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Interleukin-1β regulates metalloproteinase activity and leptin secretion in a cytotrophoblast model

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ABSTRACT: Implantation is one of the most regulated processes in human reproduction, by endocrine and immunological systems. Cytokines are involved in embryo-maternal communication and an impaired balance could result in pregnancy loss. Here we investigated the effect of interleukin 1-β on the activity of two important metalloproteinases (MMP-2 and MMP-9) that are involved in extracellular matrix remodeling as well as the secretion of leptin, one of the reproductive hormones actively regulating their activity and secretion. We found that IL-1β activates matrix metalloproteinase activity as well as increases leptin secretion. We propose that this interleukin, through the regulation of leptin, in turn activates matrix metalloproteinases which results in an increased cytotrophoblast invasion.

Introduction

The implantation process is the most critical step in achieving a successful early pregnancy. It relies on uterine-dependent and embryo-specific events, which need to be critically coordinated.

After initial attachment of the blastocyst to the endometrial lining, mononuclear cytotrophoblastic cells that surround the embryonic disc fuse to form a syncytium. These multinucleated and differentiated cells invade the pseudo-decidualized endometrium. Cytotrophoblastic cells can differentiate (Bischof and Irminger-Finger, 2005) into villous cytotrophoblastic cells, considered as stem cells, form a monolayer of polarized epithelial cells that proliferate and eventually differentiate by fusion to form the syncytiotrophoblast (Kao *et al*., 1988) or villous cytotrophoblastic cells which can also form multilayered columns of proliferating mononuclear cells that differentiate into non-polarized and invasive cytotrophoblastic cells. These motile and highly invasive cytotrophoblastic cells are found in the maternal decidua, the intima of the endometrial spiral arteries and the proximal third of the myometrium (Enders, 1968). Villous cytotrophoblastic cells are proliferating cells, in contact to one another and to the syncytiotrophoblast through the adhesion molecule E-cadherin. They express epidermal growth factor (EGF) receptors and actively secrete EGF, transforming growth factor-β (TGF-β) and interleukin-1β (IL-1β).

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The motile and highly invasive cytotrophoblastic cells, as well as tumor cells, are invasive because they secrete proteases capable of digesting the surrounding extracellular matrix. These proteases named matrix metalloproteinases (MMPs) and their inhibitors are tightly regulated. Serine proteases, cathepsins and matrix metalloproteinases have been implicated in the invasive process of tumors as well as cytotrophoblastic cells (Nagase, 1997). All matrix metalloproteinases are not equally important for trophoblast invasion; for example MMP-9 has been shown to mediate cytotrophoblastic cells invasion *in vitro*. In contrast to tumor cells, cytotrophoblastic cells are only transiently invasive (first trimester) and their invasion is normally limited only to the endometrium and to the proximal third of the myometrium (Shimonovitz *et al*., 1998). This temporal and spatial regulation of trophoblast invasion is believed to be mediated in an autocrine way by trophoblastic factors and in a paracrine way by uterine factors. Several types of regulators have been investigated such as hormones, cytokines, growth factors and extracellular matrix glycoproteins (Bischof and Irminger-Finger, 2005; Oktay *et al*., 1994). Cytokines and growth factors are known to affect the invasive behavior of cytotrophoblastic cells (Chaouat *et al*., 2005) such as several interleukins (IL-1, -6, -10, -15) as well as tumor necrosis factor, EGF, leukemia inhibitory factor, transforming growth factor-β (TGF-β), insulin-like growth factor binding protein-1 and insulin-like growth factor II and leptin have all been shown to modulate matrix metalloproteinase secretion and/or invasion in human trophoblastic cells.

Leptin appears to play a critical role in reproductive function and is linked to the inflammatory response (Gonzalez *et al*., 2000a; Moschos *et al*., 2002). Published data show that leptin could play key roles in the development of the preimplantation embryo and in the implantation process (Kawamura *et al*., 2002; Gonzalez *et al*., 2000b; Wu *et al*., 2002; Alfer *et al*., 2000; Gonzalez *et al*., 2000b; Kitawaki *et al*., 2000; Wu *et al*., 2002; Gonzalez *et al*., 2000b; Cameo *et al*., 2003; Henson and Castracane, 2006; Cervero *et al*., 2006). The down-regulation of the leptin receptor Ob-R expression at the time of implantation could play a role in subfertility (Alfer *et al*., 2000). However the leptin function in the normal implantation process is not well characterized (Yang *et al*., 2006). Leptin upregulates the expression of matrix metalloproteinases genes and enzyme related in trophoblastic invasion of mouse and human trophoblastic cells culture (Gonzalez *et al*., 2001; Schulz and Widmaier, 2004). Recent evidences indicated that leptin could promote adhesion and expansion of trophoblastic cells into the maternal endometrium *in vivo* (Yang *et al*., 2006). On the other hand, leptin up regulate the expression of relevant integrins for embryo adhesion in human epithelial endometrial cells (Gonzalez *et al*., 1999, 2001; Gonzalez and Leavis, 2001) and intensify the epithelial endometrial cell receptivity to the embryo implantation (Yang *et al*., 2006). Although leptin is regulated in several tissues by IL-1, tumor necrosis factor-α (TNF-α) and TGF-β (Sarraf *et al*., 1997), leptin may itself induce the synthesis of inflammatory cytokines *in vivo* and *in vitro* (Loffreda *et al*., 1998).

The IL-1 system composed of ligand (IL-1β), receptor type I (IL-1R I) and receptor antagonist (IL-1Ra), is produced by both preimplantation embryos and endometrium. It has been proposed to be an important factor in embryo-maternal molecular cross-talk during implantation (Sheth *et al*., 1991; Simon *et al*., 1993). IL-1β (Simon *et al*., 1997) and leptin (Gonzalez and Leavis, 2001) up-regulate β3 integrin expression (a molecular marker of endometrial receptivity) by endometrial epithelial cells. However, leptin exerts a significantly greater effect on β3 integrin up-regulation than IL-1β at similar concentrations and IL-1β stimulates leptin secretion and Ob-R expression by endometrial epithelial cells (Gonzalez and Leavis, 2001). Leptin produced and secreted locally by preimplantation embryos and endometrial epithelial cells could act in an autocrine or paracrine manner to regulate biological functions that may mediate endometrial receptivity (Gonzalez *et al*., 2000a, b).

Considering that invasion is crucial for a successful implantation and that matrix metalloproteinases actively participate in this process, our objective in the present study was to evaluate the effect of endometrial paracrine factors on trophoblastic invasion. We used *in vitro* cultured human choriocarcinoma trophoblastic cells (JEG-3) as a model of embryo implantation and we focused our attention on the effect of human IL-1β on the activity of MMP-2 and MMP-9 and on the expression/secretion of leptin in a trophoblastic cellular model (line JEG-3).

Materials and Methods

Cell Culture

Human choriocarcinoma cell line JEG-3 (American Type Culture Collection, Rockville, MD, USA) was cultured in DMEM-F12/10% (w/v) fetal bovine serum, at 37ºC in 5% (v/v) CO_2.

After 24 h, different concentrations of recombinant human interleukin-1β (IL-1β, 0-100 pg/mL) (Sigma Chemical Co., St. Louis, MO) were added to the culture, now in DMEM-F12/1% fetal bovine serum (w/v), for 3 days. Cells and conditioned media were collected separately and conserved at -20ºC.

Gelatin zymography

Gelatin zymography was used to determine metalloproteinase activity. Briefly, gelatin at a final concentration of 1 mg/mL was incorporated to a 10% SDS-PAGE with a 2% SDS-PAGE stacking gel. Aliquots (10 µl) of each sample were loaded and proteins electrophoresed for 1 h at 100 V. After electrophoresis, gels were washed five times (5 min each) in a Tris-based solution consisting of 2.5% Triton X-100 (w/v), then three times for 10 min in PBS. Then, the gel was incubated in 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.005 mol/L $CaCl_2$ (pH 7.5) at 37ºC for 24 h. Thereafter, the gels were stained with 0.5% Coomassie brilliant blue R-250 (w/v) (Sigma, St. Louis, MO) for 45 min, lightly destained in methanol: acetic acid: water (3:1:6) and finally stored in 5% acetic acid (v/v). Each gelatinase band was identified according to molecular weight, with purified human MMP-9 as standard. The presence of gelatinases was confirmed by inhibition with EDTA. Quantification of the bands was performed with the image program Image-J (NIH, Bethesda, MD). Data were expressed as fold-values of the average optical density, relative to the control band (no IL-1β) and considering the serum albumin band as load control.

Effect of IL-1β on matrix metalloproteinase activity

This was analyzed in conditioned media from JEG-3 cells, after 3 days of culture, by gelatin zymography assay. The migration of MMP-2 (72 kDa) and MMP-9 (92 kDa) specific bands was previously determined in different cell types (JEG-3/BeWo/3T3-L1) and compared to purified MMP-9 and molecular weight markers (data not shown). The presence and intensity of digestion bands (clear bands) in the gelatin-containing gel (dark background) was evaluated. Absence of activity was observed when EDTA was added at the incubation buffer (negative control).

JEG-3 cells were cultured in the presence of increasing concentrations of IL-1β (0-100 pg/mL) for 3 days and conditioned media were collected. Matrix metalloproteinase activity was evaluated in the corresponding band for each experimental condition. Digitalized area from each digested clear band was normalized against the bovine serum albumin band which appeared as a dark one, using this as a load control.

Western Blot analysis for leptin secretion

Samples from conditioned media (20 µg of protein) obtained from each experimental condition, were electrophoresed in denaturing 10% SDS-PAGE for 1h at constant voltage (100V). Samples were previously heated at 85ºC for 10 min and loading buffer containing β-mercaptoethanol and SDS was added. Then, proteins were electrophoreticaly transferred to a nitrocellulose membrane (Hybond, Amersham, Pharmacia) for 90 min at constant voltage at 4ºC. The membrane was blocked with 5% skim milk (w/v) in PBS buffer, for 30 min, at room temperature. Then, first antibody was added (polyclonal anti-leptin developed in rabbit, Sigma Co.) diluted 1:1000 in 5% skim milk (w/v) in PBS, and left overnight at 4ºC. Afterwards, 4 x 5 min-washes were performed with PBS buffer. Second antibody (anti-rabbit IgG bound to peroxidase, Sigma Co.) was added at a 1:1000 dilution in 5% skim milk (w/v) in PBS for 90 min at room temperature. Afterwards, 4 x 5 min-washes were performed, at room temperature.

The membrane was developed for chemiluminescence using ECL Western blotting system (GE Healthcare) and a bio-imaging analyzer (Fujifilm LAS-1000). Negative control did not include first antibody. BSA present in the conditioned media was used as load control. Bands were analyzed with Image-J Software (NIH, Bethesda, MD)

Total cell lysates were prepared in lysis buffer (PBS, 1% Nonidet P-40 (v/v), 0.1% SDS (w/v), and 0.01 mol/L EDTA, 0.005 mol/L Tris-HCl pH 6.8) and used as a control of total protein content. The lysates were centrifuged at 10000 x g for 10 min to remove cellular debris. Protein content was determined according to the Bradford method. Lysates were mixed with SDS-PAGE sample buffer containing 4% β-mercaptoethanol (v/v), boiled for 5 min, resolved by SDS-PAGE on a 10% gel, and stained with Coomassie Blue.

Statistics

Dose-response curves were analyzed for statistical correlation using the two-tailed Spearman test and considering the result significantly different from the horizontal line with a P value less than 0.05.

Results

Effects of IL-1β on the activity of MMP-2 and -9

MMP-9 activity increased (Fig. 1) in a dose-dependent manner with IL-1β concentrations and it was more evident at the higher concentration tested (100 pg/mL). The upper panel of the figure shows a representative gel. A similar stimulation was observed for MMP-2 activity (Fig. 2). Also in this case the upper panel shows a gel from one representative experiment.

Also, the effect of IL-1β on the expression of secreted MMP-9 was evaluated. The cytokine did not significantly change the expression of MMP-9 at any concentration tested in conditioned media (data not shown).

Effects of IL-1β on the expression of secreted leptin

Being leptin one of the reproductive hormones involved in regulation of metalloproteinase activity and secretion, we then analyzed its concentration in the conditioned media of trophoblastic cells, cultured in the presence of various IL-1β concentrations (0-100 pg/mL). Figure 3 shows the dose-response increase in leptin secretion by IL-1β.

Discussion

After oocyte fertilization has occurred, the newly formed embryo travels towards the uterus which is receptive for embryo implantation. Bidirectional interplay takes place, with stimuli coming from the endometrium affecting the embryo and, also, signals from the embryo must appear to allow for tissue invasion. The embryo must adhere and invade the endometrium in order to continue through the rest of the pregnancy. At this point, inflammatory responses take place and the immune system through a Th1/Th2 balance could prevent or assist in pregnancy development. A review by Hauguel-de Mouzon and Guerre-Millo (2006) addresses this placental cytokine network.

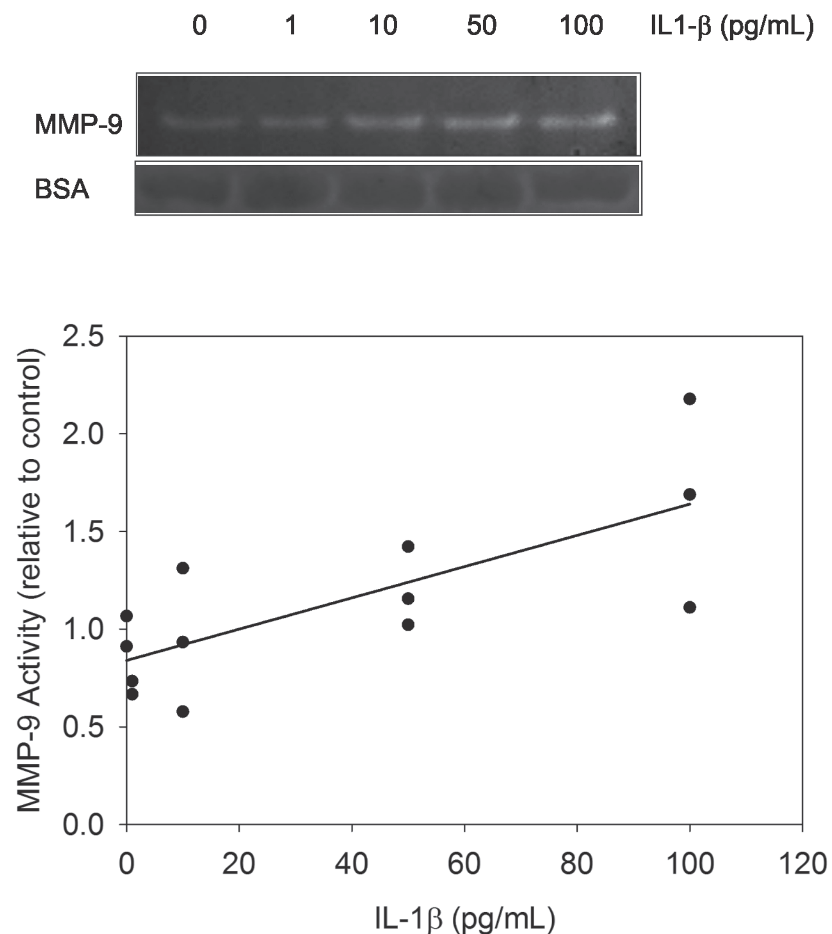

FIGURE 1. Effect of IL-1β on the enzymatic activity of MMP-9 in trophoblastic cells. The statistical test showed that the differences in MMP-9 activity from conditioned media of cells treated with IL-1β positively correlated with the different concentrations of IL-1β used (1-100 pg/mL), relative to the control group (n=3, P=0.0009). Correlation coefficient (R) was 0.7667 (Two-tailed Spearman test).

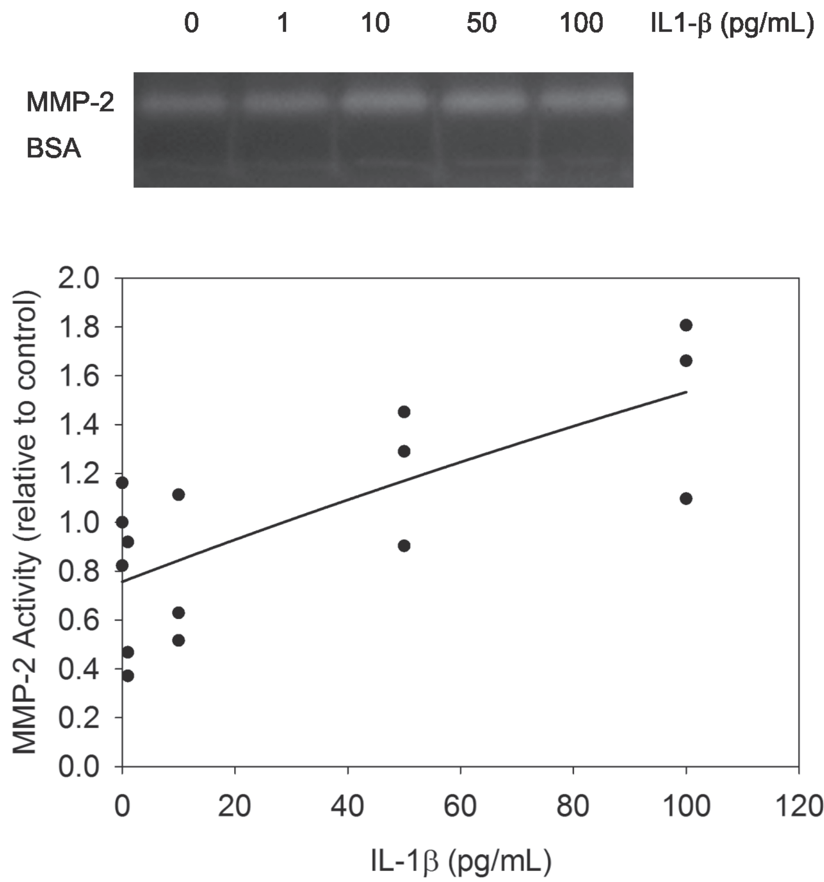

FIGURE 2. Effect of IL-1β on the enzymatic activity of MMP-2 in trophoblastic cells. The statistics test showed that the differences in MMP-2 activity from conditioned media of cells treated with IL-1β positively correlated with the different concentrations of IL-1β used (1-100 pg/mL), relative to the control group (n=3, P=0.0019). Correlation coefficient (R) was 0.7315 (two-tailed Spearman test).

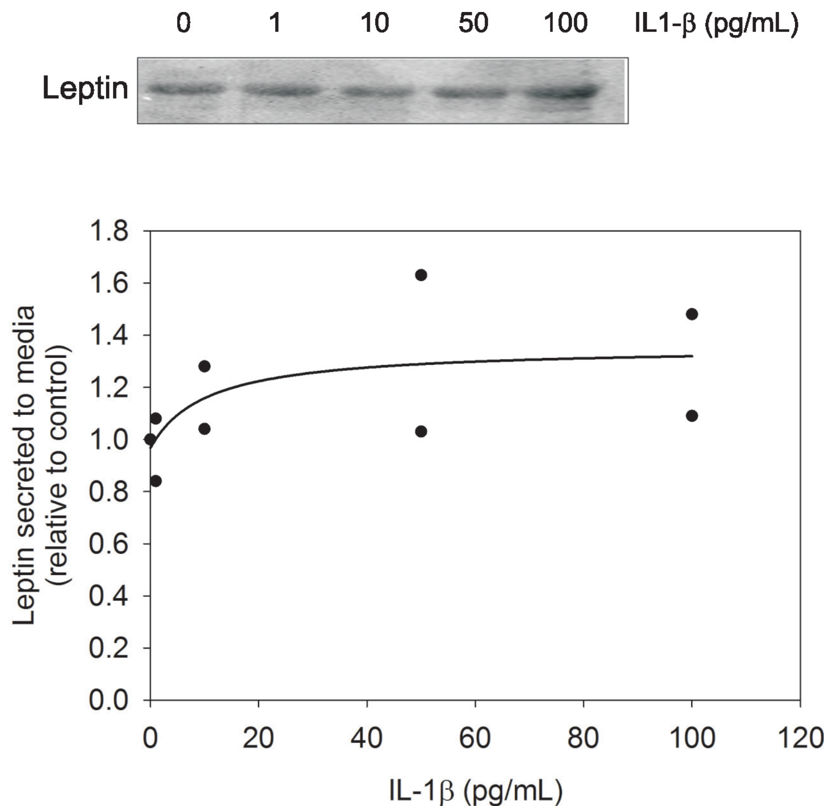

FIGURE 3. Western blot analysis of leptin secreted to the conditioned media by trophoblastic cells. The statistical test showed that the differences in leptin secretion into conditioned media of cells treated with IL-1β positively correlated with the different concentrations of IL-1β used (1-100 pg/mL), relative to the control group (n=2, P=0.0234). Correlation coefficient (R) was 0.7161 (two-tailed Spearman test).

Several cytokines are known to be produced by cells other than those related to the immune system. For example, both syncytiotrophoblast and cytotrophoblast cells are considered to produce cytokines (Guilbert *et al*., 1993) and almost all cell types of the uteroplacental tissues have been shown to participate in the cytokine network (Hunt, 1989). The IL-1 system (IL-1 isoforms and their receptors) has been connected to the leptin system (leptin and its various receptors) and, thus, leptin has been shown to trigger similar responses as IL-1β, suggesting a possible redundant system during the implantation process (Gonzalez *et al*., 2003).

We have previously shown that another cytokine, interferon-γ was deleterious to embryo implantation and development (Cameo *et al*., 1999 and Fontana *et al*., 2004).

Here we investigated the role of IL-1β on leptin secretion and metalloproteinase activation as a possible regulator of embryo invasion to the endometrial wall. We found that this cytokine was able to activate (either directly or indirectly) metalloproteinases 2 and 9 in a cytotrophoblastic model, both involved in extracellular matrix remodeling and invasion processes. When we analyzed the secretion of leptin to the culture medium, we found it increased in a dose-dependent manner with the doses of IL-1β. At this point we cannot draw a conclusion on the temporal relationship of these two events and decide whether IL-1β is acting on the enzymes through the increased expression of leptin or directly on their activity or through inhibition of the natural regulators, the TIMPs (Tissue Inhibitors of Metallo-Proteinases).

It is important to emphasize that our results parallel those found using human placental cytotrophoblast cells (Gonzalez *et al.*, 2003) indicating that this could be a good model to reproduce these effects, for future studies, without the need of primary cultures of placental tissue.

In conclusion, the present data suggest an interrelationship between IL-1β and leptin, possible through an autocrine/paracrine system which could have a profound effect on embryo implantation and pregnancy development.

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