Placenta 33 (2012) 908–913

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

The effects of oncostatin M on trophoblast cells: Influence on matrix metalloproteinases-2 and -9, and invasion activity

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ARTICLE INFO

Article history:
Accepted 17 July 2012

Keywords:
Oncostatin M
Trophoblast
Invasion
MMP-2
MMP-9

ABSTRACT

Oncostatin M (OSM), a cytokine of the interleukin-6 (IL-6) family, can either promote or inhibit cell growth in various normal and tumor cells and is expressed in rheumatoid arthritis, multiple sclerosis, multiple myeloma, and other inflammatory conditions. We investigated one of the possible mechanisms involved in trophoblast invasion using the human placental cell line derived from first trimester extravillous trophoblasts (HTR8SVneo): modulation of matrix metalloproteinase (MMP)-2 and -9 expression and enzymatic activity. And we addressed also the effects of exogenous OSM on the in vitro invasion activity of HTR8SVneo cells. We found that OSM enhanced the constitutive RNA and protein expressions of MMP-2 and MMP-9 in HTR8SVneo cell lines. Also, OSM treatment increased significantly the enzymatic activity of MMP-2 on gelatin zymography. The effects OSM on enzymatic activity of MMP-9 was not significant. We found that OSM increased invasion activities of HTR8SVneo cells in time-dependent and dose-dependent manners. This study suggests that OSM enhances invasion activities of extravillous trophoblasts during the first trimester through the increased enzyme activity of gelatinases, especially MMP-2.

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1. Introduction

In early pregnancy, the invasive nature of interstitial extravillous trophoblast cells (EVTs) is essential for the survival of the conceptus and the extent of invasion must be tightly controlled.

The invasion of the extracellular matrix by EVTs is a complex, multistep process involving the concerted action of adhesive, degradative, and migratory pathways [1]. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes capable of degrading almost all components of the extracellular matrix [2]. Because basement membranes are the major structural hindrance for invading cells, the two gelatinases, MMP-2 and MMP-9 (which cleave type IV collagen, the main component of basement membranes), are therefore regarded as key enzymes in the invasion process of EVTs. MMP expression is induced in a variety of cell types by a number of stimuli, including growth factors such as vascular endothelial growth factor (VEGF) [3], chemical agents such as the phorbol esters [4], physical stress such as hypoxia [5], and cell-matrix and cell–cell interactions [6,7].

Oncostatin M (OSM), a cytokine of the interleukin-6 (IL-6) family, can either promote or inhibit cell growth in various normal and tumor cells. It has been known that OSM has unique biological activities in inflammation, hematopoiesis, development and inhibiting the proliferation of tumor cells, remodeling of extracellular matrix, organ development, and regeneration [8]. OSM was first identified by its inhibitory activity on U937 lymphoma cells [9]. In addition, OSM inhibits the growth of many cancer cell types, including melanoma, neuroblastoma, fibrosarcoma, rhabdomyosarcoma, and other carcinoma cells [10]. However, OSM, like IL-6, is a potent mitogen for myeloma cells and stimulates the proliferation of AIDS related Kaposi's sarcoma cells [11,12] or other non-tumor cells including fibroblasts and smooth muscle cells [13,14]. Therefore, whether OSM has inhibitory or stimulatory effects on a cell is cell-type specific. OSM has shown various effects on the expressions and activities of MMPs, according to the type of cells [15–19].

Little is known about the effects of OSM on pregnancy, but OSM concentrations in the sera of pregnant women were found to be significantly higher than those of nonpregnant women. OSM molecules produced by the decidual glands and stromal cells during pregnancy seem to have a role in placental endocrine

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0143-4004/$ – see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.placenta.2012.07.014

function [20]. Previously, the significantly greater OSM expressions were identified in cytotrophoblasts, syncytiotrophoblasts and endothelium of preeclamptic placentas as compared to normal placentas, and OSM concentration of preeclamptic women's serum was significantly higher than that of normal women's plasma [21]. It is possible that OSM can affect invasion process of EVT through the various actions, including the action on the MMP-2 and -9, which are the important gelatinases during the early pregnancy.

The aim of our study was to investigate the effects of OSM on the expressions and enzymatic activities of MMP-2 and -9, as well as on the invasion activity of EVTs.

2. Materials and methods

2.1. Cell lines

The EVT cell line HTR8SVneo was kindly provided by Dr. Charles Graham (Queen's University, Kingston, ON, Canada). The cell line was produced by immortalization of HTR-8 cells, an EVT cell line from primary explant cultures of human first trimester placenta (8–10 wk gestation), with SV40 virus and selected on the basis of resistance to neomycin [22]. These cells exhibit markers of primary EVT cells in situ, including cytokeratins KRT7, KRT8, and KRT18, placental-type alkaline phosphatase, high-affinity PLAUR, human leukocyte antigen (HLA) framework antigen W6/32, insulinlike growth factor 2 (IGF2) mRNA and protein, a selective repertoire of integrins (ITGA1, ITGA3, ITGA5, ITGAV, ITGB1, ITGAVB3/B5) [23], and HLA-G when cultured on Matrigel [24]. In the present study, HTR8SVneo cells were used between passages 70 and 75.

2.2. Cell culture

HTR8SVneo cells were seeded at subconfluence density in RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 10% FBS. Cells were washed with PBS and 10^7 cells were transferred to a 100 mm culture dish with RPMI 1640 medium, and cultured for 24 h. After changing with new RPMI media, cells were treated with OSM (1–100 ng/ml) for 48 h. To evaluate the time-dependent effects of OSM, cells were treated with OSM (20 ng/ml) and incubated for 12, 24, 48 h. At the end of the experiment, cell numbers were determined using a hemocytometer, conditioned media was collected. Conditioned media was centrifuged for 10 min at 12,000 rpm and stored at −20 °C, for gelatin zymography. Cells were stored at −70 °C.

2.3. RNA extraction and real-time RT-PCR

RNA from experimental cell incubations was extracted with TRIZOL reagent (GIBCO-BRL, Int). The sequences of the primers used in real-time PCR for MMP-2 (GenBank Accession No. NM_001127891.1) and MMP-9 (GenBank Accession No. NM_004994.2) were as below; MMP-2 (Forward 5′-AAG AAC CAG ATC ACA TAC AGG ATC A-3′) and MMP-9 (Forward 5′-AGT ACC GAG AGA AAG CCT ATT TCT G-3′). To confirm the integrity of the extracted RNA, transcription of the housekeeping gene GAPDH was analyzed as a control (Forward, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′) [25].

2.3.1. cDNA synthesis

CDNA was produced using the Superscript™ || RT-PCR System (Invitrogen, Karlsruhe, Germany) according to the manufacture's recommendations for oligo $(dT)_{20}$ primed cDNA synthesis. cDNA synthesis was performed on 500 ng of RNA, at 42 °C. Finally, cDNA as diluted 1:2 prior use in quantitative PCR.

2.3.2. Quantitative TaqMan PCR

PCR was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in 384-well microtiter plates using a final volume of 10 ul. Optimum reaction conditions were obtained with 5 ul of Universal Master Mix (Applied Biosystems, Foster City, CA, USA) containing dNUTPs, MgCl2, reaction buffer and Ampli Taq Gold, 90 nM of primer(s) and 250 nM fluorescence-labeled TaqMan probe. Finally, 2 ul template cDNA was added to the reaction mixture. The primer/TaqMan probe combinations were designed on each target sequence. Amplifications were performed starting with a 10 min template denaturation step at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All samples were amplified on triplicate and data were analyzed with Sequence Detector software (Applied Biosystems).

2.4. Western blot

After washing the cultured cells with DPBS, protein was extracted using RIPA lysis and extraction buffer (Pierce, USA). 1 ml of extracted protein was centrifuged at 12,000 rpm for 10 min and quantified using BCA protein assay reagent (Pierce, USA). 50 μ of protein was mixed with 5× sample loading buffer (Fermentas, Canada) and denatured 100 °C for 5 min. Then it was subjected to 8–16% SDS-PAGE gel (Komabiotech, Korea) and electrophoresed at 125 V for 2.5 h, with subsequent electroblotting transfer onto a nitrocellulose membrane (Genescript, USA). As a loading control, we used GAPDH (Santacruz, USA) [25]. After transfer, the membrane was blocked 1 h in Noise Canceling Reagents (Millipore, USA) and then incubated overnight with 0.1 μg/ml of a mouse anti-human MMP-9 monoclonal antibody (santacruz, USA). Membranes were rinsed in TBS and 0.1% Tween 20 prior to and after incubation with horseradish peroxidase-conjugated anti-mouse IgG (ICN Biomedicals, Aurora, OH). Chemi-luminescence was detected with Luminata Crescendo Western HRP substrate (Millipore, USA) and autoradiography film (Agfa, Belgium) according to the manufacturer's instructions. The same membrane was then rinsed as above and incubated in Ez Way Antibody Erasing buffer (Komabiotech, Korea) for 15 min, with subsequent rinsing and reblocking as indicated above. The membrane was then incubated overnight with 1 μg/ml of a mouse anti-human MMP-2 monoclonal antibody (santacruz, USA) and processed as indicated above. The experiment was replicated three times. The bands from Western blotting were quantified by Gel Doc™ XR+ with Image lab software (Bio rad, USA).

2.5. Gelatin zymography

Gel zymography, with gelatin as the substrate in the gel, was used to detect the proteolytic activity of MMP-2 and MMP-9. Conditioned media from cell cultures were centrifuged, and the supernatants were mixed with 5X SDS sample buffer (Fermentas, Canada) and incubated at 37 °C for 1 h. Samples were loaded onto a 10% gelatin Zymogram-PAG 10% Pre-cast gel (Komabiotech, Korea). To enable the enzymes to renature, the gel was incubated twice for 20 min in renaturation buffer (Komabiotech, Korea) and incubated in Zymogram development buffer (Komabiotech, Korea) for 20 min, and then placed in fresh Zymogram development buffer at 37 °C for 36 h. The gel was stained with Comassie Blue R-250 (Komabiotech, Korea) for 30 min at room temperature, and then destained twice with destaining solution for 15 min. The presence of clear bands in the gels at the appropriate molecular weights reflects gelatonolytic activity of MMP-2 and MMP-9. The bands from gelatin zymography were quantified by Gel Doc™ XR+ with Image lab software (Bio rad, USA).

2.6. Invasion assay

An invasion assay was performed to examine the invasive ability of HTR8SVneo cells using a cell invasion assay kit which is coated with polycarbonate membrane (pore size: 8 μm) (CHEMICON, Billerica, USA) on ECMatrixTM. 1×10^6 cells/ml were prepared. In this experiment, 3×10^5cells suspended in the 300 μl of serum free RPMI medium were added to the upper chamber of the membrane and 500 μl of RPMI medium with 10% FBS was added in the lower chamber. Assays were carried out at 37 °C under 5% CO2 and 95% air for 72 h. At the end of the incubation, the cells on the upper filter surface were completely removed by wiping with a cotton swab. Invasive cells were stained on lower surface of the membrane by dipping inserts in the staining solution for 20 min, rinsed several times by dipping inserts in a beaker of water, and then dried in the air. Then, the number of cells passing through the membrane and migrating to the other side was counted. Cell counting was performed in triplicate and the data expressed as means.

2.7. Statistical analysis

Data are expressed as mean ± SEM. The non-parametric Mann–Whitney test and independent *t*-test were used for comparison between two groups. A *P*-value of 0.05 was considered to be statistically significant.

Each experiment was performed three times.

3. Results

3.1. The effects of OSM on mRNA expressions of MMP-2 and MMP-9

The mRNAs for MMP-2 and MMP-9 were quantified by real-time RT-PCR. We observed constitutive expression of MMP-2 and MMP-9 in HTR8SVneo cells. The expression levels of MMP-2 and MMP-9 mRNAs were compared with the internal control, the GAPDH gene. As shown in Fig. 1, OSM significantly induced MMP-2 and MMP-9 mRNA expression by 1.95-fold and 2.24-fold, respectively, after the treatment of OSM for 48 h.

3.2. The effects of OSM on protein expressions of MMP-2 and MMP-9

MMP-2 and MMP-9 proteins were constitutively expressed by HTR8SVneo cells on Western blot analysis. We found that OSM (20 ng/ml) stimulated MMP-2 and MMP-9 protein expressions to

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