Plasma kisspeptin is raised in patients with gestational trophoblastic neoplasia and falls during treatment


Kisspeptin is a 54-amino acid peptide, encoded by the anti-metastasis gene KiSS-1, that activates G protein-coupled receptor 54 (GPR54). The kisspeptin-GPR54 system is critical to normal reproductive development. KiSS-1 gene expression is increased in the human placenta in normal and molar pregnancies. Circulating kisspeptin is dramatically increased in normal pregnancy, but levels in GTN have not previously been reported. The present study was designed to determine whether plasma kisspeptin levels are altered in patients with malignant GTN. Thirty-nine blood samples were taken from 11 patients with malignant GTN at presentation during and after chemotherapy. Blood was also sampled from nonpregnant and pregnant volunteers. Plasma kisspeptin IR and hCG concentrations were measured. Plasma kisspeptin IR concentration in nonpregnant (n = 16) females was <2 pmol/l. Plasma kisspeptin IR in females was 803 ± 125 pmol/l in the first trimester of pregnancy (n = 13), 2,483 ± 302 pmol/l in the third trimester of pregnancy (n = 7), and <2 pmol/l on day 15 postpartum (n = 7). Plasma kisspeptin IR and hCG concentrations in patients with malignant GTN were elevated at presentation and fell during and after treatment with chemotherapy in each patient (mean plasma kisspeptin IR: prechemotherapy 1,363 ± 1076 pmol/l vs. post-chemotherapy <2 pmol/l, P < 0.0001; mean plasma hCG: prechemotherapy 227.191 ± 152.354 U/l vs. postchemotherapy 2 U/l, P < 0.0001). Plasma kisspeptin IR strongly positively correlated with plasma hCG levels (r² = 0.99, P < 0.0001). Our results suggest that measurement of plasma kisspeptin IR may be a novel tumor marker in patients with malignant GTN.

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tumor (33, 38). The latter three diseases are often referred to as malignant GTN or as gestational trophoblastic tumors.

All forms of GTN secrete human chorionic gonadotrophin (hCG), and serum measurement of this hormone is extremely helpful in the diagnosis, staging, and subsequent assessment of the therapeutic response of malignant GTN. After uterine evacuation of a molar pregnancy, the serum hCG in most women returns to normal. However, in those developing malignant change, the hCG plateaus and/or starts rising and requires urgent chemotherapy, which is curative in virtually 100% of cases. The onset of malignant change after a molar pregnancy can take some months to occur, and women on hCG surveillance have to avoid becoming pregnant. Clearly, earlier detection of this malignant change would be helpful, because it would enable rapid selection of patients for additional therapy. Consequently, the development of new markers for GTN may provide better predictive models of trophoblastic behavior. Moreover, many existing commercial assays for hCG detection are troubled by false positives (3) usually induced by heterophile antibodies. Heterophile antibodies are IgG immunoglobulins raised against poorly defined antigens from other species and can be found in 3–15% of healthy people. Furthermore, these assays usually do not detect all the forms of circulating hCG produced in GTN (5, 19). New markers might also help to reduce these problems.

We hypothesized that plasma kisspeptin immunoreactivity (IR) may be altered in patients with malignant GTN, since placental KiSS-1 gene expression is increased in normal and molar pregnancies (18, 35) and circulating kisspeptin is dramatically increased in normal pregnancy (14).

In the present study we used a specific radioimmunoassay (RIA) for human kisspeptin and investigated kisspeptin IR in human plasma in pregnant and nonpregnant volunteers and in patients with malignant GTN at presentation and during and after chemotherapy. Kisspeptin IR in plasma was characterized using reverse-phase fast-protein liquid chromatography (FPLC). We found that kisspeptin IR was elevated in patients with malignant GTN compared with controls and positively correlated with plasma hCG levels. Chemotherapy treatment reduced kisspeptin IR and hCG levels in each patient.

METHODS

Subjects

Eleven healthy females with normal menstrual cycles who had never been pregnant (mean age 26.1 ± 2.2 yr), five healthy females with normal menstrual cycles who had previously been pregnant (mean age 29.1 ± 3.1 yr), thirteen healthy women in the first trimester (10.4 ± 0.5 wk) of a normal pregnancy (mean age 30.7 ± 1.7 yr), seven healthy women at 38 wk of a normal pregnancy, and the same women 15 days postpartum (mean age 34.4 ± 1.1 yr) and eleven female subjects diagnosed with invasive mole (mean age 27.2 ± 3.4 yr) participated in the study. Patients were diagnosed with molar pregnancy if they had amenorrhea with a raised plasma hCG level and ultrasound confirming a molar pregnancy. Patients were diagnosed with an invasive mole if they had a persistently elevated or rising serum and urine hCG after evacuation of their molar pregnancy.

Blood was collected at first presentation (between 1 and 6 mo after removal of their molar pregnancy) and then during and after chemotherapy as their serum hCG levels fell. Patients with malignant GTN had their disease risk assessed according to the International Federation of Obstetrics and Gynaecology (FIGO) scoring system for GTN (11a).

Peptide Extraction Procedure

Peptide was extracted from plasma using Sep-Pak C18 cartridges (Waters, Hertfordshire, UK), as previously described (34). Briefly, Sep-Pak C18 cartridges were activated using 10 ml of 100% methanol and then 20 ml water. Plasma (2 ml) from patients with malignant GTN was mixed with 2 ml of 0.1 M HCl and loaded onto the cartridge. The cartridge was then washed with 10 ml of 4% acetic acid (vol/vol). The Sep-Pak-bound sample was eluted in 1.5 ml of methanol, and this eluant was dried in a Savant vacuum centrifuge and reconstituted in water plus 0.05% trifluoroacetic acid (TFA; vol/vol) for FPLC.

Reverse-Phase FPLC

Peptide extracts from plasma were dissolved in 0.6 ml of distilled water plus TFA 0.05% (vol/vol). Of this volume, 0.5 ml were fractionated by FPLC on a high-resolution reverse-phase (Pep RPC HR 1 ml) C18 column (Pharmacia, Uppsala, Sweden), as previously described (1). The column was eluted with a 24.5–29.5% gradient of acetonitrile (ACN)-water containing 0.05% (vol/vol) TFA for 40 min, and were fractions collected at 1-min intervals. The kisspeptin IR in

Subjects gave written informed consent, and ethical approval was obtained from the Hammersmith and Queen Charlotte’s & Chelsea Hospitals Research Ethics Committee (no. 04/Q0406/80). Studies were performed in accordance with the Declaration of Helsinki. Blood was taken from all subjects between 9 AM and 12 PM.

Determination of plasma kisspeptin IR in healthy female volunteers. Eleven healthy females with regular menstrual cycles who were not taking any oral contraception had blood sampled during the follicular and luteal phases of their menstrual cycle. Five healthy females with normal menstrual cycle who had previously been pregnant (mean 2.9 ± 2.4 yr postpartum) also had blood sampled during the follicular phase of their menstrual cycle. Thirteen healthy pregnant women had blood sampled during the first trimester (10.4 ± 0.5 wk) of their pregnancy. Blood was also sampled from seven healthy pregnant females at both 38 ± 8.8 wk gestation of normal pregnancy and 15 ± 6 wk postpartum. Ten milliliters of blood were taken and collected into lithium-heparin tubes (LIP, Cambridge, UK) containing 5,000 kallikrein inhibitor units (0.2 ml) of aprotinin (Trasylol; Bayer, Newbury, UK) and stored on ice. After centrifugation, plasma was immediately separated and stored at −20°C until measurement of kisspeptin IR in all subjects and hCG in the pregnant subjects.

Determination of plasma hormones in patients at diagnosis of malignant GTN, during treatment, and postchemotherapy. Blood was sampled from patients with malignant GTN at first presentation, during chemotherapy and after chemotherapy once the patients’ plasma hCG levels had fallen back into the normal range for nonpregnant females. The average length of time between the collection of the second blood sample and the initiation of chemotherapy was 21.1 ± 2.8 days. The average time postchemotherapy at which a blood sample was taken was 14.3 ± 1.4 days. Blood was collected into lithium-heparin tubes containing 5,000 kallikrein inhibitor units (0.2 ml) of aprotinin (Trasylol) and stored on ice. After centrifugation, plasma was immediately separated and stored at −20°C until measurement of kisspeptin IR, hCG, progesterone, and estradiol.

Characterization of kisspeptin IR in human plasma. Kisspeptin IR was characterized in human plasma from patients with malignant GTN (n = 4).
all fractions was determined by RIA. The remaining 0.1 ml of sample was used to calculate the percentage recovery. Recovery was calculated as kisspeptin IR (pmol) recovered from each sample, compared with kisspeptin IR loaded on to the FPLC column (pmol), multiplied by 100, and expressed as a percentage. Prolactin-releasing peptide was also run on this column to determine its elution position.

**Analytical Methods**

hCG, progesterone, and estradiol measurement. hCG was measured by the endocrine laboratory at Charing Cross Hospital (London, UK) using the UK RIA (6). Progesterone and estradiol were measured using commercially available Chemiluminescent Microparticle Immunoassays (ARCHITECT progesterone and ARCHITECT estradiol; Abbott Laboratories, Abbott Park, IL).

Kisspeptin RIA. Plasma kisspeptin was measured using an established RIA (9). Briefly, antibody GQ2 was raised in a sheep immunized with synthetic human kisspeptin-54 (Bachem, UK), conjugated to bovine serum albumin (BSA) by glutaraldehyde, and used at a final dilution of 1:3,500,000. The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and <0.01% with any other related human RFamide (arginine phenylalanine amide) peptide, including prolactin-releasing peptide, RFamide related peptide (RFRP), RFRP2, RFRP3 neuropeptide FF, and neuropeptide AF. The 125I-labeled kisspeptin-54 label was prepared using the iodogen method and purified by reverse-phase high-pressure liquid chromatography on a C18 column (Waters, Milford, MA) over a 15–45% 90-min gradient of ACN-water-0.1% (vol/vol) TFA. The specific activity of the kisspeptin label was 56 Bq/fmol. The assay was performed in duplicate using dilutions of neat plasma in 0.7 ml of 0.06 M phosphate buffer, pH 7.2, containing 0.3% BSA and incubated for 3 days at 4°C. Free and antibody-bound label were then separated by charcoal adsorption. The assay detected changes of 2 pmol/l of plasma kisspeptin IR with a 95% confidence limit. The intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

**Statistical Analysis**

All results are presented as means ± SE. The differences between plasma kisspeptin IR pre- and postchemotherapy and hCG pre- and postchemotherapy were calculated by a Mann-Whitney rank sum test. Correlations were estimated using Pearson Product Moment analysis. In all cases, P < 0.05 was considered to be statistically significant.

**RESULTS**

**Determination of Plasma Kisspeptin IR in Healthy Female Volunteers**

Plasma kisspeptin IR concentration in all nonpregnant females investigated was below the detection limit of the assay (<2 pmol/l). The plasma kisspeptin IR in pregnant females in the first trimester of normal pregnancy (n = 13) was 803 ± 125 pmol/l, and plasma hCG was 72,053 ± 10,936 U/l. The plasma kisspeptin in pregnant females in the third trimester of normal pregnancy (n = 7) was 2,483 ± 302 pmol/l, and plasma hCG was 28,818 ± 11,348 U/l. By day 15 postpartum, (n = 7), plasma kisspeptin was below the detection limit of the assay (<2 pmol/l; Fig. 1).

**Determination of Plasma Hormones in Patients at Diagnosis of Malignant GTN, During Treatment, and Postchemotherapy**

Plasma kisspeptin IR in patients with malignant GTN was elevated at presentation and fell during and after treatment with chemotherapy in each patient (plasma kisspeptin IR: prechemotherapy 1,363 ± 1,076 pmol/l vs. postchemotherapy <2 pmol/l, n = 11, P < 0.0001; Fig. 2A and Table 1). As expected, plasma hCG levels also showed a similar pattern (plasma hCG: prechemotherapy 227,191 ± 152,354 U/l vs. postchemotherapy 2 U/l, n = 11, P < 0.0001; Fig. 2B and Table 1). Plasma kisspeptin IR strongly positively correlated with plasma hCG levels (r² = 0.99, n = 39, P < 0.0001; Fig. 3). Plasma kisspeptin IR showed significant positive correlations with circulating levels of progesterone (r² = 0.92, n = 39, P < 0.0001) and estradiol (r² = 0.70, n = 39, P < 0.0001), as did hCG (r² = 0.89, n = 39, P < 0.0001 for progesterone and r² = 0.64, n = 39, P < 0.0001 for estradiol).

**Analysis of Kisspeptin IR in Human Plasma**

Reverse-phase FPLC was used to further analyze kisspeptin IR extracted from plasma by Sep-Pak cartridge. All columns had a recovery >60%. In each plasma extract the kisspeptin IR in nonpregnancy and pregnant GTN showed significant positive correlations with circulating levels of progesterone (r² = 0.92, n = 39, P < 0.0001) and estradiol (r² = 0.70, n = 39, P < 0.0001), as did hCG (r² = 0.89, n = 39, P < 0.0001 for progesterone and r² = 0.64, n = 39, P < 0.0001 for estradiol).

**DISCUSSION**

Plasma kisspeptin has been proposed as a novel tumor marker indicating the metastatic potential of specific tumors (14). However, to date no reports have investigated this possibility by examining the correlation between circulating kisspeptin concentrations and malignancy. The data described in this paper demonstrate that plasma kisspeptin IR is raised in patients with malignant GTN and falls during treatment. Our results suggest that plasma kisspeptin IR may be a novel tumor marker in patients with this disease. To ensure that the elevated kisspeptin IR levels observed were not due to levels of circulating kisspeptin persisting after
our single site RIA are lower than previously published plasma levels. The plasma kisspeptin IR in pregnant females measured using the improved assay showed a significant decrease in the kisspeptin levels with chemotherapy treatment. This suggests that, as the condition of the patients improved, the kisspeptin levels fell more. Samples taken during chemotherapy showed that, as the condition of the patients improved, the kisspeptin levels fell more. Some patients started with very high hCG levels and had chemotherapy for longer; therefore, they had more samples taken during their chemotherapy to show that, as their condition improved, the kisspeptin levels fell.

We compared plasma kisspeptin levels from malignant GTN patients to those of normal postpartum women. Blood was collected from GTN patients at first presentation between 1 and 6 mo after removal of their molar pregnancy. Plasma kisspeptin levels in normal women were below the detection limit of the assay (<2 pmol/l) as early as day 15 postpartum, suggesting that the elevated kisspeptin levels observed in GTN patients were not due to normal postpartum processes.

Although the kisspeptin/GPR54 system has recently been shown to be critical to normal reproductive development in rodents and man (7, 12, 39, 40), the role of circulating kisspeptin is presently unclear. Kisspeptin circulates in nonpregnant females at concentrations <2 pmol/l (14). However, high levels of plasma kisspeptin IR are seen in normal pregnancy. The plasma kisspeptin IR in pregnant females measured using our single site RIA are lower than previously published plasma levels using a two-site enzyme immunoassay (14). However, in accord with previously published results, plasma kisspeptin was found to be greatly increased in pregnancy and to rise between the first and third trimesters. Additional experiments are needed to determine whether the differences in absolute concentrations detected are due to, for example, the different assay methods used, the subtle differences in subject populations, or the slightly different protocols used for blood sampling and storage. There is presently no international standard protocol for measuring plasma kisspeptin, and it would be interesting to directly compare the kisspeptin assays presently available.

The source of circulating kisspeptin in pregnant women is assumed to be the placenta, where the KiSS-1 gene is highly expressed (14, 18). The role of elevated plasma kisspeptin IR in pregnancy and malignant GTN is presently unclear, and the release of kisspeptin may be regulated by different mechanisms in these conditions. Kisspeptin mediates its effects on the HPG axis via an increase in GnRH. It is therefore possible that elevated plasma kisspeptin levels in pregnancy chronically increase GnRH release, leading to a downregulation of the

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**Table 1. Results of plasma Kiss, hCG, prog, and E2 in subjects with malignant gestational trophoblastic neoplasia pre, during, and post chemotherapy**

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<th>Prog, ng/ml</th>
<th>E2, pg/ml</th>
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Kiss, kisspeptin immunoreactivity concentration; hCG, human chorionic gonadotropin; Prog, progesterone; E2, estradiol; pre, presentation; during, during chemotherapy; post, after chemotherapy. Some patients started with very high hCG levels and had chemotherapy for longer; therefore, they had more samples taken during their chemotherapy to show that, as their condition improved, the kisspeptin levels fell.

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**Fig. 2. Plasma kisspeptin IR (A) and human chorionic gonadotropin (hCG; B) in patients with malignant gestational trophoblastic neoplasia (GTN) at presentation (prechemotherapy) and after chemotherapy (postchemotherapy).** Thick black line on each graph represents mean values; n = 11. Plasma kisspeptin IR in patients with malignant GTN was elevated at presentation and fell during and after treatment with chemotherapy in each patient (plasma kisspeptin IR: prechemotherapy 1,363 ± 1,076 pmol/l vs. postchemotherapy <2 pmol/l, n = 11, P < 0.0001). Plasma hCG levels also showed a similar pattern (plasma hCG: prechemotherapy 227,191 ± 152,354 U/l vs. postchemotherapy 2 U/l, n = 11, P < 0.0001). Each symbol represents a different patient with GTN.
KISSPEPTIN IS RAISED IN TROPHOBLASTIC NEOPLASIA

HPG axis, as is seen following the administration of synthetic GnRH agonists (11). The KiSS-1 gene is an anti-metastasis gene, and transfection of melanoma cells with KiSS-1 cDNA expression dependently suppresses metastasis (21, 23). Kisspeptin-10 has been shown to inhibit trophoblast migration without affecting proliferation in vitro (1). Interestingly, placent al KiSS-1 gene expression has been shown to inversely correlate with the metastatic potential of patients with GTN. Placental KiSS-1 gene expression in molar pregnancy is elevated to a similar degree to that seen in normal pregnancy but is undetectable in choriocarcinoma (18, 35). Plasma kisspeptin IR may therefore be elevated in patients with an invasive mole to inhibit tumor metastasis and the development of choriocarcinoma (18, 35). Additional studies are required to determine whether plasma kisspeptin IR is significantly lower in patients with choriocarcinoma compared with plasma kisspeptin IR in patients with molar pregnancy. It would be of particular interest to investigate whether patients with molar pregnancy who go on to develop choriocarcinoma have different circulating kisspeptin levels from those who do not.

Measurement of placental KiSS-1 gene expression requires a placental biopsy, which is impractical in clinical practice. If plasma kisspeptin acts as a marker of tumor activity, its measurement would be more clinically applicable. We found large variations in circulating hCG and kisspeptin concentrations between GTN patients before treatment. However, plasma hCG and kisspeptin levels showed a strong correlation, and each patient with a plasma hCG level above the normal range (>5 U/l), suggesting disease activity, also had an elevated plasma kisspeptin. Because plasma hCG is one of the factors used to score disease severity in patients with GTN by the FIGO scoring system (11a), this variation in prechemotherapy plasma kisspeptin levels may also reflect differences in disease activity between subjects.

GTN is presently diagnosed by measuring hCG. Circulating levels of hCG were higher than those of kisspeptin in the GTN patients in this study. Additional work using a larger group of GTN patients would be required to confirm the sensitivity of hCG compared with kisspeptin. However, there are problems with using hCG as a diagnostic tool. Although hCG is a sensitive diagnostic tumor marker, in addition to regular hCG at least five major variants of hCG are present in serum samples. In patients with GTN, these variants of hCG can be the principal circulating form of hCG (4, 5). Some hCG assays do not detect these hCG variants, which can result in a failure to diagnose GTN (5, 19). In addition, false positive hCG IR (phantom hCG) results can also occur with hCG assays due to proteolytic enzymes that mimic hCG or heterophile antibodies in the serum that interfere with the hCG assay (3). This can lead to an incorrect diagnosis of GTN and unnecessary testing and treatment (10). New tumor markers may help to reduce these problems. Interestingly, although endogenous forms of kisspeptin-54 and -14 and 13 amino acids in length have been isolated from human placenta (20), chromatographic characterization of the circulating kisspeptin IR in GTN patients detected only a single form eluting at an identical position to synthetic kisspeptin-54. Analysis of plasma from women with normal pregnancies also shows only a single peak in the same position (data not shown). Our results suggest that measurement of plasma kisspeptin IR might be useful as a novel alternative or complementary tumor marker in patients with malignant GTN. Additional work is required to determine whether measurement of plasma kisspeptin IR correlates with disease activity in patients with GTN in whom a variant of hCG is the major circulating form or in patients with phantom hCG IR.

The high plasma kisspeptin IR concentrations in our patients with malignant GTN may simply reflect the mass of material of placental origin. Kisspeptin is expressed in trophoblasts, which are known to secrete hCG, estradiol, and progesterone (2, 14). Circulating concentrations of both kisspeptin IR and hCG were positively correlated with circulating estradiol and progesterone in patients with malignant GTN. There is also a positive correlation between progesterone and estradiol levels with kisspeptin levels in normal pregnancy (14). This might suggest that the release of kisspeptin is related to the number or mass of trophoblasts (14). However, there is evidence to suggest that circulating kisspeptin concentrations are not directly correlated to KiSS-1 expression in the placenta. Although plasma kisspeptin concentration rises throughout pregnancy and peaks at term...
(14), placental KiSS-1 gene expression is not significantly different between early and term placentas (18). In rats, KiSS-1 gene expression has been found to decrease in trophoblasts during placental maturation and gestation (1, 47).

Interestingly, although our results suggest that plasma kisspeptin IR correlates strongly and significantly with hCG in patients with malignant GTN, there is no significant correlation between plasma hCG and plasma kisspeptin concentration in pregnancy (14). Plasma hCG is markedly elevated in the first trimester of pregnancy, but lower levels are found in the second and third trimesters. In contrast, plasma kisspeptin concentrations are elevated in the first trimester of pregnancy and continue to increase to a maximum of ~7,000-fold basal levels in the third trimester of pregnancy (14).

In conclusion, we have demonstrated that plasma kisspeptin IR is raised in patients with malignant GTN and falls during and after treatment. Kisspeptin IR strongly and significantly correlated with plasma hCG levels in these patients. Our results suggest that plasma kisspeptin IR may be a novel tumor marker in patients with malignant GTN.

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