCi/well of [³H] thymidine (Amersham, UK) 18 hrs before the end of culture. When culture ended, cells were harvested onto glass fiber filter (Wallac, Finland) and the radioactivity was measured with liquid scintillation counter (Beckmann Coulter, USA).

DMEM supplemented with 10% heat-inactivated FCS was used and conditioned medium at different concentrations and L-tryptophan at the concentration of 20 µM were added according to the purposes of the experiments.

Detection of messenger RNA for IDO

To analyze the expression of messenger RNA for IDO, total RNA was isolated from chorionic villi of first trimester pregnancy and isolated trophoblasts using Trizol reagent (Invitrogen, USA). Sample RNA (1 µg) was reversely transcribed with avian myeloblastosis virus reverse transcriptase and specific fragments were amplified with the following primers: forward, 5'-TTC CTT ACT GCC AAC TCT CC-3', and reverse, 5'-TCA TAC ACC AGA CCG TCT GA-3' for IDO; forward, 5'-TTC CAG CCT TCC TTC CTG G-3' and reverse, 5'-TTG CGC TCA GGA GGA GCA AT-3' for beta-actin as an internal control. The length of the PCR product was 424 bp for IDO and 224 bp for β-actin respectively. The reaction conditions were as follows: reverse transcription 90min at 37°C, pre-denature 5 min at 94°C, denature 30 s at 94°C, annealing 30s at 53°C, and extension 45 s at 72°C. After 30 cycles, the PCR product was visualized with ethidium bromide staining on agarose gel.

Determination of cytokines and hormones

Concentrations of interleukin-2 (IL-2), IL-4, IL-10, tumor necrosis factor-α (TNF-α), human chorionic gonadotropin (HCG), human chorionic somatomammotropin (HCS), estradiol (E2) and progesterone (P) in unconditioned and conditioned media were determined by Radioimmunoassay (RIA). The RIA kits were supplied by Dongya Institute of Radioimmunoassay (Beijing, China).

Sample preparation, SDS-PAGE and In-Gel Digestion

Fifty milliliters of villous explant-conditioned media was used for proteomic analysis. Conditioned medium was concentrated 400-fold using Amicon Centriplus YM-3 centrifugal filter devices (Millipore, USA) with a 5 kDa molecular weight cut-off after centrifuged at 12000 g at 4°C for 2 hours. Protein concentration was estimated with a Bradford assay

using bovine serum albumin (BSA) as a protein standard. Concentrated samples in loading buffer (final buffer composition: 2% beta-mercaptoethanol (v/v), 1% Sodium dodecyl sulfate (SDS, w/v), 12% glycerol (v/v), 50 mM Tris-HCl, and a trace amount of bromophenol blue), boiled in water for 5 min, cooled, and 20 µg of protein were loaded per lane. Gel electrophoresis was performed on a Protean III mini-gel apparatus (Bio-Rad, Hercules, CA, USA) using 12.5% gel with 0.1% (w/v) SDS. Gel electrophoresis was carried out under a constant current of 30 mA. Because no abundance of serum proteins were present, the entire gel lane was cut, proteins were in-gel digested with trypsin over night, and peptides were extracted as described by Shevchenko et al [14], with a few minor modifications as reported previously [15]. The extracted peptides were concentrated in a Speed-Vac to 10 µL, cleaned, and desalted with C18 Zip-Tips (Millipore). The desalted peptides were dried completely in a Speed-Vac and re-dissolved in 0.6 µL of 0.1% trifluoroacetic acid (TFA) for liquid chromatography (LC) mass-spectrometry (MS)/MS analysis.

LC MS/MS shotgun analysis

1-D-LC MS/MS was performed using an LTQ linear IT mass spectrometer (Thermo, San Jose, CA, USA). The system was fitted with a C18 RP column (180 µm×150 mm, BioBasic C18.5 µm; Thermo Hypersil-Keystone, USA). Mobile phase A (0.1% formic acid in water) and the mobile phase B (0.1% formic acid in ACN) were selected. The tryptic peptide mixtures were eluted using a gradient of 2–98% B over 120 min. The LTQ linear IT mass spectrometer was set so that one full MS scan was followed by three MS/MS scans on the most intense ions from the MS spectrum with the following Dynamic Exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s.

Protein identification

The acquired MS/MS spectra were automatically searched against protein database for human proteins (EMBL-EBI proteome set for *Homo sapiens* (human), 1/28/2007 released) using the Turbo SEQUEST program in the BioWorks 3.0 software suite. An accepted SEQUEST result had to have a DCn score of at least 0.1 (regardless of charge state) and a cutoff of Rsp 4. A singly charged peptide must be tryptic, and the cross-correlation score (Xcorr) had to be at least 1.9. Tryptic or partially tryptic peptides with a charge state of 12 have an Xcorr of at least 2.2. Triply charged tryptic or partially tryptic peptides with a 13 charge state was accepted if the Xcorr was 3.75 [16].

Bioinformatic analysis

Protein identification results were extracted from SEQUEST out.file with the in-house software BuildSummary. Functional classifications were performed with GOA (<http://www.ebi.ac.uk/GOA/>) according to the accession number of proteins.

Statistic analysis

Data were presented in mean and SD. Comparison was made with Student *t* test or ANOVA where appropriate. *P*<0.05 was considered significant. Statistical software SPSS was used for data analysis.

Fig. 1. The expression of IDO mRNA. RNA was isolated from trophoblasts and chorionic villi and mRNA for IDO was amplified with specific primers. The results of RT-PCR show the expression of IDO mRNA by trophoblasts (lane 3) and chorionic villi (lane 4) and the expression of β -actin mRNA by trophoblasts (lane 1) and chorionic villi (lane 2). M: molecular marker; B: Blank.

Fig. 2. The inhibition of conditioned medium on T lymphocyte proliferation. T lymphocytes were cultured in conditioned medium for 72 hours and the proliferation was determined by [3 H]-thymidine incorporation. The proliferation of T lymphocytes was inhibited by conditioned medium in a dose-dependant manner and was partly restored by the inhibition of IDO during previous conditioning (Inhibition of IDO) or the supplementation of tryptophan to the conditioned medium (Supl of Trp). X-axis: concentration of conditioned medium, inhibition of IDO and supplementation of tryptophan; Y-axis: radioactivity of incorporated [3 H]-thymidine. Data were presented in mean and SD. *: $P < 0.001$.

Ethical consideration

The current investigation was conducted according to the protocol approved by the Hospital Ethics Committee and informed consents were obtained from all the women who provided samples.

Results

The expression and functional activity of IDO of trophoblasts

To determine the expression of IDO by chorionic villi and trophoblasts, RT-PCR was performed to detect the IDO mRNA in isolated trophoblasts and chorionic

villi. As shown in Fig 1, IDO mRNA was abundant in trophoblasts and chorionic villi. To confirm the function of IDO, conditioned medium prepared by trophoblast culture was assayed for tryptophan with high performance liquid chromatography (HPLC). The concentration of tryptophan was 19.01 $\mu\text{mol/L}$ in fresh (unconditioned) medium, 3.79 $\mu\text{mol/L}$ in conditioned medium and 15.40 $\mu\text{mol/L}$ in conditioned medium when IDO was inhibited by 1-methyl-tryptophan during conditioning, respectively, indicating that the metabolism of tryptophan via IDO is the major pathway of tryptophan depletion in this culture system.

Fig 3. Classification of identified proteins in villi-conditioned medium. The identified proteins were classified according to their cellular localizations and extracellular proteins were further classified according to their origins. A: cellular localization of proteins in conditioned medium. B: the origin of extracellular proteins.

Table 1. Identified Substances affecting T lymphocytes.

Name	Abbreviation	Origin	The effects on T lymphocytes in vitro
Estradiol	E ₂	Trophoblast	Inhibits T cell proliferation [25, 26]; Arrests T cells in S and G2/M phases of the cell cycle [25, 26]; Induces apoptosis [25, 26]; Suppresses the expression of IL-2 and its receptor [27]
Progesterone	P	Trophoblast	Inhibits T cell activity via inducing glycodeclin and progesterone-induced binding factor [28]
Human chorionic gonadotropin	HCG	Trophoblast	Suppresses PHA or mixed lymphocyte culture-stimulated T cell proliferation [31]
Human chorionic somatomammotropin	HCS	Trophoblast	Suppresses PHA or mixed lymphocyte culture-stimulated T cell proliferation [28]
Pregnancy-specific beta 1-glycoprotein	SP1	Trophoblast	Suppresses PHA or mixed lymphocyte culture-stimulated proliferation [22]
Alpha-fetoprotein	AFP	Trophoblast	Inhibits T lymphocyte proliferation [37]
Glycodeclin	-	Trophoblast	Inhibits proliferation and cytokine secretion stimulated by PHA or immobilized anti-CD ₃ mAb [33]
Transforming growth factor beta-2	TGF-β2	Trophoblast?	Inhibits T lymphocyte growth, activation and cytokine production [45]; Induces apoptosis [46, 47]
Thrombospondin-1	TSP-1	Trophoblast?	Inhibits TCR -mediated activation and cytokine production [39, 40]
Pigment epithelium-derived factor	PEDF	Trophoblast?	Blocks T cell activation, proliferation and adhesion to endothelial cells [43, 44]
Macrophage migration inhibitory factor	MIF	Trophoblast?	Inhibits cytokine-, CD3 -, and allo -induced T -cell activation [50]
Galectin-1	Gal-1	Trophoblast	controls the proliferation and survival of effector T cells [54, 55] ; antagonizes T cell activation [56]; blocks secretion of proinflammatory cytokines [57]

Lymphocyte proliferation

Conditioned medium was harvested from cultures of trophoblasts after 72 hrs. T lymphocytes were suspended in medium containing conditioned medium at different concentrations and activated with mitogen PHA. Fig 2 shows the inhibition of T lymphocyte proliferation by conditioned medium in a dose-dependent manner. The addition of tryptophan to the conditioned medium partly

restored the ability to support T cell proliferation to approximately 4 folds that of conditioned medium (Fig 2). Similarly, the addition of the pharmacologic IDO inhibitor 1-methyl-tryptophan to the previous culture of trophoblasts restored in part the ability to support T cell proliferation to about 4 folds that of conditioned medium (Fig 2). The presence of 1-methyl-tryptophan did not interfere the proliferation of T lymphocyte.

Protein profile in placental villi-conditioned media

Proteins from placental villous medium were searched against the EMBL-EBI human protein database. 1869 peptides corresponding to 548 proteins were identified. The complete list of the proteins was shown in the Supporting Material. The proteins were clarified according to their cellular components. Of the 548 proteins, 52% were intracellular or membrane proteins, 13% were extracellular proteins, 23% were cellular component unknown, and 12% were miscellaneous (Fig 3A). Of the extracellular proteins, 29% were ECM proteins, 43% were plasma proteins, 10% were proteins were of trophoblast origin and 18% were origin unknown (Fig 3B). The miscellaneous proteins were those not matched in the database or functional unknown.

Because the proteins that may affect T lymphocytes are the extracellular ones, the names of these proteins and their biological process were also listed in Supporting Material. After we searched the PubMed literature database, 9 proteins as well as estradiol and progesterone were found have effects on T lymphocytes. The names and reported effects on T cells of these substances are listed in Tab 1. No other intracellular or miscellaneous proteins were found to affect T cells in PubMed literature database.

Cytokines and hormones in conditioned medium

To validate the findings of proteomics, HCG, HCS, IL-2, IL-4, IL-10 and TNF- α in the conditioned media were measured with sensitive radioimmunoassays. HCG and HCS were detected at concentrations of 525 unit/L and 2.5 ng/ml, respectively. E2 and P were also detected at concentrations up to 56.3 pg/ml and 0.5 μ g/ml, respectively. IL-2, IL-4, IL-10 and TNF- α were not detected. All these substances were not detected in the unconditioned media.

Discussion

By conducting the experiments, we confirmed the functional expression of IDO in placental trophoblasts and found that media conditioned by placental trophoblasts strongly inhibited T lymphocyte proliferation in a dose-dependant manner and that this inhibitory effect partly depended on IDO-mediated tryptophan depletion. This conclusion follows from the observations that IDO is expressed by placental trophoblasts and is functionally active, that the inhibitory effect is in a dose-dependant

manner and that both inhibition of IDO during previous conditioning and addition of tryptophan to the conditioned media partly restore T lymphocyte proliferation. Our results indicate that tryptophan catabolism via IDO is one of the mechanisms by which trophoblasts inhibit T cell proliferation in vitro.

The immunosuppressive property of human placental trophoblast on T lymphocytes has long been recognized. Culouscou et al [5] found the immunosuppressive effect of supernatant prepared from human placental trophoblasts after a short-term culture on mitogen-stimulated human lymphocyte culture and mixed lymphocyte culture. Degenne et al [6] reported the inhibitory property of purified human syncytiotrophoblast plasma membranes (STPM) on the cytolytic activity of cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells against their target cells. Thibault et al [11] observed the inhibitory effect of STPM on IL-2 production and the expression of IL-2 receptor of Jurkat cells. Similar findings were reported with murine trophoblasts, human hydatidiform mole trophoblasts and chorionic carcinoma cells [3, 4, 10, 17]. Those findings point to the presence of immunosuppressive factors produced by human placental trophoblasts at the maternal-fetal interface. However, the immunoregulatory molecules of trophoblast origin are not known and the exact mechanism by which placental trophoblasts inhibit T lymphocytes needs to be clarified.

Munn et al [18] found that inhibition of IDO induced the loss of allogeneic concepti in mice and proposed that catabolism of tryptophan by IDO is critical for maternal tolerance to her fetus. Thus, the role of IDO in maintaining normal pregnancy was proposed. Kudo et al [19] localized IDO activity to brush border of microvillous plasma membranes of placental syncytiotrophoblast and later the same group found that human PBMC proliferation following culture in the medium previously conditioned by the culture of placental explant was inhibited, and that this inhibitory effect was enhanced when placental IDO activity was stimulated with interferon- γ (an IDO stimulator) but reversed by either supplementation of the conditioned medium with tryptophan or inhibition of IDO during the previous conditioning with pharmaceutical IDO inhibitor 1-methyl tryptophan [9]. These findings provide in vitro evidence to support the hypothesis of Munn et al that T cells could be suppressed via tryptophan catabolism at maternal-fetal interface. Later, Gutierrez et al [8] demonstrated that trophoblast supernatant inhibited activation and proliferation of T cells and the inhibition was reversed by supplement of tryptophan to the

supernatant, supporting this hypothesis as well.

Recently, this hypothesis was challenged. Gupta et al [7] found that placenta-derived soluble factors inhibited the activation, proliferation and cytokine production of T lymphocytes and these inhibitory effects were not reversed by the addition of tryptophan. They proposed that the unknown soluble factors produced by trophoblasts might inhibit T lymphocyte responsiveness. Our data in the present study revealed that both inhibition of IDO during the previous conditioning and the addition of tryptophan to the conditioned media of placental trophoblast partly restored the ability of supporting the T lymphocyte proliferation. This indicates that the catabolism of tryptophan by IDO is one of the mechanisms by which trophoblasts inhibit T lymphocytes and that there are other mechanisms underlying the inhibitory action. Further studies are needed to explore other mechanisms and effector molecules by which placental trophoblasts inhibit T cell proliferation and maternal immune tolerance is established at the maternal-fetal interface.

In the present investigation, we used proteomic technique to identify molecules that might work as inhibitory effector molecules and used radioimmunoassays to validate the findings of proteomic approaches. Proteomics, the large-scale study of proteins, offers great potential in unraveling complex biological problems such as the nature of particular molecular complexes or pathways in pathogenesis [20]. Shotgun proteomics, which was performed in the present study, is a powerful tool to separate and identify proteins from complex protein mixtures and able to identify hundreds of proteins in a single sample [20]. It provides a sensitive, rapid, high throughput, and specific analysis of peptides and proteins at molecular level [20]. With this approach, 548 proteins were identified in the supernatant of placental villi. Among these proteins are 9 proteins with immunoregulatory effects on T cells. In addition, estradiol and progesterone, which were detected by RIA, were present in the supernatant of trophoblast culture. Our data provided some candidate molecules that might inhibit T cell responsiveness and were consistent with the proposal of Gupta et al [7] that some soluble trophoblast-derived factors inhibited T cell proliferation and activation.

Proteomic analysis revealed the presence of HCG and HCS in the conditioned medium, which was validated by RIA. On the other hand, potentially interesting interleukins were not found by proteomic approaches. Meanwhile, ILs-2, 4, 10 and TNF- α were not detected with RIA. The identical findings of proteomics and RIA support each other the results of proteomic analysis and

immunoassays.

The production of estradiol (E2), progesterone (P), human chorionic gonadotropin (HCG), human chorionic somatomammotropin (HCS, formerly known as human placental lactogen, HPL), and pregnancy-specific-beta 1-glycoprotein (SP1) are characteristic of placental trophoblasts. The presence of these proteins as well as α -fetoprotein (AFP) and glycodelin were demonstrated in the present study. These pregnancy-associated hormones constitute the basis of pregnancy biology and immunology [21] because these pregnancy-associated hormones have immunoregulatory properties and alter the functions of human lymphocytes [22].

Estrogen receptor is functionally expressed in peripheral blood T lymphocytes [23, 24]. It has been shown that 17-beta-Estradiol (estrogen) inhibited T cell proliferation, arrested T cells in S and G2/M phases of the cell cycle, induced apoptosis and suppressed the expression of IL-2 and its receptor in human T cells [25-27]. In vivo, estradiol indirectly inhibits T cell proliferation and cytokine production via inhibiting dendritic cells. Progesterone induces a number of proteins such as glycodelin and progesterone-induced binding factor (PIBF), which inhibit the activity of T-cells and killer cells. HCG, HCS and SP1 were shown to suppress the proliferative T and B cell response induced by different mitogens in human and mouse [28, 29]. Human T lymphocytes from normal pregnant women express mRNA transcripts encoding the hCG/LH receptors and a receptor protein that binds 125 I-hCG, suggesting the functional expression of HCG receptor in human T lymphocytes [30]. Through its receptor, HCG exerts inhibitory effects on T lymphocytes [31]. SP1 and HCS also have the ability to suppress T cell proliferative response stimulated by PHA or mixed lymphocyte culture [22].

Glycodelin, originally isolated from human placenta and formerly named placental protein 14 (PP14) or progesterone-associated endometrial protein [32], directly inhibits T-cell proliferation and cytokine secretion stimulated by phytohemagglutinin (PHA) or immobilized anti-CD₃ mAb [33]. Alpha-fetoprotein (AFP) is mainly synthesized in the fetal liver, the yolk sac and other non-hepatic fetal tissues including placenta. Both AFP transcript and protein are expressed in placental trophoblasts [34, 35] and activated lymphocytes express AFP receptor [36]. In vitro, human AFP exhibits anti-proliferative effect on lymphocytes harvested from normal human donors and this inhibitory effect is quite significant in the same dose during blastic transformations of the

lymphocytes [37]. In vivo, AFP suppresses T cell development and T cell-dependent immune responses [38].

Extracellular matrix protein may have stimulatory or inhibitory effect on T cell activation, which depends on the interaction between T cells and extracellular matrix proteins [39, 40]. Thrombospondin-1, which is an extracellular matrix protein and is present in the supernatant of trophoblast culture as demonstrated in this study, is known as an inhibitor of angiogenesis and to have inhibitory activity in dendritic cells and thymic epithelial cells. Recently, it has been reported that thrombospondin-1 inhibited TCR-mediated activation and cytokine production of T lymphocytes and was thought to play a vital role in maintaining immune privilege of the eye [39, 40]. Thrombospondin-1 is present at the maternal-fetal interface [41], but its role in maternal tolerance to fetus has never been studied.

Pigment epithelium derived factor (PEDF), a glycoprotein that belongs to the superfamily of serine protease inhibitors, was isolated from the conditioned media of human retinal pigment epithelial cells [42]. PEDF was found to block cytokine- and growth factor-induced epithelial cell activation, angiotensin II-induced T cell proliferation by suppressing autocrine production of IL-2, and angiotensin II-induced T cell adhesion to endothelial cells by suppressing intercellular adhesion molecule-1 [43, 44]. Thus, PEDF has been considered a potent inhibitor of inflammatory response.

Transforming growth factor β (TGF- β) is the prototype for large family of growth factors with pleiotropic and multifunctional properties and affects processes ranging from regulation of cellular differentiation and growth to inflammation, wound healing and bone formation. TGF- β 2 was only growth factor detected in the conditioned media of chorionic villi. TGF- β 2 has been considered to be a potent immunosuppressive cytokine that inhibits T lymphocyte growth, activation and cytokine production both in vitro and in vivo and induces apoptosis of murine T cells [45-47].

Generally, macrophage migration inhibitory factor (MIF) is considered to play a critical role in the host inflammatory response by its ability to promote proinflammatory cytokine release. However, observations indicated that MIF has inhibitory effect on T cells [48, 49]. Recently, MIF produced by a murine neuroblastoma cell line (Neuro-2a) inhibits cytokine-, CD3-, and allo-induced T-cell activation and the inhibitory effects were reversed when the cells were transfected with dicer-generated si-RNA to MIF. These findings indicate that

MIF present in the trophoblast-conditioned media and at maternal-fetal interface may play immunosuppressive roles that need further investigation.

Galectin-1 (Gal-1), a member of galactose-binding lectins, recognizes preferentially galactose-beta 1,4-N-acetylglucosamine sequences of oligosaccharides on cell surface glycoconjugates [51-53] and can modulate immune cell functions by controlling the proliferation and survival of effector T cells [54, 55], antagonizing T cell activation [56] and blocking secretion of proinflammatory cytokines [57]. Gal-1 was expressed by placenta [51, 58], and the expression of Gal-1 was decreased in villous samples of early pregnancy loss [59], suggesting its role in sustaining normal pregnancy. Very recently, elegant work of Blois et al [52] proved that Gal-1 played a pivotal role in fetomaternal tolerance.

Unexpectedly, and further, no interleukins were found in trophoblast-conditioned media with proteomics analysis and the absence of IL-2, IL-4, IL-10 and TNF- α was confirmed with sensitive immunoassays, which is in contrast with previous reports of secretion of cytokines by trophoblasts [60]. It was noted that some substances such as leptin and resistin were not detected in the current study, but they were reported produced by trophoblasts [61, 62]. The exact reasons for these discrepancies were not known, but the possible explanations were that protein degradation might occur during the desalting and concentration and that the protein might be processed by the cells during culture [63]. The discrepancy in method of cell culture was also among the possible causes [63]. Thus, the involvement of cytokines in the immunoregulatory effect could not be completely excluded.

In summary, placental trophoblasts inhibit T lymphocyte proliferation and this inhibitory effect partly depends on IDO-mediated tryptophan depletion, indicating that tryptophan catabolism via IDO is one of the mechanisms by which trophoblasts inhibit T cells proliferation in vitro. The presence of trophoblast-derived substance including estradiol, progesterone, HCG, HCS, SP1, glycodelin, AFP, TGF- β 2, thrombospondin-1, PEDF and Gal-1, suggests that trophoblast-conditioned media might be a mixture of substances with immunoregulatory properties, that the inhibitory effect might be the result of synergistic action of these immunoregulatory substances and that maternal immune response during pregnancy could be regulated by complex mechanisms that might involve the interaction between endocrine and immune network.

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