Ghrelin and cortistatin in lung cancer: Expression of peptides and related receptors in human primary tumors and \textit{in vitro} effect on the H345 small cell carcinoma cell line

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\textbf{ABSTRACT.} Ghrelin, a natural GH secretagog (GHS) acylated peptide, and cortistatin (CST), a natural SRIF-like peptide, interfere with neoplastic growth in different cancers. We tested forty-one lung carcinomas and the H345 small cell lung carcinoma (SCLC) cell line by RT-PCR to investigate the presence of ghrelin and CST and related receptors, including type 1a GHS receptor (GHS-R1a), all SRIF-receptor subtypes (sst 1-5) and MRGX2. Moreover, the presence of ghrelin and CST peptides was studied in both tumors and H345 cells. Ghrelin and CST mRNA were present in the majority of tested tumors, but ghrelin and CST proteins were revealed only in tumors with a neuroendocrine phenotype. All the receptors mRNA had a heterogeneous expression without correlation between ghrelin (or CST) and their receptor distribution. All the transcripts, but not GHS-R1a, were expressed in H345 cells. However, ghrelin and desacyl ghrelin induced \textit{in vitro} a dose-dependent inhibition on the H345 cell proliferation and increased apoptosis. Conversely, neither CST nor SRIF affected H345 cell growth, despite the presence of their specific receptors. The anti-proliferative and the pro-apoptotic effects of ghrelin were consistent with binding experiments on H345 cell, where either acylated or des-acylated ghrelin recognized a common binding site. In conclusion, the present study indicates that: a) ghrelin and CST mRNAs are expressed in lung cancers, although some neuroendocrine tumors contain detectable amounts of the peptides; b) GHSR-1a mRNA is present exclusively in neuroendocrine tumors, whereas MRGX2 mRNA (but not peptide) is expressed in all histological types; c) both ghrelin forms inhibit H345 cell proliferation, both directly and enhancing apoptosis, despite the absence of GHS-R1a, whereas CST and its receptors do not interfere with cell growth.

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\textbf{INTRODUCTION}

Ghrelin is a recently identified gastrointestinal hormone (1, 2), with complex functions including, among others, effects on GH release, gastrointestinal motility, control of food intake and regulation of insulin secretion (3-7). It is an acyl-peptide consisting of 28 amino acids and esterified with octanoic acid on Ser3, although a form of 27 amino acids, des-Gln14-ghrelin (resulting from alternative splicing of the same gene) has also been isolated (8). Both forms exist in either acylated and, more abundantly, des-acylated forms (9), but the latter have been shown to be inactive in terms of GH-releasing activity \textit{in vivo} (10, 11). Apart from “normal” tissues (1, 12, 13), various tumors have also been reported to express ghrelin: ie, pituitary adenomas, gastrointestinal carcinoids, pancreatic neoplasms, prostate and thyroid carcinomas (13-18). The effects of ghrelin are mediated by specific receptors termed GH secretagog receptors (GHS-R): to date, two GHS-R subtypes have been identified, derived by alternative splicing of a single gene and belonging to the family of G protein-coupled receptors with seven transmembrane domains, GHS-R1a and its truncated counterpart, GHS-R1b, lacking the transmembrane domains 6 and 7 (19, 20). So far, GHS-R1a, that does not bind des-acylated ghrelin, seems to be the only functionally active receptor
for ghrelin, at least in an endocrine-regulating context: however, the role of GHS-R1b, that fails to bind acylated and des-acylated ghrelin and is not known to exhibit any GHS or ghrelin-mediated biological activity, remains to be clarified in tumors (21). Among the various non-endocrine actions of ghrelin, in neoplastic cells (as observed in prostate, thyroid, breast and adrenal tumors), modulation of cell growth seems to be variably exerted: alternatively, ghrelin has been reported to inhibit, enhance or un-affect neoplastic cell proliferation (19, 22-24). As an additional “confusing” observation, the binding pattern of GHS-R in some neoplastic tissues, such as breast and prostate tumors, (22, 23) seems to suggest the possible existence of alternative, as yet unidentified receptors (common for ghrelin and des-acylated ghrelin) (21, 24).

At present, few data are available on the GHS-R1a distribution in a small series of human lung tumors (25), but no data are available either on the expression of ghrelin in human lung carcinomas (neuroendocrine or non-neuroendocrine) or on its biological effect on small cell lung carcinoma. Cortistatin is a 17-aminoacid neuropeptide, which exhibits relevant structural homology to SRIF and binds to a specific receptor (MRGX2), to all five SRIF receptors (sst 1-5) and interestingly to GHS-R1a, suggesting a possible crosstalk between the ghrelin and the cortistatin (CST) systems (17, 26). Through these different binding sites, CST displays SRIF-like effects as well as distinct biological activities (26, 27). To date, very few data are available on a) the distribution of CST and MRGX2 in neoplastic tissues (28) and b) the biological effect of CST on neoplastic cell proliferation (29). However, as for SRIF or ghrelin, the hypothesis of a CST/MRGX2 role in “neuroendocrine” tumors is challenging and worth being looked into. Specifically, because of the frequent expression of sst in endocrine lung cancers, we were intrigued by the possibility of a CST expression and/or biological effect in small cell lung carcinoma, mediated by sst or MRGX2.

Therefore, based on the foregoing, the aims of the present study were to investigate: a) the expression of ghrelin and CST as well as of their binding sites in a series of neuroendocrine and non-neuroendocrine human lung cancers; b) the effects of both acylated or des-acylated ghrelin and CST on cell proliferation in the small cell lung carcinoma H345 cell line. We here show that mRNA of ghrelin (but not of its known receptors) as well as of CST and MRGX2 are expressed in most lung cancers and in H345 cells, despite their minimal expression at peptide level; that both ghrelin and des-acyl ghrelin, but not CST, significantly inhibit the proliferation of the small cell lung cancer H345 cell line, both directly and through apoptosis enhancement.

MATERIALS AND METHODS

Reagents

Human ghrelin (either octanoylated or des-octanoylated), human [Tyr6]-ghrelin, cortistatin-(1-14) (CST-14), Pro-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys)-Lys-NH2 were synthesized by conventional solid-phase synthesis and purified to at least 98% purity by high-performance liquid chromatography (HPLC) by NeoMPS (Strasburg, France). SRIF-(1-14) (SRIF-14, Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)] was commercially available (Bachem AG, Bubendorf, Switzerland). The tyrosine phosphatase inhibitor orthovanadate (OV), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay and all cell culture reagents were purchased from Sigma-Aldrich (Milan, Italy). [Tyr6]-ghrelin was radioiodinated ([125I]-Tyr6)-ghrelin, specific activity 2000 Ci/mmol) using a lactoperoxidase method and purified by HPLC by Amersham Biosciences (Bucks, UK).

Tissue samples and cell line

Forty-one resected lung tumors [15 adenocarcinomas (ADC), 12 squamous cell carcinomas (ScCC), 14 neuroendocrine tumors, including 9/9 typical carcinoids (TC), 3/3 atypical carcinoids (AC), 2/2 small cell carcinomas (SCLC)] were collected from the Department of Pathology of the University of Turin. Tumors were classified according to the World Health Organization (WHO) classification of lung cancer (30). All patients gave their informed consent for the research use on their tissues and the study obtained ethical approval by an independent local Ethical Committee. Human SCLC H345 cell line was purchased from the American Tissue Culture collection (Rockville, MD, USA) and was grown as a monolayer in RPMI medium (Gibco, Paisley, Scotland) with 10% fetal calf serum (Gibco) at 37 C in a 5% CO2 humidified atmosphere.

Detection of mRNA for ghrelin, GHS-R1a, CST-14, sst and MRGX2 by RT-PCR and Southern blot analysis

Total RNA was extracted from the H345 human SCLC cell line and from 41 primary lung carcinomas using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer’s recommendations. The concentration of RNA was estimated by spectrophotometry, and RNA degradation was assessed by 2% agarose gel electrophoresis. Total RNA (1 µg) was first digested, with 10 U of RNase-free DNase (Boehringer, Mannheim, Germany) in a 10 µl solution containing 2 mM MgCl2, to avoid DNA contamination. The solution was kept at room temperature (RT) for 10 min, then heated for 5 min at 70 C to inactivate DNases; 40 µM of oligodeoxynucleotide primer (oligo dT16) were added and the solution heated at 70 C for 10 min, then chilled on ice to allow primer hybridization. The final solution was reverse transcribed using 200 U of SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following manufacturer’s instructions in the presence of 40 U/µl of Recombinant RNasin Ribonuclease Inhibitor (Promega, Madison, USA). Negative control samples for further PCR amplification included omission of the reverse transcriptase enzyme.

RNA quality was assessed by amplification of β2-microglobulin mRNA; primers and probes for PCR amplification and Southern blot analysis (SBA) for β2-microglobulin, GHS-R1a, all five sst-1-5, CST-14, MRGX2 and ghrelin are reported in Table 1, whereas PCR conditions are described elsewhere (28).

PCR products were then visualized under ultraviolet (UV) light in Z 2% agarose gels containing ethidium bromide. Negative control