Posttranslational modifications of decidual IGFBP-1 by steroid hormones in vitro

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Insulin-like growth factor binding protein-1 (IGFBP-1) appears to regulate insulin-like growth factors (IGFs; IGF-I and IGF-II) biological activity within the local environment of human placenta by modulating IGFs interaction with their receptors. Considering that posttranslational modifications of IGFBP-1 such as phosphorylation and proteolysis affect its affinity for IGFs, this study was undertaken to identify the role of estrogen and progesterone in this regard. The conditioned media of steroid hormone-treated decidual cells were evaluated using different approaches using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and non-denaturing PAGE following immunoblotting as well as zymographies that contained gelatin and IGFBP-1 as substrates. Our results demonstrated that medroxy progesterone acetate (MPA) treatment increased both phosphorylation and non-denaturing PAGE following immunoblotting as well as zymographies that contained gelatin and IGFBP-1 as substrates. The results of the zymography revealed that steroid hormones regulated the activity of decidual-secreted matrix metalloproteinases (MMP)-2 and -9, in which E2 treatment up-regulated the MMP-9 activity. Finally, it was demonstrated in our study that decidual-secreted MMP-9 was capable of degrading human amniotic fluid-derived IGFBP-1. In conclusion, our data support the hypothesis that steroid hormones can modulate the function of IGFBP-1 in the interaction of IGF system activities at the embryo–maternal interface. The results of this study suggest that changes in the post-translational modifications of IGFBP-1, such as phosphorylation and proteolysis, may play a central role in the modulation of IGF system activities at the embryo–maternal interface.

Key words: decidua/IGFBP-1/phosphorylation/proteolysis/steroid hormones

Introduction

Insulin-like growth factors (IGF-I and IGF-II), their receptors (IGF-Rs) and IGF-binding proteins (IGFBPs) have vital roles in the regulation of proliferation, differentiation, migration, survival and specific functions of many cell types (Jones and Clemmons, 1995). Beside prolonging the half-life of IGFs, IGFBPs modulate IGF activities and bioavailabilities. This is due to the higher binding affinities of IGFBPs compared with IGF-Rs (Clemmons, 1997). IGFBP-1, which is a major constituent of human amniotic fluid and one of the most important decidual secretory products that important roles at the embryo–maternal interface in the regulation of placental development, embryo implantation and fetal growth. After conception, the content of decidual-derived IGFBP-1 of amniotic fluid increases from less than 1–150 μg/ml (Crossey et al., 2002). Furthermore, the concentrations of both free and complex forms of IGFBP-1 in maternal serum show a rapid increase, up to 25-fold from early to term pregnancy (Skjaerbaek et al., 2004). More interestingly, the phosphorylation profile of IGFBP-1 changes during pregnancy as well (Westwood et al., 1994). Currently, the function of this extraordinarily high level of IGFBP-1 during pregnancy is under vigorous investigation.

IGFBP-1 undergoes some forms of posttranslational modifications such as phosphorylation, glycosylation, proteolysis and polymerization, which regulate its biological functions (Jones and Clemmons, 1995; Yu et al., 1998; Sakai et al., 2001a,b). Generally, IGFBP-1 isoforms differ in their degree of phosphorylation, which is an important mechanism of an acute and reversible regulation of its function. The highly phosphorylated forms of human IGFBP-1 (pIGFBP-1) were shown to have a seven-fold higher affinity for IGF-I compared to that of the non-phosphorylated form (npIGFBP-1) (Clemmons, 1997; Westwood et al., 1997). On the other hand, proteolysis and polymerization of IGFBP-1 result in the formation of stable, lower molecular weight fragments and multimer of IGFBPs, which have either retained or lost affinity for IGFs (Gibson et al., 2001; Sakai et al., 2001a). Indeed, IGFBP proteolysis may be the primary means of exerting acute and focal control over IGF bioavailability, either inhibiting or enhancing the IGF activity in cells. In spite of the significant inroads into the detection of IGFBP proteases, their exact identity remains largely unknown.

Apparently, steroid hormones modulate several developmental, physiological and pathological events of the reproduction system in a different fashion (Lentz, 1994; Rose, 1996; Dunn et al., 2003). Meanwhile, growth factors, their receptors and their binding proteins play a central role in mediating the effects of steroid hormones; among which the role of the IGF system has been described in the literature (Badinga et al., 1999; Gielen et al., 2005). To our knowledge, no information is available concerning the effects of steroid hormones on the posttranslational modifications of IGFBP-1 with respect to the control of its biological functions. Thus, this study was designed to elucidate the effects of estrogen and progesterone on the phosphorylation alteration of decidual-secreted IGFBP-1 as well as the modification of decidual-derived IGFBP-1-degrading proteinase(s), as important regulators of IGF/IGFBP-1 interactions at the embryo–maternal interface.
Materials and methods

Isolation and culture of decidual cells

Human first trimester decidua parietalis at 8–9 weeks of gestation were collected from 24 female patients, ages between 25 and 36 years (median 32) who gave informed consent for collection and investigational use of tissues and had non-complicated pregnancies and underwent elective surgical termination of pregnancy. Ethical approval for this study was granted by the Ethics Committee of Kyorin University, School of Medicine, Tokyo, Japan. Specimens were transported immediately in ice-cold medium 199 (Gibco, Grand Island, NY, USA) containing 25 mM HEPES (Sigma Chemical, St. Louis, MO, USA) and 1% antibiotic-antimycotic mixture. After blood clots were manually removed, the decidual tissue was rinsed thoroughly in ice-cold medium 199. Then, tissue was minced into small pieces and treated to disperse the cells enzymatically. Isolation of decidual cells was performed as described elsewhere (Braverman et al., 1984), with minor modifications. In brief, the tissue was treated with 0.1% collagenase (type IA; Sigma) and 0.1% hyaluronidase (type IS; Sigma) in Ca2+- and Mg2+-free phosphate-buffered saline (PBS), while stirring at 37°C for 1 h. Then, the cell suspension was filtered through a nylon mesh with a pore size of 105 μm (Millipore, Eschborn, Germany), followed by a 40-μm nylon sieve (Becton Dickinson, San Diego, CA, USA) to remove undigested tissue debris and glandular elements, respectively. Cells were collected from the filters by rinsing and centrifugation at 8000 × g for 10 min. Then, decidual cells were washed and suspended three times and finally resuspended in medium 199 supplemented with 10% heat-inactivated and dextran-coated-treated, steroid-free fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml) (Gibco, Grand Island, NY, USA). Aliquots of decidual cell suspensions were counted by the dye exclusion test using 0.4% (vol/vol) trypan blue in PBS. The dye exclusion test showed that more than 90% of cells were viable. The decidual cells were plated at 5 × 105 cells/ml in a 35 × 10 mm plastic Petri dish (Falcon 3001, Becton Dickinson, Lincoln Park, NJ, USA). The culture medium was changed every 48 h, and cultures were maintained in a humidified 95% air/5% CO2 at 37°C, until sub-confluent. Homogeneity of the stromal cultures was verified by immunocytochemical localization of cell-specific markers, including vimentin for stroma, cytokeratin for epithelium, muscle actin for vascular smooth muscle, and intermediate filaments of factor VIII antigen for endothelium (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The contamination with endometrial epithelial or vascular cells was less than 0.1% (data not shown). Decidual cells were cultured for 5–7 days, followed by exposure to 17β-estradiol (E2) 10–8 M, and/or medroxy progesterone acetate (MPA) 10−5 M (Sigma) in a serum-free medium 199. After 48 h incubation with the designed treatments, the conditioned media (supernatants) were centrifuged at 1200 × g for 5 min to remove undesired cellular components and were concentrated using centricron-10 (Amicon Bioseparations, Millipore Corporation, Bedford, MA, USA). Then, concentrated conditioned media were immediately snap frozen in liquid nitrogen and kept at −80°C for further studies. Negative controls were obtained from conditioned media of the decidual cultures that were treated with the same volume of drug solvent, without E2 and/or MPA treatment.

Immunoblot

Concentrated conditioned media were dissolved in the lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, and 50 mM HEPES, pH 7.5, containing complete protease inhibitor). The insoluble materials were removed by centrifugation at 15 000 × g for 10 min. Then, protein concentration was measured by a protein detection kit (BioRad, Munich, Germany) according to the manufacturer and the samples were adjusted to the same amount of final protein concentrations per milliliter of supernatant. Then, supernatant was incubated overnight at 4°C with 2 μg/ml anti-MMP9 polyclonal antibody. The immunocomplexes were incubated with protein-A sepharose (Sigma) at 4°C for 2 h and immobilized complexes were pelleted by centrifugation at 10 000 × g for 1 min, washed three times with lysis buffer and were resuspended in 20 μl of Laemmli sample buffer under reducing conditions. Then, the same amount of immunoprecipitated proteins were subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under the reducing conditions and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes with 0.45-μm pore size (BioRad, Hercules, CA, USA). After blocking with Tris buffer saline (10mM Tris, 140 mM NaCl, pH 7.4) containing 3% bovine serum albumin (BSA) for 2 h at room temperature, blots were exposed to a primary antibody (monoclonal anti-MMP-9 IgG) overnight at 4°C. The secondary horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was applied for 1 h at room temperature, followed by detection with the enhanced chemiluminescence (ECL) system (Amersham, Tokyo, Japan) and exposure to a Kodak X-AR film (Eastman Kodak, Rochester, NY, USA) for 1–15 min.

To detect IGFBP-1 phosphoforms, a non-denaturing PAGE was performed, as it was described previously (Iwashita et al., 1998; Sakai et al., 2001a). Briefly, the samples were mixed with an equal volume of sample buffer (125 mM Tris–HCl, 20 mM 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 20% glycerol and 0.01% bromophenol blue, pH 6.8) and loaded onto a discontinuous polyacrylamide gel using pH 6.8 in the stacking gel and pH 7.5 in the resolving gel. Both gels contained 10 mM CHAPS, and the electrode buffer (pH 8.2) contained 1 mM CHAPS. The proteins were transferred onto a PVDF membrane, and the membranes were probed with a polyclonal rabbit anti-IGFBP-1 antibody (a gift from Dr. Clemmons, University of North Carolina at Chapel Hill) and visualized using an alkaline phosphatase-conjugated anti-rabbit IgG and a phosphatase-dependent colour development system, as previously described (Sakai et al., 2001a). In another set of experiments, to distinguish between the pIGFBP-1 and npIGFBP-1 in the concentrated conditioned media of decidual cells, samples were pretreated by incubation with or without 1 U alkaline phosphatase (Sigma) for 2 h at 37°C and were then subjected to the non-denaturing PAGE and immunoblotting, as described previously for human plasma (Westwood et al., 1997). The conditioned media of untreated cells were used as control and this experiment was repeated three times.

Zymography

Gelatinase activity in media of the steroid-treated decidual cells was detected by zymography using methods described previously, with minor modifications (Radowanowicz et al., 1994). SDS–polyacrylamide (7.5%) gels containing 1 mg/ml gelatin (type A from porcine skin; Sigma) were used and an aliquot of concentrated frozen conditioned media from different samples, containing the material released by decidual cells during 48 h, was solubilized in Laemmli sample buffer under non-reducing conditions. None of the samples were heated and all were stored on ice until they were applied to the substrate gels. An aliquot of the material extracts was removed for protein analysis, after which the samples were adjusted to the same amount of final protein concentration. Routinely, 60 μl was applied to each lane of a polyacrylamide gel. After electrophoresis the SDS was removed by twice incubating the gel for 30 min in washing buffer (2.5% Triton X-100). Then, gels were incubated in zymography digestion buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl2, 1 μM ZnCl2, 0.02% Brij 35, pH 7.6) for 16 h at 37°C. Gels were then stained (0.5% Coomassie blue R250 in 30% methanol and 10% glacial acetic acid) at room temperature for 3 h and de-stained in staining solution without Coomassie blue until the cleared bands were visible, where gelatinase activity had hydrolysed the substrate and the stacking gel was totally de-stained. The specificity of gelatinase activity as matrix metalloproteinases (MMPs) was determined by affecting the assays of inhibitors on the metalloproteinase activity, including EDTA (5 mM), a chelating agent, and tissue inhibitors of metalloproteinase-1 (TIMP-1) (Sigma) in the zymography digestion buffer, in which the gels were incubated overnight. Cell-associated and secreted proteinases from the eight different preparations of decidual cells for each experimental group were analysed in this study.

IGFBP-1 substrate zymography was performed similarly to that described for the above-mentioned gelatin substrate in gel electrophoresis, but by adding human IGFBP-1 (100 μg/ml) (Calbiochem Oncogene Research Products, San Diego, MA, USA) instead of gelatin to a 7.5% SDS–polyacrylamide gel solution before gel casting. Only two lanes of the separating portion of a minigel were used as control substrate in gel electrophoresis, but by adding two lanes of the separating portion of a minigel were used as control substrate in gel electrophoresis, but by adding

Results

Regulation of IGFBP-1 by steroid hormones

The results of SDS–PAGE and immunoblotting for total IGFBP-1 demonstrated that the concentrations of the decidual-secreted IGFBP-1...
Steroid hormones regulate decidual IGFBP-1

Detection of IGFBP-1-degrading activity in decidual secretion and its regulation by steroid hormones

In gelatin zymograms of the conditioned media of steroid hormone-treated or untreated decidual cells, a strong enzymatic activity was observed at 92 kDa, corresponding to MMP-9 molecular weight (Figure 2A). Furthermore, it was revealed that the lytic activity of this protease was regulated under steroid hormone treatment, in which E_2 clearly enhanced its activity. The inclusion of EDTA and TIMP-1, as a general metalloproteinase inhibitor, inhibited the lytic activity of this enzyme. Because the addition of EDTA to the incubation buffer abolished the enzymatic activity, it can be concluded that this enzyme belongs to the family of metalloproteinases. The disappearance of the proteolytic fragments by addition of TIMP-1 to the incubation buffer reveals that this enzyme is a member of MMPs. In another complementary experiment, immunoblotting of concentrated conditioned media was performed to identify the results of the above-mentioned enzyme as MMP-9. The result of immunoblotting, in which a specific antibody against MMP-9 was used, characterized this enzyme as MMP-9 (Figure 2B). Furthermore, to evaluate the effect of hormonal treatments on decidual cell-secreted MMP-9, SDS–PAGE and immunoblotting was performed. Our results showed that the amount of the decidual-secreted MMP-9 of the E_2-treated conditioned media was more than its amount in the MPA-treated cells and control (Figure 3B). As MMPs are secreted in a proenzyme form, which needs further activation, the increased mass of MMP-9 could be due to both the active and inactive forms of the enzyme. However, the result of the zymography shows the activated form of the enzyme.

Figure 1. (A) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting of the concentrated conditioned media of decidual cells, which were under no treatment (C), treated with estradiol (E_2) (E) or medroxy progesterone acetate (MPA) (P). These results revealed that MPA treatment increased the amount of decidual-secreted insulin-like growth factor binding protein-1 (IGFBP-1) compared with the untreated cells (lane 2). Lane 1 of panel C demonstrates that the pretreatment of conditioned media of untreated cells with alkaline phosphatase can reduce the phosphorylated isoforms, which have been shown to migrate in the absence of such treatment (lane 2 of panel C).

Figure 2. (A) Gelatin substrate in gel electrophoresis (zymography) of the concentrated conditioned media of decidual cells, which were under no treatment (C), treated with estradiol (E_2) (E) or medroxy progesterone acetate (MPA) (P). A strong proteolytic activity with a molecular weight corresponding to matrix metalloproteinase-9 (MMP-9) is seen in all above-mentioned groups. E_2 treatment clearly enhances the activity of this enzyme. (B) Insulin-like growth factor binding protein-1 (IGFBP-1) substrate in gel electrophoresis of the concentrated conditioned media of E_2-treated decidual cells (lane 1) shows that decidual-secreted MMP-9 has an IGFBP-1-degrading activity. The addition of tissue inhibitors of metalloproteinase-1 (TIMP-1) to the zymography incubation buffer (lane 2) abolished the enzymatic activity of the E_2-treated decidual conditioned media, which was capable of degrading the IGFBP-1 content of the zymography gel. These figures are representatives of triplicate experiments. C, control; E, E_2-treated; P, MPA-treated.
The main new findings of the current studies were the effect of E2 on activity, and finally, the degradation of human amniotic fluid-derived MMP-9. A previous study that showed the production of endometrial IGFBP-1 was therefore hypothesized that they might have a role in the modulation of this system by steroid hormones and the clarification of binding affinity of IGF-IGFBP binary complex. Thus, the interface. Based on the fact that progesterone and/or estrogen play important roles in the control of several interactions at this interface, it was therefore hypothesized that they might have a role in the modification of binding affinity of IGFBP-1. The results of this study revealed that the conditioned media of E2- and MPA-treated decidual cells exhibited a different pattern of IGFBP-1 phosphorylation. Although MPA treatment induced an increase in both pgIGFBP-1 and npIGFBP-1, the treatment of decidual cells with E2 reduced the pgIGFBP-1 isoforms in the extracellular fluid. Because IGFBP-1 appears to be phosphorylated inside the cell and then secreted, this result suggested that in the E2-treated decidual cells, activities of the protein phosphatase for IGFBP-1 might be increased or the protein kinase activities for IGFBP-1 might be reduced. There is convincing evidence to indicate that the estrogen pathway can interact with the insulin/IGF-I pathway and several mechanisms have been proposed to underlie this cross-talk (Dupont et al., 2000; Lai et al., 2001). Because the pgIGFBP-1 isoforms was reported to have a seven-fold higher affinity for IGF-I compared to that of the non-phosphorylated form (Clemmons, 1997), the result of non-denaturing PAGE immunoblotting of this study provides a novel mechanism through which steroid hormones affect IGFs bioavailability by modifying IGFBP-1 phosphovariants.

On the other hand, the results of zymography of this study demonstrated that the conditioned media of decidual cells exhibited an IGFBP-1-degrading activity, which was increased under E2 treatment. In a previous study, the secretion of MMP-9 by human decidual cells has been reported (Edwin et al., 2002), but there was no information with respect to its regulation by steroid hormones. Our findings revealed that E2 treatment increased the activity of decidual-secreted MMP-9. Furthermore, findings of substrate in gel electrophoresis in this study exhibited that decidual-secreted MMP-9 was capable of degrading human IGFBP-1. Because only the npIGFBP-1 isof orm was observed in the non-denaturing PAGE immunoblotting result of this study, it could be presumed that MMP-9 might selectively degrade pgIGFBP-1. Furthermore, because our antibody recognized only an intact IGFBP-1, any fragment of IGFBP-1 was not observed in the result of immunoblotting of the E2-treated conditioned media. Previously, the activity of MMPs as IGFBP-degrading proteases has been described in biological fluids and conditioned media of numerous cell lines (Fowkes et al., 1994; Thraikill et al., 1995). Our result supports a recent study, which reported that recombinant MMP-9 could degrade the decidual-secreted IGFBP-1 in vitro (Coppock et al., 2004). However, they did not examine the effect of steroid hormones in this regard. Because the enzymatic degradation of IGFBP-1 reduces its binding affinity for IGFs (Coppock et al., 2004), we concluded that both decidual-derived and trophoblastic-derived MMP-9 was capable of degrading IGFBP-1 at the embryo–maternal interface. Thus, we propose that the steroid hormones can modulate IGF/IGFBP-1 bioactivities at this interface, partly, through the regulation of IGFBP-1 proteolysis. Considering the notion that IGFBP-1 modulates IGFs effects on trophoblasts at the embryo–maternal interface (Bell et al., 1988; Takeda and Iwashita, 1993) as well as the reported roles of IGF-independent actions of IGFBP-1 in the regulation of trophoblast proliferation and migration (Hamilton et al., 1998; Irwin and Giudice, 1998; Hills et al., 2004), the regulatory effects of steroid hormones on the posttranslational modifications of IGFBP-1 may affect several physiological effects of the IGF/IGFBP-1 system. Based on the fact that the IGF system is involved in several reproductive disorders (Ning et al., 2004; Karpovich et al., 2005), the study of hormonal modulation of this system by steroid hormones and the clarification of

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the involved molecular mechanisms could reveal important clues that may be of clinical relevance in the future.

In conclusion, our findings demonstrated novel mechanisms of posttranslational modifications of IGFBP-1 under hormonal control. E2 was exhibited to be capable of stimulating the decidual-derived IGFBP-1-degrading activities and reducing IGFBP-1 phosphorylation, whereas MPA stimulated the production of both pIGFBP-1 and nplIGFBP-1 by these cells.

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References

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