IGF-II regulates metastatic properties of choriocarcinoma cells through the activation of the insulin receptor

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Choriocarcinoma is a highly malignant tumor that can arise from trophoblasts of any type of gestational event but most often from complete hydatidiform mole. IGF-II plays a fundamental role in placental development and may play a role in gestational trophoblastic diseases. Several studies have shown that IGF-II is expressed at high levels in hydatidiform moles and choriocarcinoma tissues; however, conflicting data exist on how IGF-II regulates the behaviour of choriocarcinoma cells. The purpose of this study was to determine the contribution of the receptors for IGF-I and insulin to the actions of IGF-II on the regulation of choriocarcinoma cells metastasis. An Immuno Radio Metric Assay was used to analyse the circulating and tissue levels of IGF-I and IGF-II in 24 cases of hydatidiform mole, two cases of choriocarcinoma and eight cases of spontaneous abortion at the same gestational age. The JEG-3 choriocarcinoma cell line was used to investigate the role of IGF-II in the regulation of cell invasion. We found that mole and choriocarcinoma tissue express high levels of IGF-II compared to first trimester placenta. Both IGF-I and IGF-II regulate choriocarcinoma cell invasion in a dose dependent manner but through a different mechanism. IGF-II effects involve the activation of the InsR while IGF-I uses the IGF-IR. The positive effects of IGF-II on invasion are the result of enhanced cell adhesion and chemotaxis (specifically towards collagen IV). The actions of IGF-II but not those of IGF-I were sensitive to inhibition by the insulin receptor inhibitor HNMPA(AM)³. Our results demonstrate that the insulin receptor regulates choriocarcinoma cell invasion.

Keywords: choriocarcinoma; hydatidiform mole; metastasis; IGF-II; insulin receptor

Introduction

Gestational trophoblastic neoplasias comprise a group of pregnancy-related disorders that include pre-malignant complete and partial hydatidiform mole as well as malignant lesions such as invasive mole, choriocarcinoma, and placental site trophoblastic tumour (PSTT) (Aliteri et al., 2003). Complete moles are diploid and nearly always androgenetic in origin. By contrast, partial moles are triploid, consisting of one maternal and two paternal sets of chromosomes. After uterine evacuation, 10–20% of complete and 0–5% of partial moles undergo malignant change. For complete and, to a lesser extent, partial moles this malignant change includes invasive mole, choriocarcinoma and PSTT (Soper, 2006).

Choriocarcinoma is a highly malignant tumor that can arise from trophoblasts of any type of gestational event but most often from complete hydatidiform mole (Seckl et al., 2000). The pathogenesis of these tumors is poorly understood. Due to the complete absence of maternal genome in complete hydatidiform mole, the function of paternally expressed genes has been studied in relation to choriocarcinoma development (Lustig-Yariv et al., 1997; He et al., 1998; Kim et al., 2003). IGF-II is expressed in many tissues from the paternally derived allele. Several studies have shown that IGF-II is expressed in hydatidiform moles and choriocarcinoma tissues although this expression is not related to paternal imprinting (Kim et al., 2003). Therefore, IGF-II appears to play an important role in the pathogenesis of choriocarcinoma in addition to its well known function in the regulation of normal placenta development (Kim et al., 2003).

IGF-I, IGF-II, the receptors for IGF-I, IGF-II and insulin as well as several IGF binding proteins are produced by trophoblastic cells, establishing a complex network of autocrine and paracrine actions that is yet poorly understood (Han and Carter, 2000; Constancia et al., 2002). Specifically, conflicting reports exist on how IGF-II regulates the behaviour of choriocarcinoma cells (Korner, 1995; Mckinnon et al., 2001). Initial reports implicated the Mannose 6-P/IGF-II receptor in proliferative actions of IGF-II on choriocarcinoma cell lines (Mckinnon et al., 2001) but later reports have clearly demonstrated that this receptor has mainly inhibitory effects on IGF-II actions (Li and Sahagian, 2004). Here, we have used the JEG-3 choriocarcinoma cell line to study the contribution of the receptors for IGF-I and insulin to the actions of IGF-II, specifically on cell adhesion, invasion and proliferation. Our findings indicate that IGF-I and IGF-II use different receptors to regulate the invasive properties of choriocarcinoma cells although they share downstream mechanisms involving ERK and PI3- kinase activation.

Materials and Methods

Subjects and clinical samples

Twenty-four patients (mean age 21.9 ± 7.8 years) with complete hydatidiform mole were diagnosed in gestational weeks eight through 20 with clinical,
histopathology, microsatellite and gonadotropin (hCG) analysis; and two patients diagnosed with choriocarcinoma were included in the study. Placenta from eight patients (mean age 27.4 ± 7.5 years) with non-molar abortion during weeks eight through 18 and confirmed negative for choriocarcinoma or hydatidiform mole were used in the study. All patients were selected from hospitals in Bogotá, Colombia and written consent was obtained. All procedures were approved by the local ethics committee. Blood samples were obtained and serum was separated by centrifugation. Tissues were obtained by curettage and maintained at −70°C until further analysis.

**Materials**

Recombinant human IGF-I was obtained from Genentech (San Francisco, CA), recombinant human II-IGF from Upstate Biotechnology, Inc. (Lake Placid, NY), recombinant human insulin from Sigma Chemicals (St Louis, MO) and recombinant IGFBP-1 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The monoclonal mouse antihuman IGF-I receptor antibody (αR3) and polyclonal antibodies against subunits β-IGF-IR, subunit β-insulin receptor, Akt 1/2, phospho-Akt (Ser 473), Erk 1/2 were purchased from Santa Cruz Biotechnology. A polyclonal goat antihuman α5β1 antibody (MAB1969) (Conforti et al., 1989) was purchased from Chemicon International Inc. (Mississauga, ON). U0126, a specific inhibitor of MEK kinase, was purchased from Promega (Madison, WI). LY294002, a PI3 kinase inhibitor was obtained from Cell Signalling (Beverly, MA). HNMPA(AM1) [Hydroxy-2-naphthalamidophosphonic acid tris acetoxyethyl ester], an inhibitor of insulin receptor (InsR) tyrosine kinase activity (Saperstein et al., 1989), was purchased from Alexis, Biochemicals (Alexis, San Diego, CA). Mouse monoclonal antibody (Clon 4G10) against phosphotyrosine and IGF-II neutralizing antibody S1F2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). A polyclonal rabbit antihuman anti-p-Erk 1/2 (Thr202/Tyr204) was obtained from Cell Signalling Technology (Beverly, MA). Goat antismouse IgG (H + L) was obtained from Southern Biotech (USA).

**Measurement of IGFs**

Total IGF-I, IGF-II and IGFBP-1 concentrations were determined by immunoradiometric assay (IRMA) using a commercial kit DSL (Webster, TX; IGF-I DSL-10-5600, IGF-II DSL-10-2600 and IGFBP-1 DSL-10-7800). Standards and samples were treated in duplicate. One part from tissue or serum was weighed, thawed and homogenized in two parts 0.06 M Tris-hydrochloride (pH 7.2) and 1% supplemented with protease inhibitors and protein concentration was assayed using RIPA buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1%) supplemented with protease inhibitors and protein concentration was determined with BCA Protein Assay Reagent (Pierce, USA). Equal amounts of protein extracts were denatured and separated on a 12% SDS-PAGE gel (Invitrogen Life Technologies, UK) and blotted onto a PVDF membrane (Amersham, Life Science, UK). Membranes were then blocked in a Tris-buffered saline solution with 5% BSA or 5% non-fat dry milk and probed with primary antibody. The membranes were further probed with HRP-conjugated goat anti-mouse/anti-rabbit to visualize the specific band.

**Immunoprecipitation**

Immunoprecipitations of cell extracts were performed by incubating lysates with the indicated antibodies. The reaction mixture was gently rocked at 4°C overnight. Protein G-agarose (Amersham, Life Science, UK) was used to absorb immunocomplexes that were washed extensively with ice-cold RIPA buffer containing 0.1% Triton X-100. The pellet was resuspended in loading buffer, boiled for 5 min, and centrifuged at 14 000 rpm (5 000 g) for 5 min. The supernatant was collected and subjected to western blotting.

**Matrigel invasive assay**

Matrigel invasion assays were performed as essentially described before (Chuan et al., 2006). Matrigel was purchased from Beckton Dickinson (San Jose, CA) and stored at −20°C. 0.2 ml aliquot containing 200 000 cells was seeded on the upper chamber of the Matrigel coated transwell filter. Serum-free DMEM containing IGF-I, IGF-II or IGFBP-1 was added to the lower chamber and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO2. Each assay was carried out in triplicate on at least three different occasions.

**Matrigel adhesion assay**

Matrigel adhesion assay was performed as described (Zhang and Yeh, 2004), with some modifications. JEG-3 cells were suspended in serum-free media. The cell suspension (2.5 × 105 cells in 0.5 ml medium/well) was added to 24 wells pre-coated with Matrigel and allowed to attach by incubation at 37°C for 60 min. Unattached cells were removed by repeated washing with PBS. Attached cells were stained with 0.5% Crystal Violet for 10 min. After washing with water, the stained cells were extracted with 0.25 ml of 10% acetic acid and the absorbance of the dye extract was measured at 570 nm.

**Cell proliferation assays**

Cell proliferation was assessed using the MTT assay (Mosmann, 1983). Measurements were done at the time of seeding (t = 0) and 24 h after treatment. Each plate was scanned and measured in a Spectra Shell microplate reader (SLT, Austria) at 570 nm.

**Cell motility assay**

Cell migration assays were performed using modified Boyden chambers with a 6.5-mm diameter, 10-μm thickness, porous (8.0 μm) polycarbonate membrane separating the two chambers (Transwell®; Costar, Cambridge, MA). Briefly, the lower surface of the membrane was coated with fibronectin, collagen IV, laminin or BSA (20 μg/ml in phosphate-buffered saline, pH 7.4 for 2 h at 37°C). Excess ligand was removed, and the lower chambers were loaded with medium DMEM with IGF-I (10 nM), IGF-II (10 nM), insulin (10 nM) or IGFBP-1 (10 ng/ml) for 60 min. Serum-starved JEG-3 cells were plated on the upper wells of transwell chambers and incubated at 37°C for 24 h. Cells that migrated through the upper surface of the membrane were completely removed; the cells that migrated on the lower surface of the filter were fixed in 4% formaldehyde, stained with hematoxylin and counted under a microscope. Each assay was carried out in triplicate on at least three different occasions. To verify whether IGF-I, IGF-II, insulin or IGFBP-1-stimulated migration of the JEG-3 cells were mediated through IGF-IR, IR or α5β1 integrin activation, the same procedures were performed in JEG-3 cells that were preincubated with αR3 (1 μg/ml), HNMPA(AM1)(50 μM) or MAB1969 (5 μg/ml) for 60 min.

**RNA extraction, cDNA synthesis and RT–PCR**

RNA was extracted using Trizol reagent (Invitrogen). Total RNA (5 μg) from different samples was first treated with DNase I (Promega) and then reverse transcribed using Superscript II (Invitrogen) in a reaction volume of 20 μl. The synthesized cDNA was used to measure the InsR expression by PCR. The reactions were performed in 20 μl with 1 μl of the respective cDNA sample and 0.4 μM primers, Taq DNA polymerase, buffer and nucleotides (Invitrogen). The primers used were: for GAPDH, 5′-GTTGAGGTCTGGAGTCACAAG3′ and 5′-GGTGCAACGCAGCTGGAC3′ and for the InsR, 5′-AACCAAGTGAGTATGAGGAT3′ and 5′-CCGTCCAGAGCAGAATGCT3′.
IGF-II regulation of trophoblast invasion

Results

IGF-II is highly expressed in gestational trophoblastic disease tissue

Trophoblastic cells are known to express IGF-II. Although the steady state mRNA levels of IGF-II have been measured in different trophoblastic diseases (Kim et al., 2003), no information is yet available regarding the protein levels of this growth factor in these tissues. Because we intend to study IGF-II effects on trophoblastic cells, it was important to estimate IGF-II protein concentration within the tissue. We used IRMA to analyse IGF-II and IGF-I levels in choriocarcinoma, complete hydatidiform mole as well as first trimester placenta tissue samples. As shown in Fig. 1, placenta contains an average concentration of 1.38 ± 0.21 nM (0.84 ± 0.23 ng/mg protein) of IGF-I and 2.66 ± 0.63 nM (0.43 ± 0.18 ng/mg protein) of IGF-II. Hydatidiform moles express significantly (P < 0.05) higher levels of IGF-II in comparison with placenta with levels of 7.33 ± 0.58 nM (1.12 ± 0.12 ng/mg protein). In contrast, a lower amount of IGF-I (P < 0.05) was observed in moles as compared to placenta with measured levels of 0.92 ± 0.16 nM (0.29 ± 0.11 ng/mg protein). During this investigation, two choriocarcinoma samples were available for study. The individual levels of IGF-II were 7.95 nM (1.23 ng/mg protein) and 9.37 nM (1.28 ng/mg protein), which place them in the upper range of concentration values measured in the samples. In addition, we observed high IGF-II circulating levels in sera of patients with hydatidiform mole compared to those with spontaneous abortions at the same gestational age (Fig. 1B). In summary, these results indicate that mole and choriocarcinoma tissue contain high levels of IGF-II, sufficient to saturate all known high affinity receptors (Denley et al., 2005).

IGF-II regulates choriocarcinoma cell invasion

We next studied the role of IGF-II in the regulation of choriocarcinoma cell invasion. JEG-3 choriocarcinoma cells were used since they are known to express all known receptors for IGF-I and IGF-II (Ritvos et al., 1988; O’Gorman et al., 1999). In preliminary experiments measuring ERK1/2 phosphorylation, we determined that maximal receptor activation is achieved with 5 nM of both IGF-I and IGF-II, which is in line with the known Kd for these receptors (Denley et al., 2005). The invasive properties of JEG-3 choriocarcinoma cells were measured using a matrigel cell invasion assay. As shown in Fig. 2A, both IGF-I and IGF-II treatment induced JEG-3 matrigel invasion in a dose dependent manner. Maximal effects were obtained with concentrations of 10 nM for both ligands. Similar treatment has little effect on cell proliferation within the time frame analysed (Fig. 2B). Interestingly, despite the fact that receptor saturating concentrations of IGF-I (Kd = 0.2 nM) are used (Denley et al., 2005), concomitant addition of IGF-II further increases cell invasion suggesting that IGF-II uses receptors other than IGF-IR to promote cell invasion (Fig. 2C). We also tested the effects of IGFBP-1 alone or in combination with the type I and type II IGFs. IGFBP-1 is known to be expressed in placenta and is thought to negatively regulate IGF-I activity (Crossey et al., 2002) but also to have IGF-I independent actions through direct interactions with integrin α5β1 receptor (Jones et al., 1993). Treatment with IGFBP-1 alone induced JEG-3 invasion and has an additive effect to the actions of IGF-II. In contrast, no additive effect was observed on the actions of IGF-I which is indicative of a negative interaction between the two growth factors (Crossey et al., 2002).

In order to better understand the nature of IGF-I, IGF-II and IGFBP-1 actions on choriocarcinoma cells, the effects on cell invasion of IGF-IR blocking antibody αIR3 and anti-α5β1 integrin receptor antibody were measured. As shown in Fig. 3A, treatment with αIR3 Ab completely inhibits the effects of IGF-I on cell invasion and has a small although significant effect in the actions of IGF-II. The actions of IGFBP-1 on cell invasion were not affected by treatment with αIR3 Ab. On the other hand, treatment with anti-α5β1 integrin reduces the actions of IGFBP-1 but was inactive towards IGF-I or IGF-II (Fig. 3B). An isotype Ab (IgG) did not influence basal or IGF-I/IGF-II induced cell invasion (Fig. 3C). These results strongly suggest that receptors other than IGF-I receptor or integrin α5β1 are used by IGF-II to promote choriocarcinoma cell invasion.
IGF-II induces choriocarcinoma cell invasion through the activation of the insulin receptor

The InsR, specifically the isoform A, can bind IGF-II with high affinity (Kd = 0.9 nM). Therefore, we wanted to test the effect of IGF-I and IGF-II on JEG-3 cell invasion. Fig. 4A shows that both InsR-A and InsR-B are expressed in placenta tissue. We next used JEG-3 cells to study InsR and IGF-IR activation. As shown in Fig. 4B, IGF-I treatment induced the tyrosine phosphorylation of the IGF-I receptor, an effect that was inhibited by blocking antibody aIR3. No induction of InsR tyrosine phosphorylation by IGF-I was observed. On the other hand, IGF-II treatment induced the InsR tyrosine phosphorylation with no evidence of IGF-I receptor activation. The IGF-IR blocking antibody had no discernible effect on IGF-II induced InsR activation. The IGF-IR blocking antibody had no discernible effect on IGF-II induced InsR activation. HNMPA(AM)3 was able to inhibit IGF-I treatment-induced InsR phosphorylation. This inhibition was observed from 10 to 100 nM. Similar concentrations also inhibited insulin-mediated activation of the InsR. In contrast, HNMPA(AM)3 was able to inhibit IGF-I treatment-induced InsR phosphorylation at only the higher concentration. In summary, both the direct measurement of IGF-II effects on receptor activation as well as the actions of HNMPA(AM)3 demonstrate that

**Figure 2:** Regulation of JEG-3 trophoblastic cell invasion by IGF-I, IGF-II and IGFBP-1. (A) Matrigel invasion assays to measure the treatment effect of increasing concentrations of IGF-I and IGF-II on JEG-3 cell invasion. (B) Effects of IGF-I (10 nM), IGF-II (10 nM), foetal calf serum-FCS (10%) and BSA (2%) on cell proliferation measured by MTT assay after 24 h treatment. (C) Matrigel invasion assays to measure the effects of JEG-3 cells treatments with different combinations of IGF-I (10 nM or 20 nM as indicated), IGF-II (10 nM or 20 nM as indicated) or IGFBP1 (10 ng/ml). In each case the results of at least three independent replicates are shown. Statistical analysis was performed using ANOVA. Different letters indicate statistically significant differences (P < 0.05) between cells treated with a single ligand and those treated with a combination of two growth factors.

**Figure 3:** Effects of IGF-I receptor blocking Ab, aIR-3 (1 μg/ml) (A) and integrin αvβ3 blocking Ab MAB1969 (25 μg/ml) (B) on IGF-I, IGF-II and IGFBP1 induced cell invasion; the effects of non-immune goat IgG (1 μg/ml) on IGF-I and IGF-II induced cell invasion is shown for comparison. (C) In each case the results of at least three independent replicates are shown. Statistical analysis was performed using ANOVA. Different letters indicate statistically significant differences (P < 0.05)
Figure 4: Activation of IGF-IR and InsR by IGF-I, IGF-II and insulin in JEG-3 cells (A) RT–PCR based detection of insulin receptor isoforms A and B expression in placenta from non-molar spontaneous abortions (lines 1–4 and 11), in complete hydatidiform mole (lines 5–10 and 12) and in JEG-3 trophoblastic cell line (line 13). (B) The upper panel shows the tyrosine phosphorylation of the IGF-IR and InsR upon treatment with IGF-I (10 nM) or IGF-II (10 nM) given alone or in combination with either different doses of IR inhibitor HNMPA(AM)3 or the IGF-IR blocking Ab aIR-3 (1 μg/ml). The lower panel shows the tyrosine phosphorylation of the IGF-I receptor and InsR receptor upon treatment with IGF-I (10 nM) or insulin (10 nM) given alone or in combination with either different doses of IR inhibitor HNMPA(AM)3 or the IGFR blocking Ab aIR-3 (1 μg/ml). (C) Effects of IR inhibitor HNMPA(AM)3 on IGF-I (10 nM), IGF-II (10 nM) and insulin (10 nM) induced matrigel cell invasion. Statistical analysis was performed using ANOVA. Different letters indicate statistically significant differences (P < 0.05).
the InsR is the key mediator of IGF-II actions in choriocarcinoma cells. The analysis of cell invasion (Fig. 4C) shows that HNMPA(AM)₃ treatment at concentrations (50 μM) that specifically inhibit IR activation by IGF-II also blocked ligand induced cell invasion. In contrast, IGF-I induced cell invasion was not affected by similar concentrations of HNMPA(AM)₃ although inhibitory effects are evident at 100 μM, parallel to inhibition of IGF-IR activation. As expected, IGF-I induced cell invasion was inhibited by the IGF-IR (αIR3) blocking antibody.

**IGF-II regulates cell invasion through its activation of AKT, ERK pathways and the induction of cell adhesion and motility**

In order to understand the nature of IGF-II actions, we analysed signalling pathways downstream of the IGFFR and IR, which are potentially important in the regulation of cell invasion. The activity of AKT was measured by Western Blot using anti-phospho Ser 473; while active ERK was measured using a specific Ab against Thr202/Tyr204. The results are depicted in Fig. 5. We found that treatment with IGF-II, as well as insulin, IGF-I and IGFBP-1 induced the activation of ERK as well as AKT. Similar to previous findings regarding receptor activation, IGF-IR blocking antibody, αIR3 inhibited IGF-I activation of ERK and AKT but failed to inhibit IGF-II action on these kinases (Fig. 5C). IGF-II stimulatory activity towards ERK and AKT was instead sensitive to inhibition by low concentrations (10 μM) of the IR inhibitor HNMPA(AM)₃. The relevance of ERK and AKT activation in cell invasion was next studied using matrigel invasion assays. Treatment with PI3-kinase inhibitor LY294002 and MEK inhibitor U0126, which reduce the activation of AKT and ERK respectively, resulted in significant inhibition of IGFBPs-induced cell invasion (Fig. 5D).

For cells to invade through a thick layer of matrigel a complex set of biological processes have to take place. This includes the attachment of cells to the matrigel, degradation of extracellular matrix and motility towards specific matrix protein components. In order to understand the nature of IGF-II actions on cell invasion, we measured both cell adherence as well as motility towards three different extracellular matrix proteins: collagen IV, fibrinogen and laminin (Fig. 6). We found that IGF-II treatment induces the attachment of JEG-3 cells to matrigel, an effect that was sensitive to inhibition by HNMPA(AM)₃. Similar effects could be observed when treating with insulin. IGF-I has analogous effects through the activation of the IGF-I receptor. The analysis of cell motility (Fig. 6B) showed that IGF-II treatment resulted in a 3-fold increase in motility towards collagen IV. Similar effects were observed after IGF-I treatment, but in addition, enhanced motility towards fibrinogen and laminin was also observed. Similar to what was observed when analysing cell invasion, IGF-II but not IGF-I effects were sensitive to pharmacological inhibition with the InsR kinase inhibitor HNMPA(AM)₃ used at 50 μM. The above evidence demonstrates qualitative differences in the way IGF-I and IGF-II regulate cell motility.

**Discussion**

This study showed that the circulating and tissue levels of IGF-II are elevated in gestational trophoblastic diseases, specifically complete hydatidiform moles when compared to first trimester placenta from spontaneous abortions. In addition, we have investigated the mechanisms whereby IGF-II regulates the invasive properties of trophoblastic cells. Our results show that IGF-II treatment, like IGF-I, insulin and IGFBP-1, results in enhanced invasion of JEG-3 trophoblastic cells. IGF-II employs a distinct mechanism to initiate its actions on trophoblasts, which differs from that used by IGF-I. IGF-II effects are mainly initiated through the activation of the InsR while IGF-I mostly uses the IGF-IR to initiate signalling. On the other hand, both IGFs share common intracellular mechanisms including the activation of ERK and AKT kinases, which are important for the regulation of cell invasion. The positive effects of IGF-II on invasion are the result of enhanced cell adhesion and chemotaxis (specifically towards collagen IV). In comparison to IGF-I, IGF-II actions were more sensitive to inhibition by the InsR inhibitor HNMPA(AM)₃.

The role of IGF-II in normal placental development is well established (Baker et al., 1993; Louvi et al., 1997). IGF-II is abundantly expressed in human placenta, specifically in extravillous cytotrophoblasts invading into the endometrium (Han and Carter, 2000). Consequently, a role for IGF-II in the regulation of invasive trophoblasts in normal placenta has been proposed. This study showed that the protein expression levels of IGF-I-II are highly elevated in gestational trophoblastic diseases compared to first trimester placenta from spontaneous abortions. In the two cancer tissues we obtained, high levels of IGF-II were also detected. Although, due to the scarcity of tumour cases, no statistical analysis was performed, the concentrations in choriocarcinoma are in the range observed for complete hydatidiform mole. Increased IGF-II protein levels in gestational trophoblastic disease are likely to be explained by enhanced expression of this gene. Previous results have shown that loss of imprinting of IGF-II gene locus occur in 43% of complete hydatidiform moles concomitant to increased mRNA levels of the gene (Kim et al., 2003). Therefore, enhanced local production of IGF-II can potentially contribute to progression of gestational trophoblastic diseases and choriocarcinoma pathology. In addition, increased circulating levels of IGF-II in sera may contribute to the invasive properties of choriocarcinoma cells in distant metastatic sites.

How IGF-II exerts its actions on trophoblastic cells is a matter of great interest. At least two different receptors have been previously proposed to initiate the actions of IGF-II on trophoblastic cells; the IGF-II/Mannose-6 phosphate receptor (Mckinnon et al., 2001) and the IGF-I receptor. Initial findings pointed to the IGF-II/Mannose-6 phosphate receptor based on the use of antibodies against the receptor (Mckinnon et al., 2001), but numerous studies have demonstrated that this receptor has mainly inhibitory actions on IGF-II (Ludwig et al., 1996; O’Gorman et al., 1999, 2002). Other studies have postulated that IGF-II actions are mediated by the IGF-I receptor (Pandini et al., 2002). Here we demonstrate that IGF-II signalling in JEG-3 cells is initiated mainly through the activation of the InsR. Two different lines of evidence support our findings. Firstly, IGF-II treatment of JEG-3 cells mainly induced the tyrosine phosphorylation of the InsR but little effect is observed in IGF-I receptor. In contrast, IGF-I treatment induced the tyrosine phosphorylation of the IGF-I receptor while no effect on InsR activation was observed. Secondly, although both IGFs were able to activate ERK and AKT, IGF-II but not IGF-I effects were inhibited by concentrations of HNMPA(AM)₃ that specifically inhibit the InsR (Fig. 5A). Pharmacological inhibition of the InsR with 50 μM HNMPA(AM)₃ completely blocked IGF-II induced cell invasion and motility towards collagen IV but were ineffective against IGF-I. On the other hand a blocking antibody against the IGF-I receptor has no effect on IGF-II actions in the above described parameters but effectively inhibited all IGF-I actions.

Mouse gene knockout studies first identified the important role of the InsR in mediating the growth promoting effects of IGF-II in early development (Louvi et al., 1997). The InsR exists in two isoforms that originate by alternative splicing of exon 11 in the InsR gene. The two isoforms are identical except 12 amino acids inserted.
upstream of the third last residue of the extracellular alpha subunits of the InsR-B isoform (Moller et al., 1989). The InsR-A isoform can bind insulin and IGF-II with high affinity but binds IGF-I poorly (Frasca et al., 1999; Denley et al., 2004). Therefore, it is likely that the InsR-A is the receptor mediating IGF-II actions on trophoblast cells. Indeed, previous reports show that InsR-A is highly expressed in placenta as compared with normal adult tissues such as liver and muscle, which mainly express InsR-B (Benecke et al., 1992).

Figure 5: Effect of IGF-I, IGF-II or insulin treatment on ERK and AKT activation in JEG-3 cells. Phosphorylation of ERK and AKT kinases upon treatment with IGF-I (10 nM) (A and B), IGF-II (10 nM) (A and C), insulin (10 nM) (B) or IGFBP-1 (C) given alone or in combination with either different doses of (A) the InsR inhibitor, HNMPA(AM)₃, (A and C) the IGF-IR blocking Ab, αIR-3 (1 μg/ml), (C) the MEK inhibitor UO126, (C) the PI3 kinase inhibitor LY94002 and (C) an IGF-II neutralizing antibody S1F2 (10 μg/ml). (D) Effects of MEK inhibitor UO126 and PI3 kinase inhibitor LY294002 on IGF-I, IGF-II induced cell invasion. Statistical analysis was performed using ANOVA. Different letters indicate statistically significant differences (P < 0.05).
An intriguing observation was that despite using similar concentrations of IGF-II and insulin and the latter having slightly higher affinity for InsR-A, IGF-II is significantly more potent than insulin in the promotion of cell invasion and motility. Interestingly, similar findings have been described in SKUT-1 cells in relation to cell invasion (Sciacca et al., 2002). This suggests that different signalling pathways are triggered by IGF-II and insulin despite sharing a common receptor. It is important to notice that moderate (10 nM) concentrations of IGF-II and insulin were used in the experiments, which in the case of IGF-II will preferentially activate

Figure 6: IGF-I, IGF-II and IGFBP-1 regulation of JEG-3 trophoblastic cell adhesion and motility. (A) Effect of IGF-I (10 nM), IGF-II (10 nM) IGFBP-1 (10 ng/ml) and insulin (10 nM) treatment on JEG-3 cells adhesion to matrigel. The effects of combined treatment with IGF-I receptor blocking Ab, αIR-3 (1 μg/ml), the integrin α5β1 blocking Ab, MAB1969 (25 μg/ml) and the InsR kinase inhibitor HNMPA(AM)3. (B) Results from assays measuring the effects of IGF-I (10 nM), IGF-II (10 nM) or insulin (10 nM) and IGFBP-1 (10 ng/ml) on JEG-3 cells motility towards specific extracellular matrix proteins: laminin, collagen IV and fibronectin. BSA was used as control. The effects of combined treatment with IGF-I receptor blocking Ab, αIR-3 (1 μg/ml), the integrin α5β1 blocking Ab, MAB1969 (25 μg/ml) and the InsR kinase inhibitor, HNMPA(AM)3 are also shown. In each case the results of at least three independent replicates are shown. Statistical analysis was performed using ANOVA. Different letters indicate statistical significant differences (P < 0.05)
the InsR-A isoform while insulin will activate both InsR-A and B. It has been long known that the different isoforms of the InsR have distinct signalling properties despite having identical intracellular domains (Sciaccia et al., 2003; Pandini et al., 2004). The reasons for this are not fully understood, although recent findings indicate that both receptors localized to discrete regions in the cell membrane and are differentially internalized in response to insulin (Uhles et al., 2003); thereby suggesting the involvement of additional membrane proteins in signalling. One possibility to explain the enhanced invasive activity of IGF-II is through the activation of hybrid insulin/IGF-I receptors that bind IGF-II but not insulin with high affinity. We do not favour this hypothesis because: (i) we were not able to detect IGF-I receptor activation upon IGF-II treatment and (ii) IGF-II effects on cell invasion were inhibited by HNMPA(AM)₃ used at concentrations that specifically target the InsR. To note is that, IGF-I can also bind hybrid receptors with high affinity, yet we cannot detect increased InsR tyrosine phosphorylation upon IGF-I treatment, again suggesting a minor role for hybrid receptors in signalling. Clearly, the issue of how receptor hybrids contribute to signalling requires further exploration, specially if we keep in mind that the relative effect of HNMPA(AM)₃ on hybrids is not known. Another possibility to explain the different potency of IGF-II and insulin in cell invasion assays is that InsR-A triggers different signals depending on whether it is activated by IGF-II or insulin. Indeed, support for this hypothesis comes from the study of transcriptional actions of IGF-II and insulin in fibroblasts that lack IGF-I receptor and overexpress InsR-A (Pandini et al., 2003). This study showed that although most transcriptional actions of insulin and IGF-II are shared, some ligand specific effects could be detected such as the induction of integrin αVβ3 and adhesion molecule ICAM. Interestingly, IGF-II and IGF-I but not insulin are known to bind vitronectin, a ligand for integrin αVβ3 to promote cell migration (Kricke et al., 2003). Therefore, integrin αVβ3 activation by IGF-II/ vitronectin complex can bear direct relationship to the enhanced effects of IGF-II on JEG-3 cell migration as compared to insulin. Clearly, a better understanding of how the InsR isoforms contribute to IGF-II signalling is required before the different potency of IGF-II and insulin in cell motility assays can be explained.

Despite the differences in signalling, insulin, IGF-I, IGF-II as well as IGFBP-1 have the capacity to promote cell invasion using a core set of signalling intermediares. This is evident by the fact that P13 kinase and MEK inhibitors reduce cell invasion induced by the three ligands. Consequently, in the context of the tissue, trophoblast invasion may be primarily determined by factors that influence the availability of IGFs and its binding proteins. Because we have shown that IGF-II but not IGF-I is overexpressed in placental tissue from complete hydatidiform moles and in corresponding sera, IGF-II is likely to be more influential than IGF-I in the regulation of the invasive properties of trophoblasts from complete moles. Supporting this hypothesis are previous findings showing that extravillous cytotrophoblasts, which have a very high invasive capacity, express the highest amounts of IGF-II in normal placenta while syncytiotrophoblasts express little IGF-II (Han and Carter, 2000). In order to promote cell invasion, IGF-II has to regulate complex cellular mechanisms that control adhesion, migration and extracellular matrix proteolysis. We have shown that IGF-II controls invasion by at least regulating cell adhesion and motility.

Interestingly, IGF-II but also IGF-I and insulin promote motility specifically towards collagen IV but not laminin or fibronectin suggesting a specific regulation of integrin subunits that act as collagen receptors (Denley et al., 2006). In contrast, IGFBP-1 the main secretory product of the decidua also promotes invasion towards fibronectin, in line with its known function as a ligand of the main fibronectin receptor: the α5/β1 integrin complex (Jones et al., 1993; Gleeson et al., 2001). The specific activation of fibronectin receptor by IGFBP-1 but not IGF-II also provides an explanation for its enhancing effects of IGF-II induced invasion. Future studies are needed to clarify which specific integrin receptors are influenced by IGF-II as well as the role of matrix proteolysis in IGF-II-induced trophoblast invasion.

In conclusion, we have shown that IGF-II induces trophoblast cell invasion through the activation of the InsR. Clearly, additional studies are needed to better characterize the role of the different InsR isoforms in the regulation of IGF-II actions. The development of a specific inhibitor of the IGF-II/InsR-A receptor interaction may have important applications. It could potentially be used for the pharmacological treatment of chorioncarcinomas and the several types of tumours known to overexpress IGF-II, while preserving the metabolic actions of insulin.

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