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EFFECT OF (ALA^{8,13,18})-MAGAININ II AMIDE ON HUMAN TROPHOBLAST CELLS *IN VITRO*

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Abstract : Magainins are cationic peptides with anti-bacterial and anti-tumor properties. The anti-nidatory function of a synthetic analogue of magainin, (Ala^{8,13,18})-magainin II amide, has earlier been reported, and it has been indicated that placental trophoblast cells could be a target of magainin resulting in its contragestational action. The aim of the present study was to examine the effect of (Ala^{8,13,18})-magainin II amide (100 ng/ml and 1000 ng/ml) on attachment efficiency, viability, differentiation in terms of hCG secretion and invasive function of isolated first trimester, human placental trophoblast cells grown on rat-tail collagen type-I matrix in primary cell culture. In the present experimental model, magainin was not found to affect human trophoblast cell functions *in vitro*.

Key words : attachment hCG secretion invasion magainin primary cell culture trophoblast cells viability

INTRODUCTION

Magainins belong to a broad class of anti-microbial peptides. They are found naturally in the skin of African clawed frog, *Xenopus laevis* (1). These peptides kill bacteria by permeabilizing cell membranes but do not exhibit significant toxicity against mammalian cells (2). Magainins also exhibit anti-tumour activity (3, 4). The main cellular target of magainins is thought to be the lipid matrix of cell membranes. The selective toxicity of magainins towards bacterial and tumour cells is explained by preferential interaction of magainins with anionic phospholipids abundantly present in bacterial membranes and on tumour cell surfaces (5, 6).

Recently, (Ala^{8,13,18})-magainin II amide has been shown to have anti-nidatory effect. Vaginal administration of (Ala^{8,13,18})-magainin II amide to successfully mated rhesus monkeys (*Macaca mulatta*) during the peri-implantation stage of ovulatory cycles resulted in inhibition of pregnancy establishment (7). However, estrogen and progesterone levels in the peripheral circulation and menstrual cyclicity in treatment and in post-treatment cycles

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remained unaltered (7, 8). In a study of endometrial morphology in mated monkeys exposed to (Ala^{8,13,18})-magainin II amide via vaginal route revealed focalized degeneration in few glands and in a subset of blood vessels, none-the-less, a few plaque acini were found in subluminal epithelial zone suggestive of trophoblast penetration (8). Plaque acinar cells are modified endometrial glandular epithelial cells typically found as one of the earliest endometrial responses to trophoblast penetration in the macaque and the baboon (9–11). Thus, failure of blastocyst implantation in the rhesus monkey following administration of (Ala^{8,13,18})-magainin II amide during the peri-implantation period did not arise from either the lack of ovarian steroid hormonal support, or a generalized loss of endometrial receptivity for implantation (8).

On the basis of the available evidence, it appears plausible that blastocyst implantation may have been inhibited by targeted action of the peptide on trophoblast cell functions during implantation. Trophoblast cells during their differentiation suggestively externalize phosphatidyl-serine (PS) (12, 13), and cationic peptides, such as magainin II amide, are known to bind to negatively charged phospholipids such as PS on outer leaflet of cell membrane (14). Such binding of (Ala^{8,13,18})-magainin II amide might limit trophoblast cell functions during early gestation. We hypothesized that (Ala^{8,13,18})-magainin II amide interacts with trophoblast cells resulting in loss of cell viability and/or altered functional differentiation and invasion during implantation and early stages of placentation. This hypothesis has been examined in the present study by investigating the action of (Ala^{8,13,18})-magainin II amide *in vitro* on viability, attachment efficiency, differentiation, and invasion function of first trimester, human placental trophoblast cells grown on three-dimensional rat-tail collagen type-I in primary cell culture.

METHODS

Supplies

Multiwell tissue culture plates: 4-well from Nunc (Roskilde, Denmark); 96 well from Cellstar (Grenier bio-one, Germany). DMEM, trypsin, (Ala^{8,13,18})-magainin II amide, methylthiazole-tetrazolium (MTT), Epon 812 resin and sieve cup with sieves from Sigma Chemical Co. (St. Louis, Missouri, USA); DNase1 from Roche (Mannheim, Germany); Fetal calf serum (FCS), bovine serum albumin and gentamycin from GIBCO (Grand Island, New York, USA); Penicillin, streptomycin and amphotericin B from JRH Biosciences (Lansex, Kansas, USA); Magnetic separation columns type MS and LS, CD45 microbeads from Miltenyi Biotec (Bergisch Gladbach, Germany); Millicell-PCF insert 8 μm pore size, 12 mm insert size from Millicell Corporation (Bedford, MA, USA) and Percoll from Amersham Pharmacia Biotech. Inc. (Piscataway, New Jersey, USA). The hCG Enzyme Immuno Assay kit from Bioplus Inc. (South San Francisco, CA, USA). All analytical grade reagents were purchased from Glaxo Qualigens (Mumbai, Mahrashtra, India).

Preperation of rat-tail collagen and collagen gel matries

Type-I collagen was extracted from tendon bundles of frozen rat-tails according to the method of described earlier (15). Briefly, bundles of collagen fibers were collected and washed in 80% ethanol and exposed to ultraviolet light in the laminar hood for sterilization and drying. Dried collagen was weighed and suspended at a ratio of 1 g of fiber to 300 ml of sterile 0.1% glacial acetic acid. The mixture was moderately agitated at 4°C for 72 h. Dissolved collagen was filtered through sterile muslin gauze. The mean final collagen gel concentration in a matrix gel was 2.0 ± 0.1 mg/ml.

Firm collagen gel matrix were prepared by spreading collagen solution (0.5 mg/cm^2) evenly into wells of 4 well culture plates and exposing them to ammonical fumes covered by sterile petridish for gel solidification. Culture plates with collagen gel were also prepared by spreading collagen solution (0.5 mg/cm^2) evenly into wells of 96 well culture plates and placing them in an incubator at 50°C for 2 h. All the gels in wells were exposed to UV light for 15 to 20 min for sterilization and thoroughly washed with complete medium at 37°C, and equilibrated with medium in a gas phase of 5% CO_2 in air before these were used for cell plating.

Specimens

Human first-trimester placental samples (n = 25) were obtained from healthy women undergoing medical termination of pregnancy (MTP) between 7 and 9 weeks of gestation in the Department of Obstetrics and Gynaecology, All India Institute of Medical Sciences. The samples were collected by vacuum suction. Aspirated tissue samples were collected in a sterile vacuum jar and immediately transported to the cell culture laboratory for further processing. The study was approved by the Human Ethical Committee, All India Institute of Medical Sciences, and written consents from all patients undergoing MTP were obtained.

Isolation of first trimester trophoblast cells

Trophoblast cells were isolated from freshly collected placenta according to a method described previously (16). All the manipulations were performed under sterile conditions in a laminar flow hood. Within 15 min of vacuum aspiration, the products of conception were washed with sterile cold Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS-A) containing gentamycin (50 µg/ml) and D-glucose (1 mg/ml), pH 7.4. Villous sections were dissected from chorionic membranes under stereozoom microscope and washed, minced and enzymatically digested in a enzyme mixture containing 0.25% (w/v) trypsin, 0.02% (w/v) deoxyribonuclese type-I (DNase I) (400 U/ml), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM magnesium sulfate and antibiotics-antifungal solution [penicillin 100 IU/ml, streptomycin 100 µg/ml, amphotericin-B 2.5 µg/ml] in PBS-A at 37°C for 30 min twice (10 ml of enzyme mix for 2 g of wet tissue). After each enzymatic digestion, the supernatant was passed through pre-equilibrated mesh filter (40 µm) to remove cellular debris and syncytial fragments, and isolated cells were washed

twice with cold PBS-A with 10% (v/v) FCS. Pellet of two resultant cell suspensions were pooled and suspended in 30% Percoll. The suspension was layered over a preformed Percoll gradient made with PBS-A. The gradient ranged from 30% to 70% Percoll (v/v) in 10% steps of 3 ml each by dilution of Isopercoll (9 parts of Percoll + 1 part 10 X PBS-A) with PBS-A. The gradient was centrifuged yielding two regions: a lower fraction consisting of RBCs and a band at the top, containing connective tissue elements, villous fragments and population of mononuclear cells (17). The top cellular layer was collected, washed twice with PBS-A, filtered through pre-equilibrated mesh (40 μm) to remove connective tissue elements and villous fragment and cells suspended in PBS-A containing 10% (v/v) heat inactivated FCS.

The cells were immunopurified by magnetic depletion of CD45-positive leucocytes using MACS CD45 microbeads (microbeads conjugated with monoclonal mouse antibody against CD45). Briefly, the unpurified cell suspension was passed through pre-equilibrated mesh (40 μm), washed and suspended in degassed buffer (PBS free of dissolved air, supplemented with 0.5% bovine serum albumin and 2 mM EDTA). Cell suspension was incubated with microbeads for 15 min at 10°C. After 15 min, cells were washed with degassed buffer and the cell pellet was suspended in 500 μl degassed buffer and passed through MS separation column and the negative fraction was collected as effluent. After the total suspension has passed the column, 1500 μl degassed buffer was allowed to run through the column and total effluent was collected. This method yielded an enriched trophoblast cell population in the effluent. Cell yield and cell viability were determined with trypan blue dye exclusion method (18).

Primary culture

Isolated cells were plated at a density of 1 x 10^5/cm^2 on (i) firm collagen gel matrix in multiwell culture plates for the study of attachment efficiency, (ii) dry collagen coat for viability and hormone production, and (iii) dry coats on porous membranes with 8 μm pores for invasion assay. Cells were cultured at 37°C in a humidified air atmosphere of 5% CO_2 and in complete medium. Complete culture medium consisted of DMEM, 10% (v/v) FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin-B (2.5 μg/ml).

Attachment assay

The effect of (Ala^{8,13,18})-magainin II amide on the human first trimester placental trophoblast cells attachment efficiency was assessed. Magainin at different doses [0 (group 1; control), 100 ng/ml (group 2) and 1000 ng/ml (group 3)] in complete DMEM medium was added to the wells at the time of cell seeding, the time point of seeding of cells being considered as 0 h. After 48 h of culture, wells were gently rocked for 5 min and the supernatant was collected in separate aliquot tubes. The tubes were centrifuged at low speed for 5 min, and number of unattached cells in the supernatant were counted using haemocytometer. Attachment efficiency was calculated as the percentage of cells in the supernatant against the total cells seeded on to the collagen matrix (15).

Viability assay

The cytotoxicity of (Ala[8,13,18])-magainin II amide on the human first trimester placental trophoblast cells was assessed. Isolated cells were plated with complete medium and cultured for 48 h to allow the cells to adhere and differentiate (Fig. 1). After 48 h of seeding, a washing was given to remove unattached and non-viable cells, and complete medium with magainin (group 1: control; group 2: 100 ng/ml; group 3: 1000 ng/ml) was added to wells. The experiments were run for 24 h and 48 h separately. Cell viability was assessed by the MTT method (19). At the end of culture, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml dissolved in pre-warmed PBS and filtered by 0.2 μm filter) is added to culture well and incubated for 4 h. At the end of the incubation period supernatant was removed, the cells were lysed and converted dye was solublized with acidic isopropanol (0.04 M HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm on microplate reader (Model 550, Microplate reader, Bio-Rad laboratories Inc, Hercules, CA, USA).

Hormone assay

The effect of (Ala[8,13,18])-magainin II amide on differentiation of human first trimester trophoblast cells was assayed by measuring total human chorionic gonadotropin (hCG) hormone secreted by trophoblast cells *in vitro* (20). Cells were cultured in the manner similar to viability assay as described above. After 48 h of cell seeding the cells were washed with medium to remove syncytial fragments, and fresh medium containing 10% (v/v) stripped FCS with different doses of magainin [0 ng/ml (group 1; control), 100 ng/ml (group 2) and 1000 ng/ml (group 3)] was added. Selective adhesion of cytotrophoblast cells to tissue culture surfaces, as shown in Figure 1, was an effective step toward enriched cytotrophoblast cultures for hCG assay (21). The experiments were run for 24 and 48 h separately. At the end of the experiment the supernatant from each well were stored separately in aliquots at −20°C.

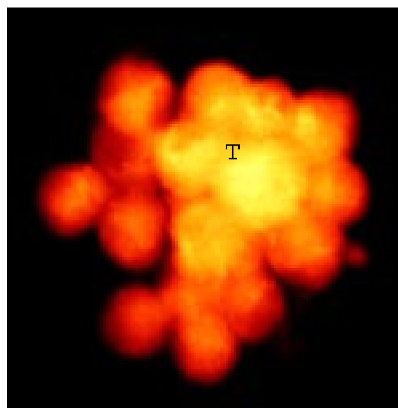

Fig. 1 : Fluorescent image of cytotrophoblast cell cluster (T) after 48 h of culture, stained with aqueous eosin, exhibiting apparent cell-cell and cell-matrix adhesion under confocal laser scanning microscopy (CLSM). 40X.

Total hCG was measured by enzyme immunoassay (EIA) method (22). Standardization of the culture method to detect hCG in trophoblast cell culture conditioned medium involved the use of activated charcoal stripped serum to remove hormones. The average concentration of hCG as measured by Enzyme Immunoassay (EIA) was as follows: media with stripped serum without cells: <5 mIU/ml; human trophoblast cell conditioned medium: 124

mIU/ml; peirpheral serum of woman at 8 weeks of gestation: > 300 mIU/ml; and in peripheral serum of adult male hCG was undetectable. The assay is based on antibody sandwich assay method. The mean absorbance value (A_{450}) of the samples, reference controls (hCG positive serum sample from a pregnant woman and hCG negative serum sample from a male volunteer) and standards (0-300 mIU/ml) were measured spectrophotometrically at 450 nm with a microplate reader (Model 550, Microplate reader, Bio-Rad laboratories Inc, Hercules, USA). The absorbance (A_{450}) values of the standards were used to plot the standard curve. The concentration of hCG is directly proportional to the mean absorbance value (A_{450}) of the test sample. The range of detectable concentration of hCG by this assay is 2–300 mIU/ml and within-assay coefficient of variation was 7%. All samples were estimated in one run of assay.

Invasion assay

The effect of ($Ala^{8,13,18}$)-magainin II amide on the invasive capacity of human first trimester placental trophoblast cells *in vitro* was assessed. To quantify invasive function of trophoblast cells *in vitro*, a method described by Librach et al (23) with some modification was employed. Briefly, first trimester trophoblast cells were cultured on Boydens' chamber (Millicell-PCF, invasion chamber inserts) with 10 µg/cm^2 collagen coated polycarbonate membranes with 8 µm pores. The inserts were pre-equilibrated with complete medium, 2 h before cell seeding. 2–5 x 10^4 cells/insert were seeded on the collagen-coated membrane of insert, the time point of cell seeding being considered as 0 h. After 48 h, the complete medium containing either no magainin (group 1) or 1000 ng/ml of ($Ala^{8,13,18}$)-magainin II amide (group 3) was added, and they were further cultured for 48 h. The upper surface of the chamber insert membranes were then gently scrubbed with cotton swab soaked with DMEM. The cells invaded on the lower surface of the membrane were fixed with 4% neutral buffered paraformaldehyde at room temperature for 10 min and the membranes were removed, washed and stained with 2% aqueous eosin. Invasion of collagen coated Millicell inserts by first trimester human cytotrophoblast cells was studied using Confocal laser scanning microscope (CLSM, TCS SP2, mounted on DMIRE-2 inverted microscope, Leica Microsystems Heidelberg GmbH, Mannheim, Germany). The total surface area of cell which have traversed the membrane were quantified with Leica microscope and QWIN Quantimet-500C+ image processing and analysis system (Leica, Cambridge, England).

Statistical analysis

Statistical analysis of data between different groups for parameters was done by Kruskal-Wallis test. The analysis between the groups of data for two different time periods 24 h and 48 h for hCG secretion and viability was done by Friedman test. The invasion assay data was analyzed by Mann-Whitney test (24). Data are shown as means ± s.e.m.

RESULTS

Human placental samples (n = 25) were obtained from first trimester (7–9 weeks

of gestation) medical terminations. Placental villi were immediately dissected and a part of the villous sample was evaluated for its morphology, and parallel samples were used for trophoblast cell isolation.

Placental villous samples were used to isolate cytotrophoblast cells using trypsin digestion followed by Percoll purification and immunopurification methods. The yield and viability of isolated human first trimester trophoblast cells was $2.2 \pm 1.0 \times 10^5$ trophoblast cells/g of placental tissue and $91 \pm 4\%$, respectively in 25 placental samples used in this study.

Attachment efficiency

Attachment of human first trimester trophoblast cells revealed no significant difference between cells allowed to attach on collagen biomatrix for 48 h *in vitro* in control condition (group 1; $83 \pm 11\%$) and cells allowed to attach for 48 h in presence of 100 ng/ml (group 2; $79 \pm 12\%$) or 1000 ng/ml (group 3; $75 \pm 12\%$) ($Ala^{8,13,18}$)-magainin II amide added at the time of cell seeding.

Viability

Viability of cultured human trophoblast cells was assessed by reduction of methylthiazole tetrazolium (MTT) salt by active mitochondrial dehydrogenase enzyme present in viable cells. The mean optical density value (from the triplicate wells) of the control wells was taken as 100% viability and the mean optical density value of other two groups were compared to control value to calculate the percentage change in the viability of trophoblast cells. ($Ala^{8,13,18}$)-magainin II amide addition to trophoblast cells in cultures did not result in statistically significant effect on the viability of the cells at both doses tested, and at both time points, 24 h and 48 h (Table I). However, in three out of six experiments, an exposure to 1000 ng/ml of magainin II amide for 24 h and in one experiment for 48 h, the trophoblast cell viability in vitro was found to be higher by

TABLE I : Effect of ($Ala^{8,13,18}$)-magainin II on first trimester human placental trophoblast cells grown in primary culture on rat-tail collagen type-I

Parameter	*Group 1 (Control)*		*Group 2 (Magainin-treated, 100 ng/ml)*		*Group 3 (Magainin-treated, 1000 ng/ml)*	
	24 h	*48 h*	*24 h*	*48 h*	*24 h*	*48 h*
Viability [%] (n=6)	100	100	91±12	96±11	139±16	105±12
HCG secretion [mIU/ml] (n=5)	121±16	128±19	105±23	124±19	113±17	141±18
Invasion efficiency [$\mu m^2.10^{-3}$] (n=5)	ND	23±1	NA	NA	ND	20±2

Values are shown as means ± s.e.m. Each experiment was done in triplicate.
ND, not detected. NA, experiment not done.

two fold in treated samples than in the control, though this was not statistically significant.

HCG secretion

The concentration of hCG in culture media obtained from trophoblast cells conditioned media was evaluated by enzyme immunoassay (EIA). The profile of hCG secretion showed a wide range of values from 25–362 mIU/ml hCG in all the groups tested. ($Ala^{8,13,18}$)-magainin II amide addition to trophoblast cell cultures did not affect hCG secretion by cells at both doses tested, as well as, at 24 h and 48 h following addition of ($Ala^{8,13,18}$)-magainin II amide to cell cultures (Table I).

Invasion efficiency

96 h after trophoblast cells were seeded on collagen coated Millicell inserts, trophoblast cell invasion through pores (8 μm) of Millicell inserts membranes was recorded following eosin staining of membranes and analysed using Leica microscope attached with image analysis software to examine the area of trophoblast

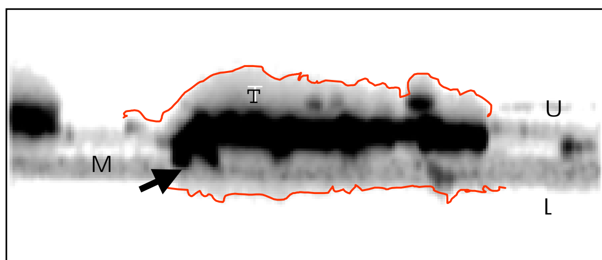

Fig. 2 : Three-dimensional reconstruction of trophoblast cell (T) and Millicell membrane following serial (1 μm) optical sectioning by CLSM. Trophoblast cell processes from upper portion (U) were found to invade through pores (↗) and seen on the lower side (L) of the insert membrane (M).

cell processes invaded to the lower surface of Millicell insert (Fig. 2). Exposure of human trophoblast cells for 48 h to magainin amide did not significantly affect the invasive function of trophoblast cells *in vitro* (Table I).

DISCUSSION

It has earlier been reported that isolated cytotrophoblast cells from human placenta undergo differentiation to form syncytia in primary culture, and these cells exhibited characteristics of differentiated trophoblast cells, such as secretion of human chorionic gonadotropin (hCG), human placental lactogen and progesterone in a regulated fashion, and following cell plating these cells attached and invaded the extracellular matrix (17, 25). The method of trophoblast cell culture on rat-tail collagen as employed in the present study appears to be a robust model to study trophoblast cell differentiation *in vitro* as cells exhibited a very high percentage (~80%) of attachment on collagen biomatrix, and subsequently differentiated and secreted hCG. This primary cell culture model appears potential for studies designed to delineate human trophoblast cell behaviour and functions. In the present study, we have employed this model to examine the hypothesis that ($Ala^{8,13,18}$)-magainin II amide exposure affects viability, differentiation and invasive efficiency of trophoblast cells.

Magainins are toxic to bacteria and this toxicity is due to preferential interactions of peptides with anionic phospholipids abundant in bacterial cell wall (2). Eukaryotic cells are not susceptible due to preferential presence of negative

phospholipids on the inner membrane of cells. Due to the presence of negatively charged phospholipids on their outer leaflet of cell membrane, tumour cells are however susceptible to magainins (3, 26). Cytotrophoblast cells during their differentiation for cell fusion and formation of syncytiotrophoblast suggestively externalize PS on their outer leaflet of cell membrane (27). This similarity in the structure between the invasive tumour cells and differentiating cytotrophoblast cells could make the latter cell type a target of magainin action with possible loss of their viability and functions.

In the present study, no statistically significant change in the viability of trophoblast cells exposed to magainin II amide either for 24 or 48 h *in vitro* was observed as compared to control treatment. However, the trophoblast cell viability, in some cases, was found to be higher following exposure to 1000 ng/ml *in vitro* compared with that in the control treatment group. Since the MTT test measures the activity of mitochondrial dehydrogenase enzyme, it appears that such higher degree of human trophoblast cell viability might have resulted from either an increase in the numbers of mitochondria and/or mitochondrial enzyme activity resulting from the stress following exposure to magainin. Magainin compounds have been shown to interfere with the transduction of free energy and synthesis of ATP in *E.coli*, isolated rat liver mitochondria, hamster spermatozoa (28, 29). Magainin can dissipate the electrical potential across various energy-transducing membranes and thus uncouple respiration from other free energy requiring processes. This response is evident at only higher dose of the peptide reflecting the cooperativity required in pore forming action of magainins (28, 29). This may result in an increase in the respiratory rate due to enhanced rate of substrate oxidation to replenish the ATP pool via glycolysis and citric acid cycle (30). Thus, it appears feasible that mitochondrial dehydrogenase enzyme activity and substrate oxidation increased in trophoblast cells exposed to 1000 ng/ml of magainin amide resulting in higher MTT breakdown.

Although mRNA of hCG subunits has been detected in cytotrophoblast, the synthesis of the α and β subunits of hCG occurs in syncytiotrophoblast layer (31). It has been reported that there occurs externalization of PS from the inner to the outer leaflet of the cell membranes during differentiation and fusion of cytotrophoblast cells. PS and some fusogenic proteins together form a proteolipid compound which signals the fusion between cytotrophoblast cells (27). Antiphospholipid antibodies against PS have been found to disrupt this process of syncytiotrophoblast formation *in vitro* as measured in the amount of hCG secreted (12, 32). hCG production by syncytiotrophoblast is also responsive to signal transduction through membrane bound protein kinase C whose activity is dependent on membrane phospholipid, particularly PS (33, 34). Antiphospholipid (aPL) antibodies against PS can also interfere with signal transduction and prevent the induction of hormone production by syncytiotrophoblast (35). We therefore hypothesized that interaction between magainin and trophoblast could similarly result in loss of differentiation and fusion

of cytotrophoblast cells due to the action of magainins on PS in their outer leaflet of cell membrane. Magainin II amide causes flip-flop of PS from external to inner leaflet (36) and thus may also interfere with hCG hormone production by blocking event in signal transduction through membrane bound protein. In the present study of human trophoblast cells grown on collagen type-I matrix, no significant difference was observed in the secretion of hCG upon exposure to magainin II amide as compared to the control group.

During initial stages of blastocyst implantation, trophoblast cells interact closely with the extracellular matrix of the maternal endometrium (20). It is now well established that adherence to extracellular matrix is required for trophoblast cell differentiation and invasion of maternal decidua (37). Adhesion and invasion of trophoblast cell is mediated by number of cell adhesion molecules expressed in a spatially and temporally regulated manner by trophoblast, as well as, by endometrial cells. Among these trophoblast cell adhesion molecules, α1 and α5 integrins and E cadherins are regulated by phospholipids (38). Alteration in the repertoire of these cell adhesion molecules is considered to be one of the causes of recurrent miscarriage and infertility seen in the patient of antiphospholipid syndrome (39). In such patients, antiphospholipid antibodies against PS and cardiolipin are found to be circulating in the serum (12,39). We thus hypothesized a similar interaction between externalized PS on the human trophoblast cells and magainin II amide could have led to failure of embryo adhesion to and invasion of maternal endometrium toward successful implantation (7, 8). In the present study, however, no adverse effect of magainin II amide on the attachment and invasive efficiency of trophoblast cells on collagen type-I biomatrix *in vitro* was observed.

Thus, in the present study, primary human trophoblast cell culture grown on collagen biomatrix showed no significant difference in cell viability, attachment efficiency, hCG secretion and invasiveness following exposure to magainin II amide as compared to control treatment. It is likely that such failure of magainin II amide, a potent cationic peptide, in affecting trophoblast cell viability *in vitro* could have resulted from insufficient dose of magainin used *in vitro* (2–4, 40). It is possible that collagen as a cell substratum might have substantially quenched positively charged magainin II amide molecule, because collagen contains a large amount of negatively charged hydroxyproline, resulting in an apparent lack of its actions on human trophoblast cells cultured on collagen biomatrix *in vitro*. It will be of interest to examine the effect of different concentrations of magainins on placental trophobalst cells cultured on extracellular matrices of varying thickness (25).

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REFERENCES

1. Zasloff M. Magainin, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 1987; 84: 5449–5453.
2. Zasloff M, Brian M, Chen HC. Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc Natl Acad Sci USA* 1988; 85: 910–913.
3. Cruciani RA, Baker JL, Zasloff M, Chen HC, Colamonici O. Antibiotic magainins exert cytolytic activity against transform cell lines through channel formation. *Proc Natl Acad Sci USA* 1991; 88: 3792–3796.
4. Baker MA, Zasloff M, Jacob LS. Anticancer efficacy of magainin 2 and analogue peptides. *Cancer Res* 1993; 53: 3052–3057.
5. Matsuzaki K, Sugishita K, Fujii N, Miyajima K. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. *Biochemistry* 1995; 34: 3423–3429.
6. Zasloff M. Antimicroboal peptide of multicellular organisms. *Nature* 2002; 415: 389–395.
7. Dhawan L, Ghosh D, Lalitkumar PGL, Sharma DN, Lasley BL, Overstreet JW, Sengupta J. Anti-nidatory effect of vaginally administered ($Ala^{8,13,18}$)-magainin II amide in the rhesus monkey. *Contraception* 2000; 62: 39–43.
8. Ghosh D, Dhawan L, Lalitkumar PGL, Wong V, Rosario JF, Hendrickx AG, Lasley BL, Overstreet JW, Sengupta J. Effects of vaginally administered ($Ala^{8,13,18}$)-magainin II amide on endometrium in the rhesus monkey. *Contraception* 2001; 62: 39–43.
9. Hendrickx AG, Houston ML. *Embryology of baboon.* University of Chicago, Chicago, 1976.
10. Enders AC, Hendrickx AG, Schlafke S (1985) Implantation in the rhesus monkey: endometrial rsponses. *Am J Anat* 1985: 173: 147–169.
11. Ghosh D, Dhara S, Kumar A, Sengupta J. Immunohistochemical localization of receptors for progesterone and oestradiol-17 beta in the implantation site of the rhesus monkey. *Hum Reprod* 1999; 14: 505–514.
12. Adler RR, Ng AK, Rote NS. Monoclonal antiphosphatidylserine antibody inhibits intercellular fusion of the choriocarcinoma line, JAR. *Biol Reprod* 1995; 53: 905–910.
13. Vogt E, Ng AK, Rote NSA. Antiphosphatidylserine antibody removes annexin-V and facilitates the binding of prothrombin at the surface of a choriocarcinoma model of trophoblast differentiation. *Am J Obstet Gynecol* 1997; 177: 964–972.
14. Matsuzaki K, Harada M, Funoakoshi S, Fujii N, Miyajima K. Physicochemical determinants for interactions of maigainins 1 and 2 with acidic lipid bilayers. *Biochem Biophys Acta* 1991; 1063: 162–170
15. Ghosh D, Danielson KG, Alston JT, Heyner S. Functional differentiation of mouse uterine epithelial cells grown on collagen gels or reconstituted basement membrane. *In Vitro Cell Dev Biol* 1991; 27A: 713–719.
16. Bischof P, Friedli E, Martelli M, Campana A. Expression of extracellular matrix-degrading metalloproteinases by cultured human cytotrophpblast cells: effects of cell adhesion and immunopurification. *Am J Obstet Gynecol* 1991; 165: 1791–1801.
17. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF. Purification characterization and in vitro differentiation of cytotrophoblast from term placenta. *Endocrinology* 1986; 118: 156–182.
18. Freshney RI. Culture of animal cells - a manual of basic technique. Wiley-Liss Inc, New York, 2000.
19. Mosmann T. Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J Immunol Methods* 1983; 65: 55–63.
20. Fisher SJ, Cui TY, Zhang L, Hartman L, Grahl K, Zhang G, Tarpey J, Damsky CH. Adhesive and

degradative properties of human placental cytotrophoblast cells in vitro. *J Cell Biol* 1989; 109: 891–902.

21. Guilbert LJ, Winkler-Lowen B, Sherburne R, et al. Preparation and functional characterization of villous cytotrophoblast free of syncytial fragments. *Placenta* 2002; 23: 175–183.

22. Ghosh D, Stewart DR, Nayak NR, Lasley BL, Overstreet JW, Hendrickx AG, Sengupta J. Serum concentrations of oestradiol-17beta, progesterone, relaxin and chorionic gonadotrophin during blastocyst implantation in natural pregnancy cycle and in embryo transfer cycle in the rhesus monkey. *Hum Reprod* 1997; 12: 914–920.

23. Librach CL, Werb Z, Fitzgerald ML, et al. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J Cell Biol* 1991; 113: 437–449.

24. Siegel S, Castellan NJ Jr. Non parametric statistics for behavioural sciences. New York: McGraw Hill, 1988.

25. Kliman HJ, Feinberg RF. Human trophoblast-extracellular matrix (ECM) interactions in vitro: ECM thickness modulates morphology and proteolytic activity. *Proc Natl Acad Sci USA* 1990; 87: 3057–3061.

26. Utsugi T, Schroit AJ, Connor J, Bucana CD, Fidler IJ. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res* 1991; 51: 3062–3066.

27. Pötgens AJ, Schmitz U, Bose P, Versmold A, Kaufmann P, Frank HG. Mechanisms of syncytial fusion: a review. *Placenta* 2002; 23 (Suppl A): S107–S113.

28. Westerhoff HV, Hendler RW, Zasloff M, Juretic D. Interactions between a new class of eukaryotic antimicrobial agents and isolated rat liver mitochondria. *Biochem Biophys Acta* 1989; 975: 361–369.

29. Westerhoff HV, Juretic D, Hendler RW, Zasloff M. Magainins and the disruption of membrane-linked free-energy transduction. *Proc Natl Acad Sci USA* 1989; 86: 6597–660.

30. Sammut IA, Harrison JC. Cardiac mitochondrial complex activity is enhanced by heat shock proteins. *Clin Exp Pharmacol Physiol* 2003; 30: 110–115.

31. Muyan M, Boime I. Secretion of chorionic gonadotropin from human trophoblasts. *Placenta* 1997; 18: 237–241.

32. Katsuragawa H, Kanzaki H, Inoue T, Hirano T, Mori T, Rote NS. Monoclonal antibody against phosphosphatidylserine inhibits in vitro human trophoblastic hormone production and invasion. *Biol Reprod* 1997; 56: 50–58.

33. Gelicher N, Harlow L, Zilberstain M. Regulatory effect of antiphospholipid antibodies on signal transduction: a possible model for auto-antibody-induced reproductive failure. *Am J Obstet Gynecol* 1992; 167: 637–642.

34. Reza F, Igarashi K, Tokita S, et al. Anti-idiotypic monoclonal antibody recognizes a consensus recognition site for phosphatidylserine and protein kinase C. *FEBS Lett* 1994; 339: 229–233.

35. Di Simone N, Decarolis s, Lanzone A, Roisvalle E, Giannice R, Caruso A. In vitro effect of antiphospholipid antibody-containing sera on basal and gonadotropin release hormone dependent human chorionic gonadotropin release by cultured trophoblast cells. *Placenta* 1995; 16: 75–83.

36. Matsuzaki K, Murase G, Zasloff M. Antimicrobial peptide, magainin II, induces rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. *Biochemistry* 1996; 35: 11361–11368.

37. Damsky C, Sutherland A, Fisher S. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *FASEB J* 1993; 1320–1329.

38. Di Simone N, Castellani R, Caliandro D, Caruso A. Antiphospholid antibodies regulate the expression of trophoblast cell adhesion molecules. *Fertil Steril* 2002; 77: 805–811.

39. Manson JJ, Isenberg DA. Antiphospholipid syndrome. *Int J Biochem Cell Biol* 2003; 35: 1015–1020.

40. Chen HC, Brown JH, Morell JL, Haung CM. Synthetic magainin analogues with improved antimicrobial activity. *FEBS Lett* 1988; 236: 462–466.