CLINICAL STUDY

Expression of functional KISS1 and KISS1R system is altered in human pituitary adenomas: evidence for apoptotic action of kisspeptin-10

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Abstract

Context: KISS1 was originally identified as a metastasis-suppressor gene able to inhibit tumor progression. KISS1 gene products, the kisspeptins, bind to a G-protein-coupled receptor (KISS1R, formerly GPR54), which is highly expressed in placenta, pituitary, and pancreas, whereas KISS1 mRNA is mainly expressed in placenta, hypothalamus, striatum, and pituitary.

Objective and design: KISS1/KISS1R pituitary expression profile, coupled to their anti-tumoral capacities, led us to hypothesize that this system may be involved in the biology of pituitary tumors. To explore this notion, expression levels of KISS1R and KISS1 were evaluated in normal and adenomatous pituitaries. Additionally, functionality of this system was assessed by treating dispersed pituitary adenoma cells in primary culture with kisspeptin-10 and evaluating intracellular calcium kinetics and apoptotic rate.

Results: Both KISS1 and KISS1R were expressed in normal pituitary, whereas this simultaneous expression was frequently lost in pituitary tumors, where diverse patterns of KISS1/KISS1R expression were observed that differed among distinct types of pituitary adenomas. Measurement of calcium kinetics revealed that kisspeptin-10 elicits a remarkable increase in $[Ca^{2+}]_i$ in individual cells from four out of the five GH-producing adenomas studied, whereas cells derived from non-functioning pituitary adenomas (NFPA, $n=45$) did not respond. In contrast, kisspeptin-10 treatment increased the apoptotic rate in cells derived from both GH-producing and NFPA.

Conclusions: These results provide primary evidence that KISS1 and KISS1R expression can be differentially lost in pituitary tumor subtypes, where this system can exert functional, proapoptotic actions, and thereby offer novel insights to investigate the biology and therapeutic options to treat these tumors.

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Introduction

Pituitary adenomas are relatively rare, regularly benign neoplasms, which exhibit a wide range of biological behaviors in terms of hormone production and cell proliferation, and ultimately result from health complications (1). In most instances, early surgical intervention provides the best chance for either the cure or the control of pituitary tumor progression and its side effects (2). To date, medical strategies are strongly dependent

on the specific tumor, and the options are limited by the reduced number of effective molecular targets that can be employed. Accordingly, it is of high interest to identify and characterize new molecules that could serve as potential targets for pituitary tumor treatment.

The KISS1 gene encodes a precursor protein that is processed into several related peptides, generically named kisspeptins (3–5), where the major product appears to be a 54-amino acid peptide, named kisspeptin-54 or metastin. In addition, three natural

peptides of 14-, 11-, and 10-amino acids have been also identified, sharing a common 10-amino acid C-terminal region (5). All kisspeptins have been reported to exhibit a similar affinity and efficacy to bind KISS1R (formerly GPR54; (5)), a previously orphan receptor mainly expressed in brain, testis, ovary, spinal cord, placenta, and pituitary (4–6). Kisspeptins were originally identified by virtue of their ability to inhibit tumor metastasis in human melanoma cells through KISS1R (6–10), thus their ‘metastatin’ name. However, the kisspeptin/KISS1R system has recently emerged as an essential player in the neuroendocrine regulation of the reproductive axis, as it was already suggested by the severe hypogonadotropism observed in humans displaying mutated KISS1R (11, 12), and in animal models lacking this receptor (12). Available evidence indicates that kisspeptins stimulate gonadotropin secretion primarily through an indirect mechanism involving activation of hypothalamic GnRH release (13). Nevertheless, recent data from our group and others have demonstrated that kisspeptins also act directly upon the pituitary by inducing both LH and GH release, possibly explaining the functional significance of the reported presence of KISS1R in the rat pituitary (14, 15), jointly with its ligands (3, 5, 15). These findings prompted us to question whether KISS1 and KISS1R were expressed in the human pituitary and whether they may exert a putative role in the regulation of pituitary tumor behavior and/or progression. To answer these questions, we examined the expression of KISS1/KISS1R system in different pituitary tumors, and evaluated the direct action of kisspeptin-10 on cells from several types of pituitary adenomas by measuring its effects on free cytosolic calcium levels ($[Ca^{2+}]_i$). Finally, we also evaluated the effect of kisspeptin-10 on apoptosis in cells from non-functioning pituitary adenomas (NFPA) and GH-producing adenomas.

Materials and methods

The study was carried out in accordance with the Declaration of Helsinki and approved by the University Hospital Ethics Committees participating in this research. Written informed consent was obtained from each patient.

Chemicals

Unless otherwise indicated, chemical products and tissue culture reagents were purchased from Sigma Chemical Co.

Pituitary samples and cell culture

A total of 119 pituitary adenoma samples removed by transsphenoidal resection were included in this study: 34 from patients showing GH-secreting tumors and acromegaly with elevated serum GH and/or insulin-like

growth factor 1 levels; 19 from patients presenting prolactin (PRL)-secreting adenomas with elevated serum PRL levels; 20 ACTH-secreting pituitary tumors causing Cushing’s disease; 2 TSH-omas, and 45 samples from patients screened out as having clinically nonfunctioning adenomas with no evidence of hormone hypersecretion. Samples from local Hospitals were immediately transferred to sterile cold (4 °C) culture medium (DMEM). Under sterile conditions, each sample was minced into 1–2 mm³ pieces, and two to five of them were snap frozen in liquid nitrogen for posterior RNA isolation. When possible, remaining tissue pieces were processed as previously described (16).

For measurements of $[Ca^{2+}]_i$, 50 000 dispersed cells/cover slip were plated in 10% serum containing medium. For apoptosis determination experiments, 30 000 to 50 000 cells/culture dish were seeded. Medium was freshly replaced every 48 h.

KISS1 and KISS1R expression in pituitary samples

Expression of KISS1 and KISS1R were analyzed in 119 adenomatous pituitary fragments and additionally in 2 normal pituitary tissues obtained from different commercial sources (Clontech and CliniSciences, Montrouge, France). Total RNA was isolated using Trizol reagent (Invitrogen) as recommended by the manufacturer’s instructions. RNA was reverse transcribed to cDNA by using PowerScript (BD Bioscience, Erembodegem, Belgium) reverse transcriptase following the manufacturer’s manual. PCR analysis was employed to assess the expression of KISS1 (GenBank accession no. NM_002256), and its receptor KISS1R (GenBank accession no. NM_032551) was assessed by using an iCycler IQ thermocycler (Bio-Rad). Temperature profiles were as follows: 94 °C/15 s, 65 °C/15 s, and 72 °C/15 s for 35 cycles. PCR products were electrophoresed in 2% agarose gel containing ethidium bromide and extracted using QIAquick Gel Extraction kit (Qiagen). Identities of amplicons were confirmed by sequencing at University Facilities. To control the integrity of RNA and the efficiency of the RT reaction, the expression of 18S was determined in each sample.

Measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells

The effect of kisspeptin-10 administration on $[Ca^{2+}]_i$ kinetics in single adenomatous pituitary cells in culture was evaluated using a microfluorimetric technique previously described in detail (16), in which kisspeptin-10 was administered through pipette ejection (16) and its effect on $[Ca^{2+}]_i$ was measured every 5 s by using MetaFluor Software (Imaging Corporation, West Chester, PA, USA).

Immunofluorescence

Primary cultured pituitary adenoma cells (20 000/well) were seeded in 8-well chamber slides (Lab-Tek Chamber Slide System, Nalgene Nunc International, Naperville, IL, USA) and incubated for 30 min in a humidified atmosphere at 37 °C with a rabbit anti-human GPR54 antibody (1:100; MBL International Corporation, Woburn, MA, USA). The cells were then fixed in methanol–acetone (1:1) for 10 min at –20 °C, blocked for 1 h with a blocking buffer (5% goat serum in PBS), and incubated with a secondary FITC-conjugated goat anti-rabbit antibody for 45 min at room temperature. Slides were then mounted, visualized, and photographed as previously reported (17). Preimmune serum and antigen-adsorbed antibody were used as controls of antibody specificity.

DAPI staining

Cells were grown in 35 mm diameter culture dish at a seeding density of 30 000 to 50 000 cells/dish in DMEM+10% FCS for 24 h prior treatment. The day of the experiment, cells were washed and treated with kisspeptin-10 (10^{-6} , 10^{-7} , and 10^{-9} M) for 12, 24, or 48 h in DMEM+0.5% FCS. Following, adenomatous cells were fixed in Bouin's solution for 30 min, and then stained with 1 µg/ml DAPI for 30 min. Treatments were randomly examined by an operator in a blind manner at 40× magnification in a fluorescence microscope (Zeiss) at 340–380 nm light intensity. Apoptotic cells were characterized by chromatin condensation and fragmentation, whereas cells considered as normal exhibited a typical morphology with smooth nuclear and cellular membranes (18).

Results

KISS1 and KISS1R expression in pituitary samples

PCR amplification of *KISS1* gave the expected fragment size of 119 bp in 35.5% of NFPA (16 out of 45), in 35% of corticotrope adenomas (7 out of 20), in 38.24% of GH-producing adenomas (13 out of 34), and in 47.37% of prolactinomas tested (9 out of 19; Fig. 1B–E). DNA sequencing of the purified band confirmed the identity of the amplified transcripts, thus demonstrating that *KISS1* is expressed in the above-mentioned pituitary tumors. Furthermore, *KISS1R* transcript was expressed in 51.1% of NFPA (23/45), in 40% of corticotrope adenomas (8/20), in 41.18% of GH-producing adenomas (14/34), and in 57.89% of tested prolactinomas (11/19; Fig. 1B–E). The amplified product presented the expected size for the *KISS1R* cDNA fragment (198 bp), and the nucleotide sequence matched the published sequence for the respective human *KISS1R*.

Figure 1 *KISS1* and *KISS1R* expression profile in normal and different adenomatous pituitaries. Each panel illustrates the percentage of samples expressing *KISS1* (K), *KISS1R* (KR), *KISS1* and *KISS1R* simultaneously (K/KR) and the absence of both *KISS1* and *KISS1R* transcripts (lack), respectively, from 2 normal pituitary samples, 45 NFPA, 20 ACTH-omas, 34 GH-omas, 19 PRL-omas and 2 TSH-omas.

As shown in Fig. 1A, *KISS1* and *KISS1R* transcripts were expressed simultaneously in normal pituitary samples. Similar synchronized expression of both transcripts was also confirmed in 26.6% of studied NFPA (12 out of 45), in 15% of corticotrope adenomas (3/20), in 14.7% of GH-producing adenomas (4/34), and in 37% of prolactinomas (7/19). Thus, the vast majority of pituitary tumors studied lacked simultaneous expression of *KISS1* and *KISS1R* (Fig. 1B–E). Particularly, in 40% of NFPA (18/45), in 40% of ACTH-producing adenomas (8/20), in 38.2% of GH-producing adenomas (13/34), and in 31% of PRL-secreting tumors (6/19), the concomitant expression of both *KISS1* and *KISS1R* transcripts is defective. Additionally, we include two cases of TSH-producing adenomas in which *KISS1* and *KISS1R* transcripts were completely absent (Fig. 1F). Statistical comparison using a χ^2 method indicated that the frequency distribution patterns of *KISS1/KISS1R* expression displayed by the different types of pituitary adenomas differ significantly.

A basic set of information regarding patient age, gender, tumor size and recurrence, as well as *KISS1* and *KISS1R* expression could be collected in 89 out of the 119 cases studied, and has been included in Supplementary Table 1, see section on supplementary data given at the end of this article. Analysis of the data

collected did not reveal any apparent association between these parameters and *KISS1/KISS1R* expression.

Measurement of $[Ca^{2+}]_i$ in single cells

$[Ca^{2+}]_i$ was evaluated in cells derived from five different GH-producing adenomas in which *KISS1R* expression was previously confirmed by PCR analysis. A prominent, significant rise in $[Ca^{2+}]_i$ in response to kisspeptin administration was observed in cells from four of them. A detailed analysis of $[Ca^{2+}]_i$ recordings from all responsive cells ($n=71$ cells), showed that 1 μ M kisspeptin induced a biphasic increase in $[Ca^{2+}]_i$ in GH-producing cells (Fig. 2A). This response was characterized by a rapid and prominent $[Ca^{2+}]_i$ spike that gradually declined to near-basal levels. In contrast, the percentage of cells that responded to kisspeptin administration with a rise in $[Ca^{2+}]_i$ was variable among the four different examined cases (Fig. 2A inset). Quantitative analysis of different parameters that characterize the response to kisspeptin-10 administration reveals that all cases showed a similar $[Ca^{2+}]_i$ increment as well as a comparable time to reach the maximal response (Fig. 2A inset).

On the other hand, cultured cells derived from NFPA in which *KISS1R* expression was previously demonstrated by RT-PCR did not respond to kisspeptin administration with changes in their $[Ca^{2+}]_i$ basal levels. Immunocytochemical staining of *KISS1R*

on individual adenomatous pituitary-derived cells using fluorescent secondary antibody demonstrated its localization at the plasma membrane level, thus confirming the presence of *KISS1R* on studied cells (Fig. 2B).

Apoptotic rate in pituitary tumors upon kisspeptin administration

The effect of kisspeptin-10 on apoptosis was tested in cultured cells derived from NFPA and GH-producing adenomas expressing *KISS1R*. In NFPA, 12, 24, and 48 h incubation with increasing doses of kisspeptin-10 (10^{-9} to 10^{-6} M) were tested. After 12 h incubation in the presence of 10^{-6} M kisspeptin-10, a significant increase in the number of apoptotic adenomatous cells was observed (Fig. 3). Such effect was also noticeable after 24 h treatment with all kisspeptin doses tested. In contrast, no significant effect of kisspeptin-10 after 48 h treatment was observed for any of the evaluated doses on NFPA-derived cells (Fig. 3A). On the other hand, in GH-producing adenomatous cells, 24 h incubation with 10^{-6} M kisspeptin-10 elicited a significant increase in the number of apoptotic cells (Fig. 3B). As can be observed in a representative micrograph, several cultured adenomatous cells exposed to 10^{-6} M kisspeptin-10 for 24 h showed the typical DNA fragmentation that occurs during apoptosis as evidenced by DAPI staining (Fig. 3D).

Figure 2 (A) Representative profile of the $[Ca^{2+}]_i$ kinetics observed in response to 1 μ M kisspeptin-10 treatment (arrow) in single adenomatous somatotropes obtained from Case JA-0702. The table below illustrates the percentage of kisspeptin-responsive tumor somatotrope cells, the $[Ca^{2+}]_i$ maximal increase, and the time to reach the maximal rise in every studied case. (B) Primary cultured NFPA cells (2×10^4 /well) were incubated with the specific *KISS1R* antibody, fixed, incubated with the secondary FITC-conjugated antibody, mounted with the antifade containing reagent, and photographed with a $60\times$ magnification objective. FITC, NFPA cells observed with the FITC filter, revealing *KISS1R* immunofluorescence; u.v., NFPA cells observed with the u.v. filter, revealing nuclear immunofluorescence; u.v. + visible: merged u.v. and visible images.

Figure 3 (A and B) The time (12, 24, and 48 h) and dose (10^{-6} , 10^{-7} , and 10^{-9} M) effects of kisspeptin-10 on apoptotic rate of human NFPA and GH-producing cultured cells, respectively, are shown. Tumor cells were cultured *in vitro* in the absence (control) or presence of kisspeptin-10. One thousand cells were randomly counted per treatment and determined to be normal or apoptotic upon morphological characteristics. Results are expressed as percentage of apoptotic cells (mean $\pm$ s.e.m.) from at least seven and a maximum of ten NFPA tumors and from at least three somatotropinomas. * $P < 0.05$, compared with control. (C and D) Representative micrograph of apoptotic cells derived from pituitary tumor exposed to 10^{-6} M kisspeptin-10 for 24 h. Arrows indicate apoptotic cells in which chromatin condensation/nuclear fragmentation has been induced by kisspeptin treatment.

Discussion

Earlier reports showed that the KISS1/KISS1R system plays an important role in tumor progression in a wide variety of tumor types (reviewed in (19)). More recently, its presence at the pituitary level and its direct action on adenohypophyseal hormone release have been demonstrated in several species (14, 20, 21). However, to date, the presence and potential role of the KISS1/KISS1R system in pituitary adenomas has not been reported yet. Our present data show an altered expression of genes encoding kisspeptin and its receptor in different types of pituitary adenomas, which contrasts to their concomitant expression in normal human pituitary. Furthermore, we provide evidence on functional responses elicited by kisspeptin in cultured adenomatous pituitary cells, in which kisspeptin-10 directly acts on GH-producing adenomatous cells by increasing $[Ca^{2+}]_i$ and by inducing apoptosis in KISS1R-expressing pituitary tumors.

A compelling body of evidence indicates that the participation of KISS1/KISS1R tandem is critical for the neuroendocrine regulation of reproduction, primarily acting through GnRH secretion at the hypothalamus (11, 12, 22, 23). Although initial reports documented

high KISS1R levels at the pituitary (3–5), its putative physiological roles as well as those of their natural ligands, kisspeptins, in this gland have remained elusive and focus of speculation. Recently, our group and others demonstrated that kisspeptins also act directly at the pituitary level by releasing both LH and GH (14, 20, 21), thus offering new evidence to understand the functional significance of the reported presence of KISS1R at the pituitary, where it is also accompanied by the expression of its ligands (14, 24). Furthermore, on the basis of data from gene expression analyses on pituitary as well as direct functional responses at both the individual and population cell level, there is now evidence suggestive that KISS1/KISS1R system is not only involved in the control of gonadotropes, but can also regulate somatotropes, thereby expanding the potential neuroendocrine functions for this neuropeptide system (5, 14, 25). In this context, the concomitant expression of both KISS1 and KISS1R transcripts in normal pituitary samples reported herein further substantiates the contention that autocrine and/or paracrine interactions of kisspeptin and its receptor may occur in normal human pituitary gland, similar to that previously documented for KISS1/KISS1R system in an invasive tissue as placenta (4) and in papillary cancers (26).

Interestingly, expression of KISS1 and KISS1R is altered in pituitary adenomas, with a high proportion of them being defective in the simultaneous expression of both transcripts. This partial or complete loss of expression of the KISS1/KISS1R system in pituitary adenomas suggests that a functional involvement of these genes in the etiology of pituitary tumors should not be discarded, as it has been reported elsewhere for several neuropeptides as galanin, neurotensin, vasopressin, and cholecystokinin (27, 28). Likewise, expression levels of somatostatin receptors often show pathophysiologically relevant alterations in pituitary tumors (29), and it will be of interest to analyze the possible relationship between these two inhibitory systems, KISS1/KISS1R and somatostatin/somatostatin receptors, at the pituitary level, especially in GH adenomas. In line with this, although the precise relevance and underlying mechanism of the uneven expression of KISS1/KISS1R in human pituitary adenomas are yet to be elucidated, it is not unreasonable to propose that understanding the impaired expression of this molecular tandem may result, in the future, of therapeutic or clinical relevance in the outcome of patients with pituitary adenomas, as it has been already anticipated in several malignancies, including melanoma (30, 31), carcinoma of the ovary (32), stomach (33), urinary bladder (34), and esophagus (35). In support of this view, our present results are indicating that all the required elements for the KISS1/KISS1R system to be functional are in place in the human pituitary.

To the best of our knowledge, the present study is the first to characterize one of the elements of the signaling

pathway triggered by KISS1R upon kisspeptin-10 administration in single cultured adenomatous cells. Specifically, kisspeptin-10 administration elicited a rapid and prominent $[Ca^{2+}]_i$ increase in GH-producing adenoma cells. Previous studies have shown that metastatin and shorter kisspeptin forms induce a potent and specific increase in $[Ca^{2+}]_i$ in different types of cells (36), including cell lines transfected with KISS1R as CHO-K1 cells (5), HEK293 (4), Cos-7 cells (37), human trophoblasts (38), cultured hippocampal neurons (25), and in primary rat gonadotropes and somatotropes (14). Our present data provide strong evidence that kisspeptin-10 directly acts on human GH-producing adenomatous cells by increasing $[Ca^{2+}]_i$, a well-known, pivotal second messenger directly and necessarily linked to hormone release (39, 40). Perhaps the tight link between Ca^{2+} signaling and secretion may help explain the unique lack of response shown by the studied NFPAs in terms of $[Ca^{2+}]_i$ dynamics, in that these type of tumors are often refractory to stimulation and hardly respond to releasing factors by secreting significant levels of hormones, as opposed of the intrinsic sensitivity of GH-producing adenomas, which secrete massive GH levels in response to an exogenous stimulus most often via Ca^{2+} signaling.

Of note, however, our data reveal that the functional capacity of the KISS1/KISS1R system in pituitary adenomas is not restricted to Ca^{2+} signaling, but can be associated to a more clinically relevant process in these tumors, i.e. apoptosis. Indeed, it is known that pituitary tissue homeostasis results from the balance between cell proliferation and programmed cell death or apoptosis. Consequently, the impairment in cell death mechanisms derives in tumor genesis and progression (41). In the present work, we analyzed the potential effect of kisspeptin administration on the apoptotic rate in KISS1R-expressing pituitary adenomas, particularly on GH-producing and NFPA. This revealed that kisspeptin-10 exposure for 12 or 24 h (for NFPA) or 24 h (for GH-producing adenomas) caused a significant increase in the number of apoptotic cells. Thus, the ability of kisspeptin-10 to induce apoptosis shown here, coupled to the reported evidence for increased basal level of apoptosis following KISS1R activation described in other cell systems as human mammary carcinoma cell line (42), HEK293 or Jurkat cells (43), and in light of the originally documented metastasis inhibiting properties of KISS1 gene (3), makes the KISS1/KISS1R tandem an attractive target for drug design and research, as a potential new therapeutic target for the management of certain pituitary adenomas.

In summary, our results provide evidence that the expression pattern of the KISS1/KISS1R system is distinctly altered in pituitary adenomas; also, that kisspeptins directly stimulate Ca^{2+} signaling in GH-producing adenoma cells; and finally, that kisspeptins augment apoptotic rate in cells from both GH-producing and NFPA. These findings emphasize the importance of

developing additional studies to attain a more complete understanding of the KISS1/KISS1R system, their gene products, and functional capacities as a new, potentially relevant player in normal human pituitary and in pituitary adenomas.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-10-0905>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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