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Short Communication

Effects of ligands or substrate of insulin-regulated aminopeptidase (IRAP) on trophoblast invasion

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Abstract: Insulin-regulated aminopeptidase (IRAP) activity increases during placentation and in the invasive tumor cell of trophoblast suggesting a role for this peptidase in the invasiveness of normal and malignant trophoblast. To investigate this hypothesis, we studied the effects of substrate (OT) and inhibitors (angiotensin peptides and LVV-H7) of IRAP on the first trimester trophoblast proliferation and invasion. Addition of these peptides in the culture medium of trophoblastic cells significantly decreased metalloproteinase-9 activity and cellular invasiveness while no effect was observed on cell proliferation. The peptide IRAP inhibitors could exert their effect on cytotrophoblastic cell invasiveness by inhibition of its enzymatic activity, and thus increasing half life of the known placental peptide substrate of IRAP, OT. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: OT; angiotensin IV; LVV-H7; IRAP; invasion; trophoblastic cells

INTRODUCTION

Cytotrophoblastic cells (CTB) of the human placenta proliferate, migrate, and invade the pregnant uterus to allow implantation and placentation [1]. The invasive property of trophoblastic cells is limited in time and space and depends on their ability to secrete proteases.

Insulin regulating aminopeptidase (IRAP), or oxytocinase, also known as placental leucine aminopeptidase, is involved in the maintenance of homeostatic mechanisms during pregnancy by regulating OT levels [2]. The mRNA is mostly expressed in syncytiotrophoblast and its levels in human placenta are increased with advancing gestation [3,4]. Maternal serum level of IRAP is also increased during pregnancy, reaching a peak at 38 weeks of gestation indicating secretion of the membrane bound form of the enzyme [5]. Many studies investigated the regulation of P-LAP gene expression in trophoblast. Activator protein-2 (AP-2) is a main activator of IRAP promoter [2], and Ikaros could cooperate with this transcription factor for maximal expression of IRAP gene [6]. It was already observed that Ikaros is predominantly expressed in extravillous cytotrophoblast cells. The suppression of Ikaros transcriptional function in these cells lead to a decrease in migration and invasion of extravillous cytotrophoblastic cells, suggesting a possible role of its transactivator in migration and invasion of trophoblastic cells in early placentation. Moreover, an immunohistochemical study of normal and invasive tumor placental tissues showed that IRAP was predominantly expressed in the invasive phenotype of extravillous trophoblasts (EVTs). The expression of IRAP in the EVTs peaked during the late first to second trimesters of pregnancy, and decreased in the third trimester [7]. The increase in IRAP expression during placentation and in the invasive tumor cell of trophoblast suggests an involvement of this peptidase in invasiveness of normal and malignant trophoblasts [7].

Angiotensin IV (Ang IV) and LVV-hemorphin 7 (LVV-H7) are specific peptide inhibitors of IRAP which bind to its catalytic site to inhibit its catalytic activity [8]. These inhibitors are ideal pharmacological tools to investigate the physiological roles of IRAP in the different tissues. Cumming and Harding suggested that Ang IV and its analogs are capable of regulating metalloproteinases (MMPs) expression in glial and fibroblasts (unpublished observation), thereby playing a role in extracellular matrix remodeling [9]. We hypothesized that IRAP could play a role in the invasion of trophoblastic cells by alteration of MMP expression. The aim of this study was first to test this hypothesis by studying the effect of the IRAP inhibitors on trophoblastic cells.

MATERIAL AND METHODS

Reagents

Dulbecco's modified Eagle's medium high glucose (DMEM), Ham F12, antibiotic mixture (penicillin, streptomycin), were

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products of Invitrogen (Basel, Switzerland). Fetal calf serum (FCS) was from Biochrom AG (Oxoid AG, Basel, Switzerland). Lysis buffer (5X) and Cell Titer 96 Aqueous one solution cell proliferation assay were purchased from Promega (Catalysts AG, Wallisellen, Switzerland), and BIO-RAD protein assay was from Bio-Rad (Munich, Germany). OT, Vasopressin, and Collagen I were from SIGMA (Sigma-Aldrich, Switzerland).

LVV-H7 was synthesized by Mimotopes (Clayton, Australia), and Norleucine1-Ang IV (Nle^1-Ang IV) and Divalinal-Ang IV (Dival-Ang IV) were purchased from Auspep (Parkville, Australia).

CTB Purification

Placental tissues were obtained from patients who voluntarily and legally chose to terminate pregnancy during the first trimester (7–12 weeks of gestation). Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local ethic committee.

Cytotrophoblast cells were isolated from the first trimester placentas as described [10]. In brief, fresh tissue specimen were isolated and washed several times in sterile Hanks buffered salt solution (HBSS). The tissue was then enzymatically digested four times for 20 min at 37 °C (0.25% trypsin, 0.25 mg/ml Dnase I). Single cells were collected, trypsin cocktail was neutralized with FCS, and cells were then resuspended in DMEM. This cell suspension was filtered on 100 μM filter, laid onto Percoll gradient (70–5% Percoll diluted with HBSS) and centrifuged for 25 min at 1200 *g*. The 30–45% bands containing cytotrophoblastic cells were collected, washed and suspended in DMEM. Cells were then immunopurified.

Cytotrophoblastic Cells Culture

HIPEC 65 (cell line described by Pavan *et al.*, [11]) and cytotrophoblastic cells were grown in DMEM high glucose/F-12 containing 10% FCS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were treated with peptides during 24 h before lysis (Lysis buffer, Promega). Protein concentration was assayed with Bio-Rad assay.

Proliferation Assay

Cell proliferation assay was performed according to the instructions of the manufacturer. The ligands (Nle^1-Ang IV, Dival-Ang IV, and LVVH7) and substrate (OT) of IRAP were tested at 0.1 μM on HIPEC 65. Data were expressed as the percentage of treated cells to the untreated (controls) cells. Three independent experiments, each run in triplicate, were performed and the results expressed as means ± standard error of the mean (SEM). Paired Student's *t* test was used to compare treated and untreated cells.

Invasion Assay

Cell invasion assay was performed in an invasion chamber based on the Boyden chamber principle. Each insert was fitted with an 8 μM pore size polycarbonate membrane (Coastar) precoated with rat's tail collagen I (5 $\mu g/cm^2$). Inserts were washed in DMEM and incubated for 30 min at room temperature. For each well, 2×10^4 cells in 100 μl of serum free media, in the presence or absence of peptides (0.1 μM for Ang IV peptides and LVV-H7, 10 μM for OT), were added to the upper compartment of the transwell chambers. A volume of 500 μl of complete media was added to the lower chamber. Cells were incubated for 72 h at 37 °C in a CO_2 (5%) incubator. After incubation, the supernatant was discarded and viable cells that invaded collagen were stained with crystal violet cell stain. After washing, the stain was extracted with a solution of 1% acetic acid : 50% ethanol for 15 min at room temperature. A measure of 100 μl of the dye mixture was transferred to a 96-well microtiter plate for colorimetric measurement at 560 nm. Data were expressed as the percentage of treated cells that invaded the collagen-coated membrane relative to the untreated (controls) cells. Three independent experiments, each run in triplicate, were performed and the results expressed as means ± SEM. Paired Student's *t* test was used to compare treated and untreated cells.

Zymography

Proteolytic activity of culture supernatants were assayed using gelatin-substrate gel electrophoresis as described previously [12]. Zymograms were scanned with an Epson Perfection 1200 Photo scanner and the surface of the digestion bands measured by the Kodak 1D Image analysis software (Kodak, Rochester, NY). Three independent experiments, each run in triplicate, were performed and the results expressed as means ± SEM. Relative MMP-9 activity were calculated by arbitrarily setting the activity of the control as 100. Paired Student's *t* test was used to compare treated and untreated cells.

RESULTS

Effects of IRAP Inhibitors on Proliferation of HIPEC65

As an antiproliferative effect of LVV-H7 was already described on L929 cells [13], we tested the effect of LVV-H7 and Ang IV peptides (Nle^1-Ang IV and Dival-Ang IV) on HIPEC 65 cell line, which is an extravillous CTB cell line able to proliferate in contrast to primary CTB, but none of these peptides were able to influence the proliferation of these cells (Figure 1).

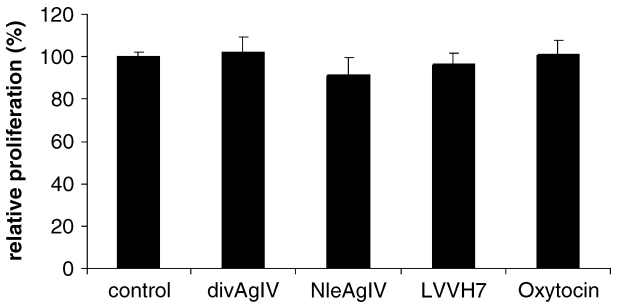

Figure 1 Proliferative effect of IRAP inhibitors and substrate on HIPEC 65 cells.

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Effects of IRAP Inhibitors or Substrate on Cytotrophoblastic Cells Invasiveness

We next examined the effect of these peptides on CTB invasiveness. Ang IV peptides (Nle[1]-Ang IV and Dival-Ang IV) and LVV-H7 were added to the culture of primary CTB in boyden chamber. It was observed that all the peptide IRAP inhibitors tested could significantly inhibit invasion of CTB by 40% at 0.1 μM (Figure 2). Among the peptides that are cleaved by IRAP, the neurohypophyseal nonapeptide hormone OT is known to play a role as a trigger for the initiation of labor through its actions on myometrial contractility. To determine its effect on trophoblastic cells invasion, primary CTB was cultured in boyden chamber in the presence or absence of OT. As shown in Figure 2, OT significantly decreased the invasion of CTB by over 25%. This result is in agreement with those observed in ovarian carcinoma cells *in vitro* and *in vivo* [14] and reinforces the idea that first trimester trophoblasts behave like cancer cells [15].

Effects of IRAP Inhibitors or Substrate on MMP-9 Activity

As MMP-9 is the major metalloproteinase involved in CTB invasion, we tested the effect of IRAP inhibitors and substrate on MMP-9 activity of CTB. HIPEC 65 cells were treated with vehicle control, Ang IV peptides or LVV-H7 for 24 h. MMP-9 activity was assayed on culture supernatant and corrected for intracellular protein concentration. As shown in Figure 3(A), these peptides significantly inhibit the MMP-9 activity by about 40% either at 0.1 and 1 μM. Same results were observed with cytotrophoblastic cells treated with OT (Figure 3(B)).

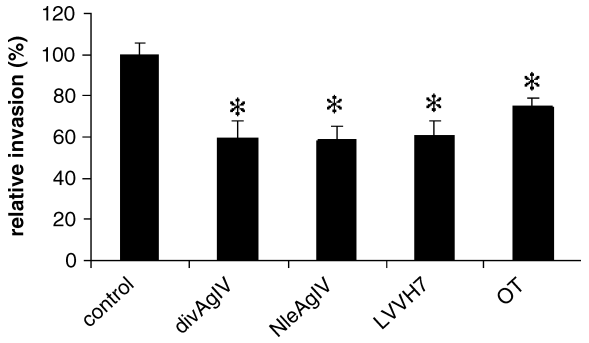

Figure 2 Effect of IRAP inhibitors and substrate on CTB invasion. *, $P < 0.005$.

DISCUSSION

Here, we provide the first evidence for the involvement of IRAP in CTB invasiveness, probably by regulation of MMP-9 expression. This observation is important for reproduction since CTB have to proliferate, migrate, and invade the uterus to allow embryo implantation [1]. This step of the embryology is highly regulated both in time and in space. The invasive behavior of CTB is mainly due to the capacity of MMPs to degrade extracellular matrix [10]. MMPs regulation is intensively studied and is established at different levels: at transcriptional level depending on various cis-elements present in their gene promoter, and various soluble factors including cytokines, hormones, growth factors at their secretion level, and at their protein level depending on their state of activation, inhibition or glycosylation [16].

IRAP, the enzyme that degrades OT is also identified as the marker protein that is predominantly found in GLUT4 vesicles in insulin responsive cells. It is also intensively studied notably in the brain for its involvement in learning and memory [9,17]. IRAP inhibitors (LVV-H7 and Nle1-Ang IV) accelerate spatial learning and facilitate memory when administered into the brain. These peptide IRAP inhibitors were thought to influence MMP expression, resulting in modification of extracellular matrix composition and changes in intracellular signaling systems leading to altered synaptic plasticity [9]. IRAP, called oxytocinase due to its ability to cleave OT efficiently, is also known

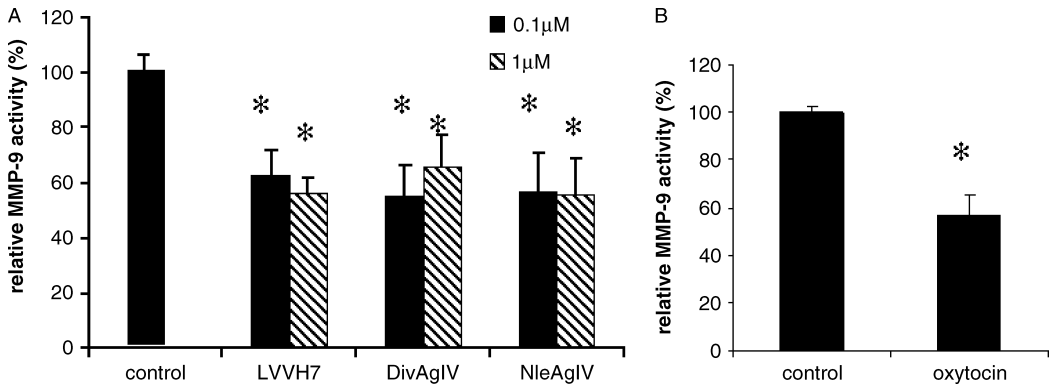

Figure 3 Effect of IRAP inhibitors (A) and substrate (B) on MMP-9 activity of first trimester trophoblastic cells (HIPEC 65 and CTB, respectively). *, $P < 0.005$.

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to regulate OT level in maternal serum and in the placenta during pregnancy [2]. The high concentration of OT induces uterine contraction at the onset of labor. However, the rising levels of OT have to be regulated in the third trimester so as not to trigger premature labor. A study on Ikaros, a transactivator of IRAP expression showed that this factor is present in the placenta and could influence invasiveness of extravillous CTB [3], thus leading to the suggestion of another role for IRAP in pregnancy. Both IRAP [3,4], its substrate (OT) [18], and possibly its peptide inhibitors [19,20] have been described in the placenta. Addition of the peptide inhibitors of IRAP in the culture medium of trophoblastic cells decreased the MMP-9 activity and invasiveness of cells while there is no effect on cell proliferation. Interestingly, addition of the peptide substrate of IRAP, OT to the same system elicited a similar effect of cell invasiveness. This confirms our hypothesis that the peptide IRAP inhibitors exert their effect on CTB invasiveness by inhibition of its enzymatic activity, and thus increasing the half life of the known peptide substrate of IRAP, OT. However, in view of the close association of IRAP with GLUT4 in insulin responsive tissues, the effect of IRAP inhibitors in regulating glucose uptake in the placenta could not be totally discounted. The presence of GLUT4 was recently found in the first trimester syncytiotrophoblast [21]. Moreover, the involvement of glucose transporter (GLUT1 and GLUT3) in early pregnancy for successful implantation by trophoblast invasion has been recently described [22]. So the evaluation of involvement of GLUT4 in both trophoblastic cells and cancer cells invasion could be of great interest.

In conclusion, it is more and more probable that IRAP could play a role in trophoblast cell invasion. Peptide inhibitors and substrate of this protein could thus decrease the invasiveness of CTB.

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