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ORIGINAL PAPER

Effects of recombinant H2 relaxin on the expression of matrix metalloproteinases and tissue inhibitor metalloproteinase in cultured early placental extravillous trophoblasts

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Abstract Relaxin promotes softening of the uterine cervix and inhibits uterine contractility in rats, mice and pigs. Little information, however, is available about the role of relaxin in humans. In 2002, LGR7 and LGR8 were discovered to be receptors for relaxin and those receptors were identified in the human placenta. Thus, in this study, effects of recombinant H2 (rH2) relaxin on human early placental extravillous trophoblasts (EVTs) were examined. Isolation of EVTs from early placental trophoblasts was performed using the procedures established in our laboratory. After 48-h subculture, the presence of relaxin receptors in cultured EVTs was characterized by RT-PCR and immunoblotting. The cultured EVTs were treated with different doses (0.3–3 ng/ml) of rH2 relaxin for 24 h. The effects of rH2 relaxin on MMP-2, -3, -9 and TIMP-1 mRNAs levels were examined by real-time RT-PCR. RT-PCR and immunoblotting revealed that relaxin receptors are present in early placental EVTs. Treatment with rH2 relaxin increased MMP-2 and -9 mRNAs levels and decreased TIMP-1 mRNA levels in cultured EVTs, whereas rH2 relaxin did not affect MMP-3 mRNA levels. These results suggest that relaxin may promote the invasive potential of early placental EVTs through up-regulating MMP-2, -9 mRNAs and down-regulating TIMP-1 mRNA in EVTs.

Keywords Relaxin · Extravillous trophoblast · Early placenta · MMP · TIMP

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Introduction

In humans three forms of relaxin have been identified; H1, H2, and H3 relaxin. H1 relaxin is immunolocalized in the human decidua, placenta, and prostate, but not in the ovary [1]. H2 relaxin is immunolocalized in the corpus luteum [2], placenta [1], and deciduas [3]. H3 relaxin is mainly immunolocalized in the brain [4]. Their biological functions in humans, however, have not been elucidated yet. In rodents, relaxin is well known to inhibit uterine contractility and induces cervical ripening and lengthening of the interpubic ligament prior to parturition and also promotes the growth of the mammary glands and nipples as a prerequisite for successful lactation [5]. A considerable part of the biological actions of relaxin is based on its ability to induce modifications of stromal tissue components. Relaxin leads to thinning of collagen fibers in murine mammary glands. In human scleroderma fibroblasts, relaxin decreases the synthesis of collagen [6]. The expression and catalytic activities of collagenase-1 (MMP-1), gelatinases A and B (MMP-2 and -9) and stromelysin-1 (MMP-3), members of the matrix metalloprotease (MMP) family, are increased by relaxin in breast cancer cells [3], lung fibroblasts [7], and fetal membrane [3, 8]. In 2002, two orphan guanine nucleotide binding protein (G protein)-coupled receptors, LGR7 and LGR8 were discovered to be receptors for relaxin [7]. It is recently recommended that LGR7 and LGR8 be known as relaxin family peptide receptors 1 (RXFP1) and 2 (RXFP2) [9]. The expression of LGR7 and LGR8 mRNA has been identified in the human placenta [10]. The identification of the relaxin receptors, LGR7 and LGR8, in the human placenta suggests that relaxin may take a physiological role in the placenta.

The serum levels of relaxin in human are the highest during the first trimester of pregnancy when the function of

corpus luteum is most active, and decline throughout the remainder of pregnancy [11, 12]. The human early placenta is characterized by the invasion of extravillous trophoblasts (EVTs) to the decidua, leading to direct contact between trophoblasts and maternal blood in process known as hemomonochorial placentation. During the invasion process, EVTs express matrix metalloproteinases (MMPs), which are zinc-dependent proteolytic enzymes that cleave all the constituents of the extracellular matrix [13]. Thus, we conducted the present study to investigate the direct effects of human relaxin (recombinant H2 relaxin) on the expression of MMPs in human early placental EVTs cultured in vitro.

Results

RT-PCR analysis of LGR7 and LGR8 mRNA in cultured EVTs

The mRNA expression of LGR7 and LGR8 in chorionic villi, cultured EVTs and HTR-8/SV neo trophoblast cells of human EVT cell line after 48 h subcultures was examined by RT-PCR analysis. The 228-bp band corresponding to LGR7, 221-bp band to LGR8 and 218-bp to β-actin were observed in chorionic villi, cultured EVTs and HTR-8/SV neo cells after 48 h subcultures (Fig. 1A). All bands were sequenced to be correct.

Densitometric analysis revealed that the fold increase of LGR7 mRNA in cultured early placental EVTs and HTR-8/SV neo cells was 0.69 ± 0.1 ($P < 0.05$) and 0.92 ± 0.03 ($P = 0.3$), respectively, relative to the abundance in chorionic villi (Fig. 1B). The fold increase of LGR8 mRNA in cultured early placental EVTs and HTR-8/SV neo cells was 0.81 ± 0.07 ($P < 0.05$) and 1.04 ± 0.12 ($P = 0.3$), respectively, relative to the abundance in chorionic villi (Fig. 1C). Densitometric values of LGR7 and LGR8 mRNAs in cultured early placental EVTs were significantly lower ($P < 0.05$) compared to those in chorionic villi (Fig. 1B, C). Densitometric value of LGR8 mRNA in early placental EVTs was 1.25 ± 0.06 ($P < 0.05$) relative to the abundance of LGR7 mRNA in those cells (Fig. 1D).

Immunocytochemical analysis of LGR7and LGR8 in cultured EVTs

To confirm the presence of LGR7 and LGR8 proteins in cultured EVTs after 48 h subculture, immunocytochemical analysis was performed using their specific antibodies. LGR7 and LGR8 were positively immunostained in cultured EVTs. The replacement of the primary antibody with blocking peptide resulted in a lack of positive immunostaining (Fig. 2).

Effect of rH2 relaxin treatment on MMP-2, -3, -9 and TIMP-1 mRNA levels in cultured EVTs

Real-time RT-PCR analysis revealed that the treatment with rH2 relaxin of 0.3, 1.0, and 3.0 ng/ml increased MMP-2 mRNA levels in cultured EVTs, to $171 \pm 34.4\%$ ($P = 0.1$), $242 \pm 9.4\%$ ($P < 0.05$), and $402 \pm 93.8\%$ ($P < 0.05$), respectively, compared with untreated cultures (Fig. 3A). The treatment with rH2 relaxin did not affect MMP-3 mRNA levels in cultured EVTs, compared with untreated cultures. The treatment with rH2 relaxin of 0.3, 1.0, and 3.0 ng/ml resulted in $131 \pm 83.9\%$ ($P = 0.3$), $188 \pm 30.6\%$ ($P = 0.3$), and $88 \pm 19.4\%$ ($P = 0.3$), respectively, compared with untreated cultures (Fig. 3B). The treatment with rH2 relaxin of 0.3, 1.0, and 3.0 ng/ml increased MMP-9 mRNA levels in cultured EVTs, to $211 \pm 0.3\%$ ($P < 0.05$), $366 \pm 239\%$ ($P = 0.2$), and $429 \pm 178\%$ ($P < 0.05$), respectively, compared with untreated cultures (Fig. 3C). On the other hand, the treatment with rH2 relaxin of 0.3, 1.0, and 3.0 ng/ml decreased TIMP-1 mRNA levels in cultured EVTs, to $25.3 \pm 4.7\%$ ($P < 0.05$), $16.8 \pm 3.1\%$ ($P < 0.05$), and $16.1 \pm 3.8\%$ ($P < 0.05$), respectively, compared with untreated cultures (Fig. 3D).

Discussion

The controlled invasion of trophoblast into the decidua is an essential process for early placental development and the maintenance of early pregnancy. For a successful implantation, trophoblast needs to cross the basal membrane of the decidua, migrate through the decidual interstitium and finally reach the vasculature. During these processes, MMPs play major roles [14]. Relaxin production and action during pregnancy and parturition are known to be highly species dependent. In humans, relaxin is both a systemic pregnancy hormone secreted by the corpus luteum and an autocrine/paracrine hormone at the maternal-fetal interface formed by the decidua, placenta and fetal membranes [8]. Relaxin receptors (LGR7 and LGR8) have been identified in human term amniotic epithelium, decidua and placenta [7, 10, 15], especially LGR7 is observed in the villous cytotrophoblast [7, 10, 15]. The possible presence of an autocrine/paracrine system based on relaxin and its receptors in the decidua parietalis and in villous cytotrophoblasts was suggested [3]. In the present study, we have demonstrated for the first time the presence of relaxin receptors (LGR7 and LGR8) in the human early placental EVTs which were distinguished from villous cytotrophoblasts. Densitometric analysis of RT-PCR indicated that the LGR7 and LGR8 mRNAs in chorionic villi containing cytotrophoblasts and syncytiotrophoblasts were

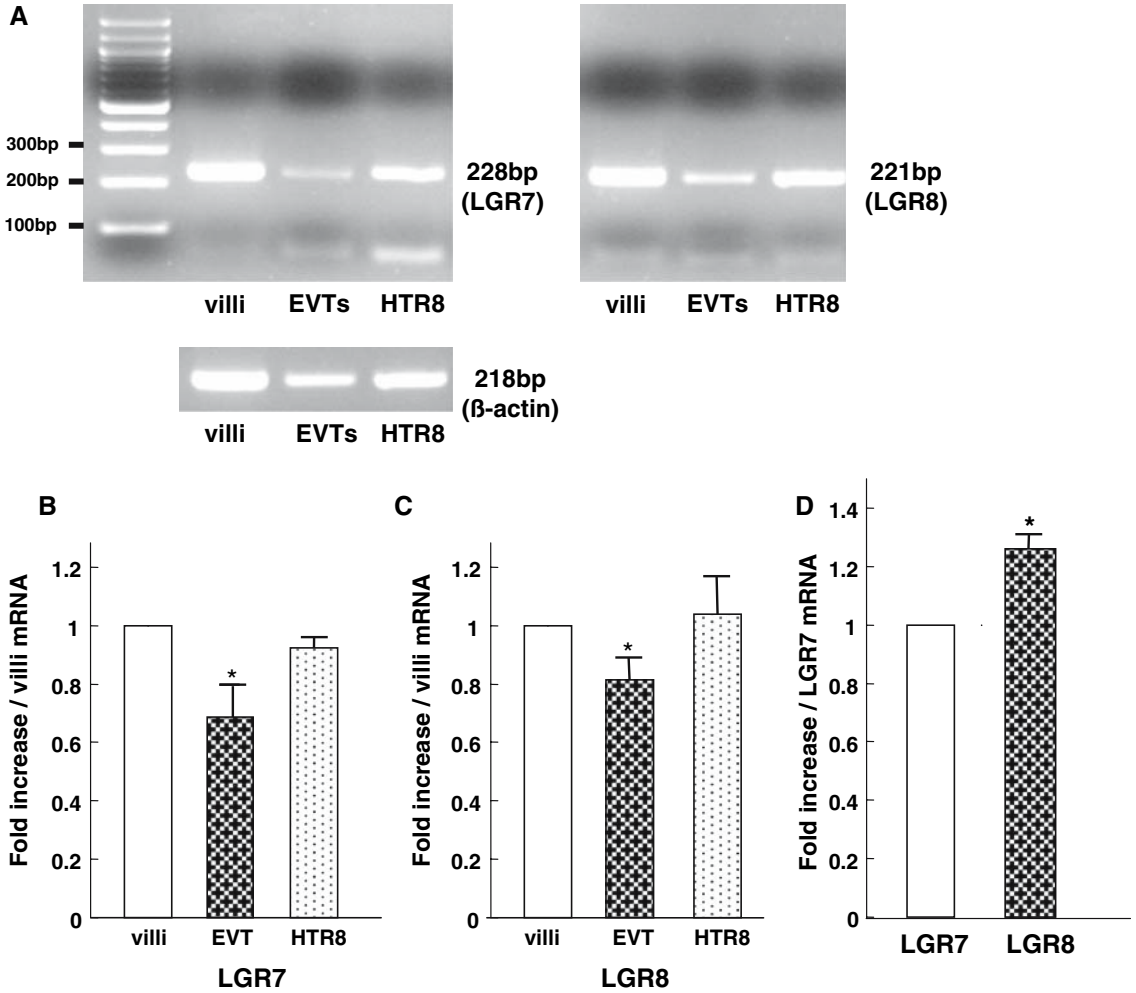

Fig. 1 LGR7 and LGR8 mRNA expressions in chorionic villi, cultured early placental EVTs and HTR-8/SV neo cells. The RT-PCR products for LGR7 (228 bp), for LGR8 (221 bp) and for β-actin (218 bp) are shown in each lane (**A**). Densitometric values of LGR7 mRNA (**B**) and LGR8 mRNA (**C**) in chorionic villi, early placental EVTs and HTR-8/SV neo cells were expressed relative to the abundance of β-actin mRNA. Data were presented as the fold increases over the average intensity of chorionic villi LGR7 mRNA (**B**) and LGR8 mRNA (**C**). LGR7 mRNA and LGR8 mRNA expression in the early placental EVTs were significantly lower compared to those in chorionic villi. Data represent the mean ±SD of three independent experiments. *$P < 0.05$ vs. LGR7 mRNA (**B**) and LGR8 mRNA (**C**) in chorionic villi. Furthermore, densitometric values of LGR8 mRNA in early placental EVTs were expressed relative to the abundance of LGR7 mRNA (**D**). LGR8 mRNA expression in the early placental EVTs was significantly higher compared to LGR7 mRNA in those cells. Data represent the mean ± SD of three independent experiments. *$P < 0.05$ vs. LGR7 mRNA in early placental EVTs

more abundant compared with those in cultured early placental EVTs. Furthermore, in cultured early placental EVTs, densitometric values of LGR8 mRNA were significantly higher compared to those of LGR7 mRNA.

Relaxin is a positive regulator of MMP-1 and MMP-3 expression and inhibits TIMP-1 expression in fibroblasts in the human lower uterine segment [16, 17]. Although there are several studies on relaxin as a positive regulator of MMPs in term placenta [3, 17, 18], no data have been described regarding the possible interaction between relaxin and MMPs in early placenta. The present study is the first to describe the effects of rH2 relaxin on the expression of MMP-2, -3, -9 mRNAs and TIMP-1 mRNA in early placental EVTs cultured in vitro.

In the early stage of pregnancy, EVTs detach from the basement membrane form cell columns and invade the endometrial stroma deeply up to one-third of the uterine myometrial thickness [19]. Around the invasive trophoblasts, the extracellular matrix shows a mosaic pattern, including different components such as collagen IV, laminin, and heparin sulfate [13]. Various steps in trophoblast invasion include the attachment to the basement membrane, the detachment from the basement membrane matrix and the breakdown of basement membrane components,

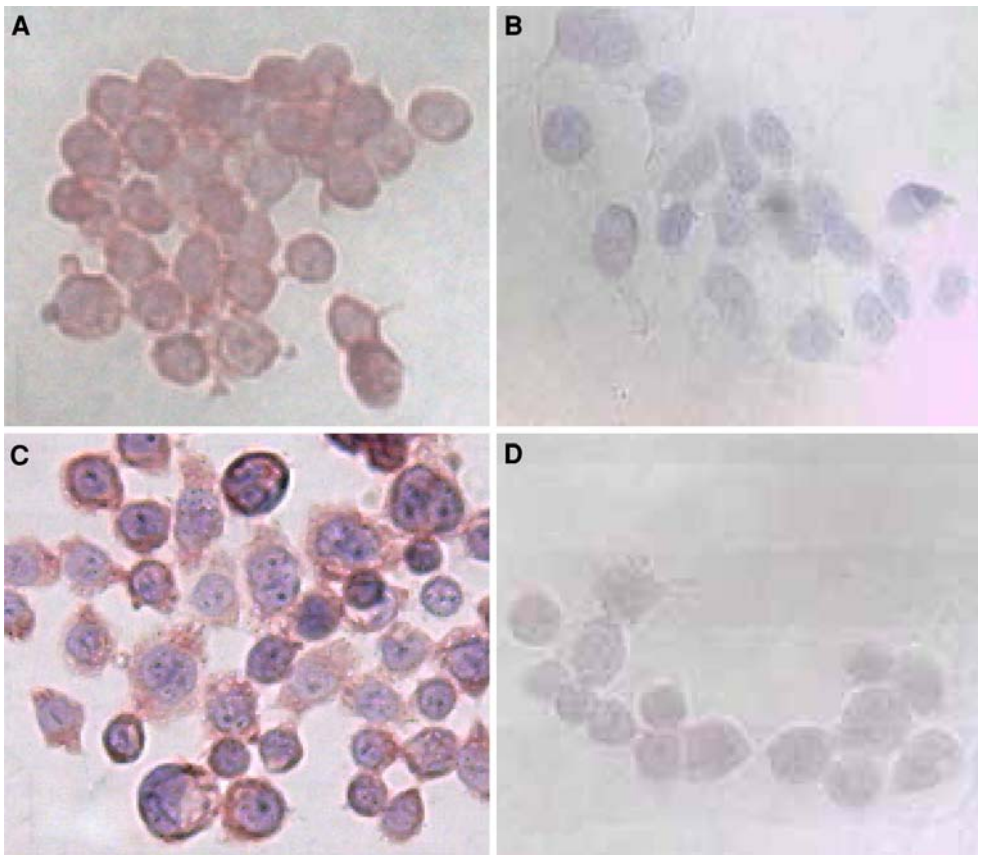

Fig. 2 Immunocytochemical analysis of LGR7 (**A**) and LGR8 (**C**) in cultured early placental EVTs attached to FN-precoated dishes after 48-h subculture. Strong immunostaining of LGR7 (**A**) and LGR8 (**C**) was detected in cultured early placental EVTs. The replacement of the primary antibody with blocking peptide resulted in lack of positive immunostaining of LGR7 (**B**) and LGR8 (**D**). Original magnification, 400×

and these steps are mediated by MMPs and serine proteases [20]. MMPs are still growing family of enzymes known to be involved in the degradation of extracellular matrix. This MMP family can be classified into four groups according to the substrate specificity and localization [21]: gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, -8, and -13), stromelysins (MMP-3, -7, -10, -11, and -12) and MMPs containing a transmembrane domain near their carboxyl terminus (MMP-14, -15, -16, and -17). The activity of MMPs is tightly regulated by several inhibitors, known as TIMP-1 to -4 [13, 21–23]. Of the multiple MMPs produced by the human placenta, MMP-2 and MMP-9 expressed primarily by EVTs have been assigned key roles in promoting the invasive capacity of cytotrophoblasts, because they degrade native collagen IV around the invasive trophoblasts [13, 24]. In the present study, our attention was paid to MMP-2, -3, and -9, because it is known that these three MMPs can degrade collagen IV which is abundant in the interstitial part of the decidua. Moreover, the levels of MMP-9 expression before eighth weeks of pregnancy are much lower as compared with MMP-2 levels, suggesting that MMP-2, rather than MMP-9, plays an important role in the stage of human embryo implantation. After the eighth weeks of pregnancy, the levels of MMP-9 expression increase with the development of the placenta during the first trimester of pregnancy, while the levels of MMP-2 expression decline during the same period. The levels of MMP-9 expression coincided with the invasive potential of EVTs, suggesting that MMP-9 expression can be involved in the positive regulation of the invasiveness of those cells [25–27]. The production of these two MMPs by the placenta is down-regulated during the third trimester of pregnancy, paralleling the decline in trophoblast invasiveness associated with the advance of gestational age [25, 27, 28]. On the other hand, Pro-MMP-3 is involved in the proteolytic activation of pro-MMP-2 and pro-MMP-9 that are produced by trophoblast cells, and able to degrade fibronectin, laminin, and type IV collagen [29]. The activity of MMPs is regulated by TIMP-1 which can inactivate all MMPs in activated form compared with other TIMPs [22]. In the present study, we demonstrated that rH2 relaxin up-regulated both MMP-2 and -9 mRNA expression, down-regulated the TIMP-1 mRNA expression and did not regulate MMP3 mRNA expression in the cultured early placental EVTs.

Regulation of the expression of MMPs in early placental EVTs may be mediated in a paracrine manner by endometrial factors and in an autocrine manner by trophoblastic factors. Endometrial factors are leukemia inhibitory factor, tumor necrosis factor, transforming growth factor beta, interleukin-1, -6 and insulin-like growth factor binding protein-1 [30], while trophoblastic factors are hCG and

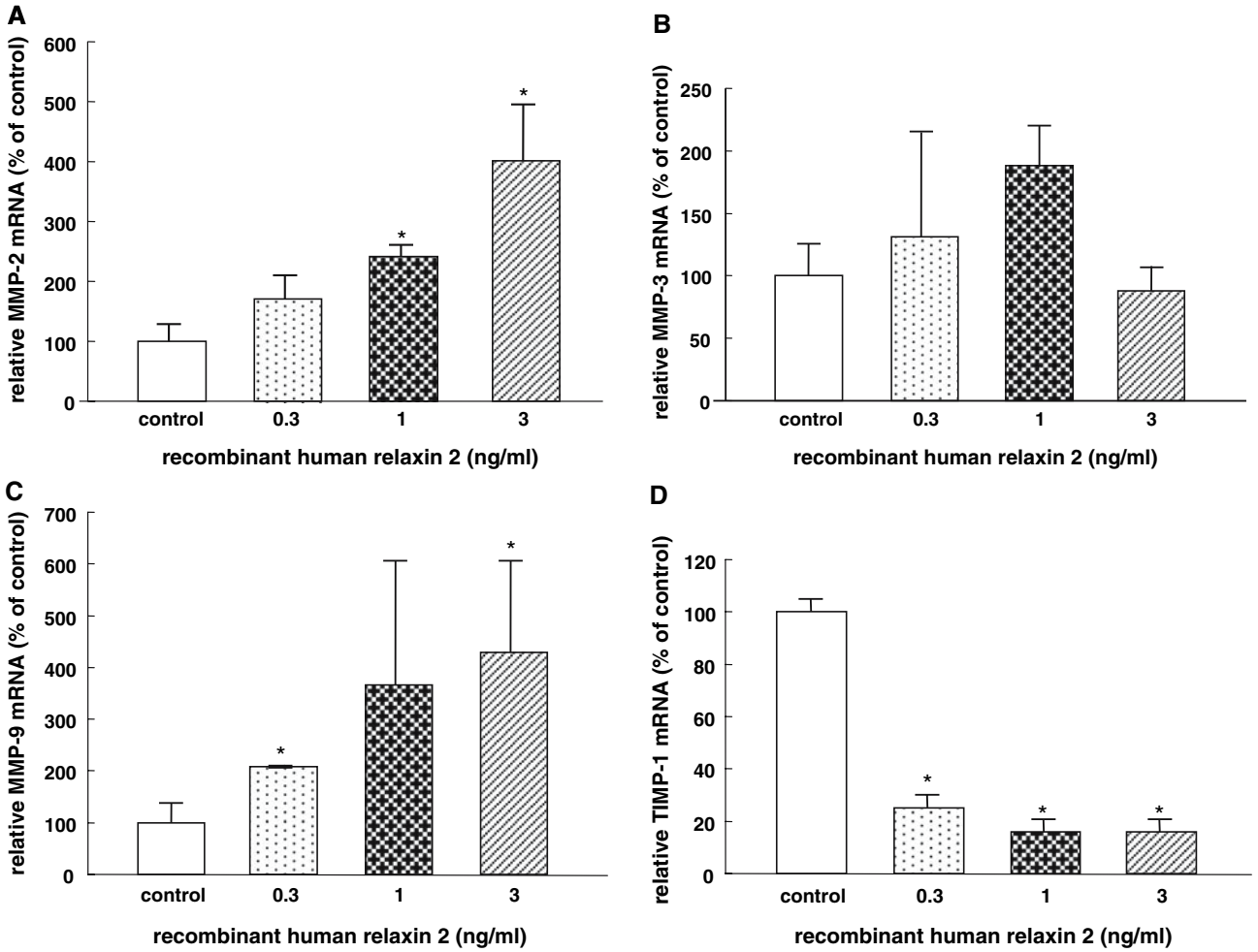

Fig. 3 Effect of exogenous rH2 relaxin on the expression of MMP-2, -3, -9 and TIMP1 mRNA in cultured early placental EVTs. The amount of MMP-2, -3, -9 and TIMP1 mRNA was extrapolated from a standard curve by real-time quantitative RT-PCR, and the results are expressed as a ratio of the MMP-2 (**A**), -3 (**B**), -9 (**C**), and TIMP1 (**D**) mRNA compared to the GAPDH mRNA. Values from untreated cultures were set at 100, and data from relaxin-treated cultures were expressed as a percentage of the untreated control. Data represent the mean ± SD of three independent experiments. $^*P < 0.05$ vs. untreated control cultures

leptin [30]. All these factors are reported to affect the secretion and activation of MMP-2 and MMP-9 in trophoblasts [30]. On the other hand, relaxin produced in the decidua [3] may affect the regulation of MMP-2, MMP-9, and TIMP-1 in early placental EVTs in a paracrine manner, whereas relaxin produced in EVTs as reported previously [1] may exert the effects on EVTs in an autocrine manner. Our previous report have suggested thyroid hormone (T3) play a vital role in up-regulating the invasive potential of EVTs in an endocrine manner through increasing the level of MMPs expression in cultured early placental EVTs [31]. In a similar manner, relaxin produced by the corpus luteum may regulate MMP-2, -9 and TIMP-1 mRNA expression in early placental EVTs in an endocrine manner. Since MMP-2 and MMP-9 have been shown to participate in the positive regulation of the invasive potential of cultured EVTs into the matrigel [24, 32], the results obtained in the present study suggest that rH2 relaxin may promote the invasive potential of early placental EVTs by up-regulating MMP-2 and -9 expression and down-regulating TIMP-1 expression through the interaction with relaxin receptors (LGR7 and LGR8) in those cells.

Materials and methods

Clinical material

Normal early placental tissues were obtained from 32 patients who underwent elective abortion at 5–8 weeks of gestation for psychosocial reasons. The gestational age of the placenta was determined by estimating the duration of pregnancy from the date of the patient's last menstrual period and by ultrasound examination. Informed consent

was obtained from each patient before surgical intervention for use of placental tissues for the present study. The study was approved by the institutional review board.

Cell culture

Trophoblasts differentiating into EVTs were isolated and purified according to the techniques using the enzymatic digestion of anchoring chorionic villi, with the use of human fibronectin (FN)-precoated culture dishes, as described previously in our laboratory [31]. Trophoblasts from the cell column were directly accessible to enzymatic digestion and were released from the tissues in aggregates, whereas the isolation of villous cytotrophoblasts required more intensive enzymatic digestion of chorionic villi [31]. Purified FN 10 μg/ml (ICN Biomedicals, Aurora, OH) was incubated at 37°C for 1 h in 5% CO_2, and then 0.5 ml FN was placed into the dishes. Chorionic villi was incubated in PBS containing 0.125% trypsin (Sigma–Aldrich Corp., Tokyo, Japan), 4.2 mM $MgSO_4$, 25 mM HEPES, and 50 Kunitz unit/ml deoxyribonuclease type IV (Sigma–Chemie, Saint-Quentin, Fallavier, France) at 37°C for 15 min without agitation. After tissue sedimentation, the supernatant was filtered (100 μm pore size). The collected cells were sedimented twice with PBS. Trypsin digestion was stopped with 5% FBS (BioWhittaker, Walkersville, MD). The cells obtained were centrifuged at 300 *g* for 10 min, diluted to a concentration of 5 to 6 × 10^5 cells/2 ml, and then plated on FN-precoated, six-well, 35-mm culture dishes (BD Biosciences, Oxnard, CA). The cells were maintained in bicarbonate-buffered RPMI Medium 1640 (Invitrogen Life Technologies, Grand Island, NY), supplemented with 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and incubated in 5% CO_2 at 37°C. For 3 h, the cells were washed three times and were cultured for 48 h at 37°C in 5% CO_2 in bicarbonate-buffered RPMI Medium 1640 supplemented with 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After 48 h subcultures, the cells attached to FN-precoated dishes were characterized by immunocytochemical analyses of cytokeratin-7 (CK7), human placental lactogen (hPL), ErbB1, and ErbB2 to identify the trophoblastic origin of the cells [31]. The cells were also characterized by RT-PCR analyses of ErbB1 and ErbB2 after 48-h subcultures. After 48 h subcultures, more than 99% of the cultured cells adherent to FN-precoated dishes expressed CK7, hPL, and ErbB2 protein, but not ErbB1 protein, indicating the characteristic features of EVTs (data not shown). In those cells, the expression of LGR7 and LGR8 was examined by RT-PCR and immunocytochemical analysis. Thereafter, the cultured cells were stepped down to FBS-free condition by incubating in RPMI Medium 1640 supplemented with 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cultured cells were treated for the subsequent 24 h in the absence or presence of the different doses (0.3–3 ng/ml) of rH2 relaxin (kindly provided by Dr. Elaine Unemori, CA). After 24 h of subsequent cultures, the expression of MMP-2, -3, -9 and TIMP-1 in cultured EVTs was examined by real-time quantitative RT-PCR.

The HTR-8/SV neo trophoblast cells of human EVT cell line were kindly provided by Dr. Benjamin K. Tsang, University of Ottawa, Ottawa, Canada. The cells were cultured in bicarbonate-buffered RPMI Medium 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were seeded into six-well, 35-mm culture dishes. The cells were incubated in 5% CO_2 at 37°C. After 48 h subcultures, the expression of LGR7 and LGR8 in those cells was examined by RT-PCR.

Total RNA extraction and cDNA synthesis

After 24 h of subsequent cultures in the absence or presence of rH2 relaxin, total RNA was isolated from cultured EVTs using RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA of chorionic villi was also isolated. First strand cDNA was synthesized from 4 μg total RNA using a Transcriptor 1st Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Total RNA extraction and cDNA synthesis from HTR-8/SV neo cells after 48 h of subcultures were performed in the same procedure as described above.

RT-PCR

PCR was performed using 0.1 μg cDNA as a template in a 25-μl reaction buffer [10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100] containing 1.0 pM of each primer, 2.5 mM deoxy-NTPs, and 2.5 U Taq DNA polymerase (Promega Corp., Madison, WI). Reactions were amplified by a Gene Amp PCR System 9600-R (Perkin-Elmer, Norwalk, CT) using the following thermal profile: initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final elongation of 72°C for 5 min. The specific oligonucleotide primers designed to amplify the sequences of LGR7, LGR8, and β-actin. LGR7 forward primer was 5′-GGTATTAATTTGGCCGCATTTATCATCA-3′ and reverse primer was 5′-TATGGTACCTGGTATTTCTACC TGAAGC-3′. LGR8 forward primer was 5′-TGCACAGA GAGCACAGCAGAATGGCTC-3′ and reverse primer was

5′-GGACAGTGCAACCCGATGTGAAAGACC-3′. β-actin forward primer was 5′-AAGAGAGGCATCCTCACC CT-3′ and reverse primer was 5′-TACATGGCTGGGGTG TTGAA-3′. 10 μl of the reaction mixture was electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide. Distilled water was used as a negative control for all of the reactions in place of the cDNA (data not shown). The resultant PCR products were cloned into TA cloning vector (Invitrogen, San Diego, CA) and sequenced (Bio Matrix Research, INC, Chiba, Japan). The sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information. The signals of specific bands of LGR7 and LGR8 in chorionic villi, cultured early placental EVTs and HTR-8/SV neo cells were scanned and determined by dividing the signal intensity compared with the corresponding β-actin to correct for any loading difference between lanes. The intensity of the bands was quantified by Spot Densitometry Software, Image J [33].

Immunocytochemical analysis

Immunocytochemical staining was performed using the avidin–biotin immunoperoxidase method with the use of Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Briefly, cultured cells attached to the FN-precoated chamber slides were washed three times with PBS at room temperature and were fixed in 1.0% formaldehyde for 10 min. The fixed cells were preincubated with 3% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity, and then blocked with 5% blocking serum in PBS for 30 min to saturate nonspecific binding sites. The primary antibodies against LGR7 and LGR8 were diluted with PBS containing 5% blocking serum. After the fixed cells were incubated overnight at 4°C and washed three times for 20 min with PBS, the cells were incubated with biotinylated polyvalent antibody, and thereafter, chromogenic reaction was developed by incubation with AEC (Nichirei, Tokyo, Japan). Negative controls were performed by substituting the primary antibody with specific peptides (Phoenix Pharmaceuticals, Belmont, CA). The cells were counterstained with Harris hematoxylin, and mounted with glycerol vinyl alcohol (Nichirei, Tokyo, Japan). The primary antibodies used were the specific antibodies against LGR7 and LGR8 (Phoenix Pharmaceuticals, Belmont, CA).

Real-time quantitative RT-PCR

After 24 h of subsequent cultures in the absence or presence of rH2 relaxin, total RNA was isolated and synthesized cDNA was obtained from cultured EVTs. The ratio of MMP mRNA to GAPDH mRNA copy number was measured with real-time quantitative RT-PCR using a LightCycler™ system and SYBR green I dye (Roche Molecular Systems, Indianapolis, IN). The parameter specific commercial primer sets of MMP-2, -3 -9 and TIMP-1 primers and their standard cDNAs were purchased from Search-LC (Heidelberg, Germany).

The reaction mixture contained 2 μl DNA Master SYBR Green I, 2 μg of GAPDH or MMP-2, -3, -9 and TIMP-1 LightCycler™-Primer Set (Search-LC, Heidelberg, Germany), and 10 μl cDNA with dilution at 1:500. The final volume was adjusted to 20 μl with H_2O. The PCR conditions were programmed according to the primer supplier's instructions. Fluorescent products were measured by a single acquisition mode after each cycle. To distinguish specific products from non-specific products and primer dimers, a melting curve was obtained.

Statistical analysis

Results were expressed as the mean ± SD of three independent experiments. Statistical analyses were carried out by one-way ANOVA and post hoc Student's *t*-test. A difference with a $P < 0.05$ was considered statistically significant.

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