Kisspeptin is released from human prostate cancer cell lines but plasma kisspeptin is not elevated in patients with prostate cancer

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Abstract. Kisspeptin, the product of the *KiSS-1* gene, inhibits metastasis and stimulates the hypothalamo-pituitary-gonadal axis. Kisspeptin is therefore a putative target in the treatment of hormone-sensitive malignancies. Prostatic carcinoma remains a significant cause of mortality despite improvements in therapy. The role of kisspeptin in prostatic carcinoma remains undefined. We therefore aimed to investigate release of kisspeptin by prostatic cancer cell lines; investigate expression of *KiSS-1* in human prostate tissue; investigate whether patients with prostate carcinoma have elevated plasma kisspeptin. 1) Culture medium from prostatic carcinoma cell lines LNCaP, DU145 and PC3 was assayed for kisspeptin immunoreactivity (-IR). Kisspeptin-IR release was detectable from all three cell lines. The effect of hydroxyflutamide, gefitinib and resveratrol on kisspeptin-IR release from these cell lines was also investigated. No effect of the drugs tested on release of kisspeptin-IR was observed. 2) Expression of *KiSS-1* in human prostate tissue (n=4) was investigated using *in situ* hybridisation. Expression of *KiSS-1* was detected in human prostate tissue. 3) Plasma kisspeptin-IR was compared in 92 patients with prostatic carcinoma and 73 male controls. Kisspeptin-IR was not detected in the plasma of either patients with prostate cancer or control patients. We have therefore shown for the first time the release of kisspeptin-IR by prostatic carcinoma cell lines. We have also shown that *KiSS-1* is expressed in human prostate tissue, and that circulating levels of kisspeptin-IR are not elevated in patients with prostatic carcinoma. Further work is required to determine the role of kisspeptin in the prostate.

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Introduction

For more than two decades, prostate carcinoma has remained the most common cancer afflicting men in the USA, and the second highest cause of cancer-related death (1). This despite an overall fall in mortality from prostate carcinoma between 1992 and 2002, attributable to a number of changes in both diagnostic and therapeutic approach (2). The identification of novel points of therapeutic intervention may therefore yield further advances in the treatment of this major source of morbidity and mortality.

Kisspeptin is a 54 amino acid peptide encoded by the gene *KiSS-1* (3). In addition to the full length peptide, kisspeptin also exists *in vivo* as the truncated fragments, kisspeptin-14 and -13. The minimum sequence required for receptor activation is the C-terminal decapeptide kisspeptin-10 (3). Kisspeptin signals via the G-protein-coupled receptor, GPR54 (3). Kisspeptin is critical to the functioning of the hypothalamo-pituitary-gonadal (HPG) hormonal axis. Mice and humans lacking a functional GPR54 or mice null for *KiSS-1* do not mature sexually and have low circulating gonadotrophins and sex hormones (4-10). GPR54, but not *KiSS-1*, is expressed in GnRH neurones in the hypothalamus (6,11). Kisspeptin is thought to modulate sex hormone secretion predominantly via GnRH, but may also exert direct effects on the pituitary and gonads (12-17). Continuous administration of GnRH has been known for some time to cause downregulation of the HPG axis. The principle mechanism is thought to be downregulation of the GnRH receptor (18). Continuous administration of kisspeptin desensitises the HPG axis to its effects, although in this case, desensitisation is thought to occur at the level of GPR54 (19-21).

Kisspeptin/GPR54 signalling is also thought to be important in tumour biology. Kisspeptin was originally known as metastin because it was implicated in suppression

of tumour metastasis in a number of human cancers (22-31) and circulating kisspeptin has been proposed as a tumour marker for gestational trophoblastic disease and pancreatic cancer (32,33). However, the levels of kisspeptin in prostatic cancer have not previously been investigated.

Although GPR54 mRNA has been identified in human prostate cDNA by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), there is disagreement over the expression of *KiSS-1*. Muir and co-workers (34) failed to detect *KiSS-1* expression in cDNA from human prostate, whereas Ohtaki and colleagues (23) did detect *KiSS-1* expression, albeit at comparatively low levels. Furthermore, the expression of *KiSS-1* in prostatic cancer has not been investigated. We therefore examined the release of kisspeptin from both androgen sensitive and insensitive human prostate cancer cell lines, and investigated the effects of three common therapeutic agents for prostate cancer on kisspeptin release from these cell lines. We investigated whether *KiSS-1* expression could be detected in samples of human prostate tissue using *in situ* hybridisation (ISH). We also investigated for the first time the circulating levels of kisspeptin in patients with prostate cancer.

Materials and methods

All chemicals and reagents were obtained from Invitrogen (Paisley, UK) unless otherwise stated.

Cell lines and culture. We used the androgen sensitive prostatic carcinoma cell line LNCaP and the androgen independent cell lines DU145 and PC3. All cell lines were kindly donated by Dr Tahereh Kamalati, Division of Surgery, Oncology, Reproductive Biology and Anaesthetics, Imperial College London, UK.

All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% fetal bovine serum and 1% antibiotic (100 IU/ml penicillin and 100 μg/ml streptomycin). Cells were cultured at 37˚C, 95% O_2, 5% CO_2. The medium was changed every 2-3 days and cells were passaged on reaching 80-90% confluence.

Kisspeptin release by human prostate cancer cell lines. LNCaP, DU145 and PC3 cells were cultured as above. Prior to experimental manipulation, cells were plated onto 24-well multidishes pre-coated with Nunc Nunclon surface (Nunc International, Roskilde, Denmark). Cells were allowed to grow to confluence and serum starved in RPMI-1640 medium with 100 IU/ml penicillin and 100 μg/ml streptomycin (serum-free medium, SFM) for 2 h. In order to investigate the time course of kisspeptin release from human prostate cancer cell lines, 500 μl/well fresh SFM was then added (T=0). Cells were incubated at 37˚C and medium was collected at T=2, 4 or 24 h (n=4 wells/group) and stored at -20˚C until measurement of kisspeptin immunoreactivity (-IR) using an established radioimmunoassay (RIA) (32,35-37). The RIA antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and <0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43, neuropeptide FF, and neuropeptide AF. 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.

Characterisation of kisspeptin-IR released by LNCaP cells. The form of kisspeptin released from LNCaP cells was investigated by reverse phase chromatography as previously described (32,38). Kisspeptin-IR was extracted using Sep-Pak C18 cartridges from 2-ml culture medium taken from LNCaP cells grown to confluence in a flask. The extracted kisspeptin-IR was freeze-dried and resuspended in distilled water with 0.1% trifluoroacetic acid (TFA). It was then loaded onto a reverse-phase fast protein liquid chromatography (FPLC) column and eluted with a 10-40% gradient of acetonitrile (AcN) with 0.1% TFA over 60 min at a flow rate of 1 ml/min/fraction. The fractions collected were dried down, reconstituted in assay buffer and kisspeptin-IR measured by RIA.

The effect of hydroxyflutamide, gefitinib and resveratrol on kisspeptin release by human prostate cancer cell lines. The effect of a number of therapeutic agents used in the treatment of prostate cancer on kisspeptin release by LNCaP, DU145 and PC3 cells was also investigated. Cells were plated as in study 2 above and serum starved for 2 h. At T=0, the culture medium was changed and replaced with 500 μl SFM or with 500 μl SFM containing hydroxyflutamide (an androgen receptor antagonist), gefitinib (a tyrosine kinase inhibitor) or resveratrol (which influences the cell cycle at a number of points). Increasing doses of the therapeutic agents were used (0.1, 1 or 10 μM; n=6 wells/group). At T=2 h, culture medium was removed and stored at -20˚C until it was assayed for kisspeptin-IR as above.

In situ hybridisation (ISH) to investigate expression of kisspeptin in prostate tissue. The expression of *KiSS-1* in the human prostate has previously been examined using cDNA libraries, with conflicting results (23,34). This is in contrast to the expression of GPR54 mRNA, which was identified in prostate cDNA in both studies (23,34). We therefore examined the expression of *KiSS-1* mRNA by direct investigation of human tissue samples.

Samples of anonymised prostate tissue (n=4) were provided by the Tissue Bank at Hammersmith Hospital. Samples were cancer-free prostate sections from patients who had undergone open prostatectomy but were subsequently diagnosed with benign prostatic hypertrophy. Ethical approval was obtained from the Hammersmith and Queen Charlotte's & Chelsea Hospitals Research Ethics Committee (reference no. 05/Q0406/154). Samples were prepared by the Department of Histopathology, Hammersmith Hospital. Briefly, samples 3-5-mm thick were prepared by a series of treatments with increasing concentrations of ethanol and xylene before immersion in wax. Wax embedded samples were sliced on a microtome to produce 5-μm slices and were mounted on to glass slides for ISH.

ISH was performed as previously described (39). Briefly, sense and antisense, corresponding to nucleotides 212-492 of the human *KiSS-1* gene (accession no. NM002256), were radiolabelled with [^{35}S]-cytosine triphosphate (37 MBq)

Table I. Clinical characteristics of prostate cancer patients subclassified by tumour-nodes-metastasis (TNM) classification T value.

	TNM classification, T value (primary tumour)				
	T1	T2	T3	T4	Tx
n	11	31	28	11	11
Mean age (years) ± SEM	68.2±3.0	71.5±1.1	72.4±1.5	77.0±2.4	76.7±2.9
Metastatic disease, n (%)	0 (0)[c]	1 (3.2)[c]	4 (14.3)[c]	5 (45.5)[c]	11 (100)[c]
Antiandrogen therapy, n (%)	1 (9.1)[b]	2 (6.5)[b]	5 (17.9)[b]	5 (45.5)[b]	4 (36.4)[b]
GnRH agonist therapy, n (%)	3 (27.3)	5 (16.1)	7 (25.0)	5 (45.5)	5 (45.5)
Mean PSA (ng/ml) ± SEM	2.31±1.2	14.03±4.22	28.18±9.00	14.82±8.29	419.3±186.3[a]

The number of patients from each group undergoing therapy with antiandrogenic drugs or a GnRH agonist at the time of blood sampling is also shown. n, number; SEM, standard error of the mean; GnRH, gonadotrophin releasing hormone; PSA, prostate specific antigen. Mean age and PSA analysed by One-way ANOVA with Bonferroni post-test. Presence of metastases, use of antiandrogen therapy and use of GnRH therapy analysed by Chi-squared test. [a]P<0.001 Tx samples vs. T1, T1, T3 and T4 samples. [b]P<0.05 by Chi-squared test for equal distribution of antiandrogen therapy across different T values. [c]P<0.001 by Chi-squared test for equal distribution of presence of metastases across different T values.

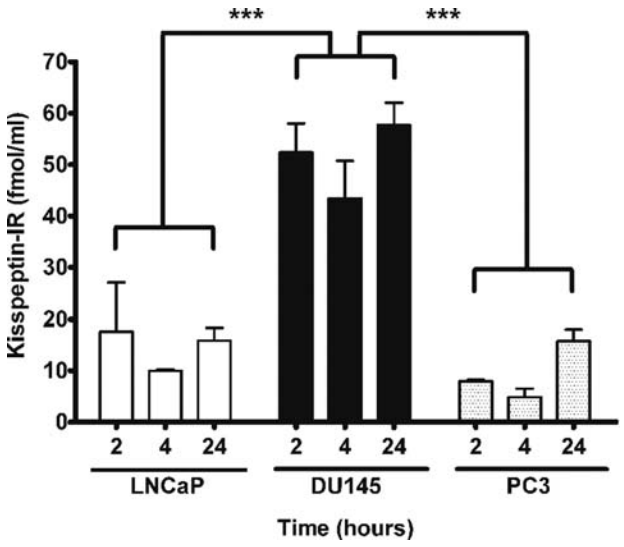

Figure 1. Kisspeptin-IR release from human prostate cancer cell lines LNCaP, DU145 and PC3. Cells were investigated after a 2-h serum starvation, following which fresh serum-free medium (SFM) was added and then removed 2, 4 or 24 h later. No kisspeptin-IR was detected in SFM (data not shown). ***p<0.001 (Two-way ANOVA with Bonferroni post-test).

(Amersham Biosciences, Buckinghamshire, UK). Hybridisation buffer was supplemented with probe at a rate of $1x10^5$ Bq/μl and incubated overnight at 60˚C. Slides were then RNAse-A treated, washed, air-dried and exposed to autoradiographic film (Bio-Max Film MR, Kodak, Hemel Hempstead, UK). After exposure, the film was developed and *KiSS-1* expression determined by observation of specific hybridisation.

Investigation of plasma kisspeptin-IR in patients with prostate cancer. Blood samples were collected from 92 patients with prostate cancer. Table I shows the clinical characteristics of these patients subclassified by the T value assigned by the tumour-nodes-metastasis (TNM) classification (40). Plasma was also obtained from 73 male controls who attended the oncology clinic with a diagnosis of malignancy other than prostatic carcinoma (mean age ± standard error of the mean, SEM: 70.4±3.4 years). Levels of kisspeptin-IR was measured by RIA in the plasma of patients and controls. Volunteers gave written informed consent and the study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Hammersmith and Queen Charlotte's & Chelsea Hospitals Research Ethics Committee (reference no. 04/Q0406/80).

Results

Kisspeptin release by human prostate cancer cell lines. Kisspeptin-IR was detected in the culture medium of all three cell lines investigated (Fig. 1). There was no statistically significant effect of time on the release of kisspeptin-IR within any of the cell lines, up to 24 h. DU145 cells released significantly more kisspeptin than either LNCaP or PC3 cells [mean kisspeptin-IR (pmol/l) ± standard error of the mean (SEM) at T=2 h: LNCaP cells 17.6±19.5, DU145 cells 52.4±5.6 (p<0.001 vs. LNCaP and PC3 cells), PC3 cells 7.9±0.3; at T=4 h: LNCaP cells 9.9±0.2, DU145 cells 43.5±7.2 (p<0.001 vs. LNCaP and PC3 cells), PC3 cells 4.9±1.6; at T=24 h: LNCaP cells 15.9±2.9; DU145 cells 57.7±4.4 (p<0.001 vs. LNCaP and PC3 cells); PC3 cells 15.8±2.2]. Statistics by Two-way analysis of variance (ANOVA) with Bonferroni post-hoc test.

Characterisation of kisspeptin-IR released by LNCaP cells. Reverse phase FPLC was used to characterise further the kisspeptin-IR detected in the culture medium of LNCaP cells. Recovery was >60% for all columns. A single immuno-reactive peak was detected in each sample of culture medium

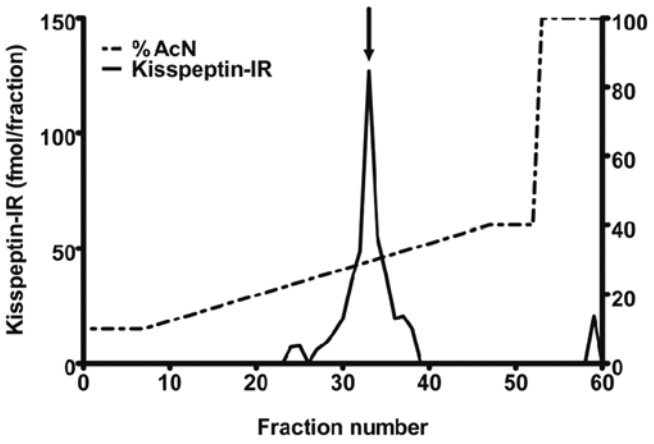

Figure 2. Representative elution profile of kisspeptin-IR extracted from 2 ml of LNCaP medium by Sep-Pak cartridge and fractionated by reverse-phase FPLC. Dotted line indicates % acetonitrile (AcN); arrow indicates elution position of synthetic human kisspeptin-54.

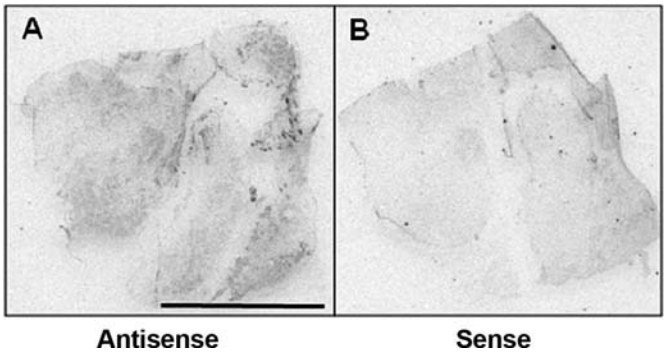

Figure 3. Representative autoradiograph of *in situ* hybridisation indicating *KiSS-1* mRNA expression in human prostate tissue using an antisense riboprobe (A) and a sense (control) riboprobe (B). Dark staining indicates hybridisation. Scale bar indicates 5 mm.

tested, corresponding to the elution point of synthetic human kisspeptin-54 (Fig. 2).

The effect of hydroxyflutamide, gefitinib and resveratrol on kisspeptin release by human prostate cancer cell lines. The effects of the drugs hydroxyflutamide, gefitinib and resveratrol on release of kisspeptin-IR by LNCaP, DU145 and PC3 cells were investigated. The drugs were each tested at a range of doses: 0, 0.03, 0.1, 0.3, 1 and 10 μM. No significant effect on kisspeptin-IR at any of the doses tested was observed, compared with basal levels of kisspeptin-IR in both the androgen sensitive and insensitive cell lines (data not shown).

ISH to investigate expression of kisspeptin in prostate tissue. Kisspeptin expression in prostate tissue was investigated using ISH. The *KiSS-1* antisense riboprobe resulted in significantly greater staining than the sense (control) probe, throughout prostate tissue in all samples examined (n=4). This indicates expression of *KiSS-1* mRNA in human prostate tissue (Fig. 3).

Investigation of plasma kisspeptin-IR in patients with prostate cancer. Plasma kisspeptin-IR was not detectably raised in patients with prostate cancer, independent of the TNM classification and treatment status of the patient (mean kisspeptin-IR <2 pmol/l; mean kisspeptin-IR in plasma of healthy male controls <2 pmol/l). There was no correlation with serum PSA level and plasma kisspeptin-IR.

Discussion

Kisspeptin was first characterised in relation to its anti-metastatic properties. Loss of kisspeptin expression has been associated with increased metastasis and cancer progression in a number of human cancers, including melanoma, phaeochromocytoma, oesophageal squamous cell carcinoma and cancers of the bladder, breast, stomach and pancreas (22-31). In addition, circulating levels of kisspeptin-IR have been proposed as a tumour marker in gestational trophoblastic disease and pancreatic cancer (32,33). However, its role in prostate cancer has not been determined.

Furthermore, kisspeptin is critical in regulation of the hypothalamo-pituitary-gonadal (HPG) axis (8-12,16,35,36,41,42). Current treatment strategies for prostatic carcinoma include modulation of the HPG axis with antiandrogenic drugs and downregulation of HPG axis function with continuous administration of GnRH analogues. Kisspeptin is thought to act upstream of GnRH in its effects on the HPG axis (12-14,16,41-44) and frequent or continuous administration of kisspeptin has also been shown to cause a reduction in HPG axis function in rodents and primates (19-21). Thus, establishing a role for kisspeptin in prostate carcinoma may indicate possible novel therapeutic approaches.

Kisspeptin-IR was detected in culture medium from all three cell lines investigated. It has previously been noted that prostate cancer cells produce GnRH-like peptides and these have been implicated in the autocrine regulation of prostate cancer (45). In a similar fashion, it is possible that kisspeptin may also act as an autocrine agent, regulating tumour growth. The identification of alternative, novel regulators of tumour growth may suggest possible points of therapeutic intervention, particularly in those patients undergoing therapy with GnRH agonists who escape control. However, all three cell lines used originate from patients with metastatic disease. The detection of kisspeptin-IR released by these cells might therefore seem to conflict with published data, which has associated metastatic potential and disease progression with loss of *KiSS-1* expression in other cancers (22-31). Further work, such as studies of the effect of kisspeptin on the response of prostate cancer to growth factors, would further illuminate the implications of local production of kisspeptin for cancer growth and spread.

In order to better understand the significance of the release of kisspeptin-IR by the three cell lines investigated, we investigated the effect of a number of drugs on release of kisspeptin-IR by LNCaP, DU145 and PC3 cells. The drugs chosen act via different mechanisms. Hydroxyflutamide is an androgen receptor antagonist used in the treatment of prostate cancer (46); gefitinib is an inhibitor of the tyrosine kinase domain of the epidermal growth factor receptor (47); and resveratrol is a phytoalexin, acting at a number of points

in the cell cycle, and has been associated with reduction of prostate cancer progression in mice (48). The lack of an effect of these drugs on kisspeptin-IR release in all three cell lines investigated suggests that kisspeptin does not play a role in mediating the clinical effects of these drugs. This lack of effect was independent of the androgen sensitivity status of the cell line tested.

In order to investigate whether the release of kisspeptin-IR by these cell lines was confined to carcinomatous cells, or whether there might be a role for kisspeptin in normal prostate physiology, we also performed ISH for *KiSS-1* mRNA in tissue samples taken from patients suffering from benign prostatic hypertrophy. *KiSS-1* mRNA was present in human prostate tissue. Previous studies of human cDNA libraries have either failed to show expression of *KiSS-1* mRNA in prostatic tissue (34), or have shown expression at comparatively low levels (23). In contrast, there is greater agreement on the presence of GPR54 mRNA in human prostate cDNA (23,34). Although not quantitative, our data, derived directly from tissue, support the expression of *KiSS-1* mRNA in the human prostate. Kisspeptin may thus play a novel autocrine or paracrine role in normal prostate physiology. The nature of this role requires further investigation.

Our data show that kisspeptin-IR is released by a number of prostatic carcinoma cell lines and that *KiSS-1* mRNA is present in prostate tissue. We therefore went on to investigate whether kisspeptin-IR was measurable in the circulation of patients with prostate carcinoma and whether this correlated with disease stage, the presence of known metastases and treatment modality. Our data suggest that circulating kisspeptin does not play a role in the pathology of prostate cancer. Further work is required to determine whether kisspeptin plays a paracrine or an autocrine role for kisspeptin in prostate cancer.

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