Knocking down gene expression for growth hormone-releasing hormone inhibits proliferation of human cancer cell lines

N Barabutis1,2 and AV Schally*,1,2
1Veterans Affairs Medical Center and South Florida Veterans Affairs Foundation for Research and Education, Miami, FL 33125, USA; 2Department Of Pathology and division of Hematology/Oncology, Department of Medicine, University of Miami, Miller School of Medicine, Miami, FL 33125, USA

Dysfunction of cell differentiation and cell cycle regulation define and promote carcinogenesis. Growth hormone-releasing hormone (GHRH) was first isolated from human pancreatic tumours and only subsequently identified in human and animal hypothalami (reviewed in Schally and Varga, 2006). The full intrinsic biological activity of GHRH is retained by the NH2-terminal 29 amino acid sequence. Growth hormone-releasing hormone is secreted by the hypothalamus and upon binding to the specific GHRH-Receptors (GHRH-R) on somatotrophs regulates the release of Growth Hormone (GH) from the anterior pituitary gland. In turn, GH stimulates the production of insulin-like growth factor-I (IGF-I), which functions as a cell cycle stimulator (Macaulay, 1992; Westley and May, 1995).

Growth hormone-releasing hormone receptors (GHRH-R) is a class II G protein-coupled receptor and contains seven transmembrane domains (Mayo, 1992). Growth hormone-releasing hormone receptor is homologous with the receptors for the vasoactive intestinal peptide (VIP), the pituitary adenyl cyclase activating peptide and calcitonin (Gaylinn et al, 1993). Recently, peptide receptors that mediate the effects of GHRH and its antagonists on tumours were identified. The isolation and sequencing of cDNAs which correspond to the tumoral GHRH receptor mRNA revealed that they are splice variants (SVs) of the pituitary GHRH receptors (pGHRH-R) (Rekasi et al, 2000).

Splice variant 1 of GHRH receptor is a functional receptor, which differs from the pGHRH-R only in the N-terminal extracellular domains. The first 89 amino acids of the pGHRH-R are replaced in SV-1 receptor by a different 25-amino acid sequence (reviewed in Schally and Varga, 2006). Some tumours also express pituitary type of GHRH receptor (Havt et al, 2005; Christodoulou et al, 2006). Besides its ligand-dependent activity, a ligand-independent activity of SV1 has also been demonstrated (Kiaris et al, 2003). A recent study showed the stimulation and proliferation of MCF-7 breast cancer cells after the transfection of SV1 (Barabutis et al, 2007). The expression of mRNA for GHRH and the presence of biologically or immunologically active GHRH were demonstrated in several established cancer cell lines and human tumours. Collectively, those data suggest that GHRH may function as a growth factor among a large class of mitogens involved in tumorigenesis. In an endeavour to develop new methods for cancer treatment, we developed the antagonists of GHRH (reviewed in Schally and Varga, 2006).

Growth hormone-releasing hormone antagonists suppress the in vivo growth of various experimental cancers such as prostatic (Zarandi et al, 2006; Stangelberger et al, 2007), mammary (Buchholz et al, 2007), ovarian (Chatzistamou et al, 2001), renal cell carcinomas (Halms et al, 2000), small cell lung carcinomas...
MATERIALS AND METHODS

Peptides and chemicals

Growth hormone-releasing hormone antagonists JMR-132 [PhAc, DArg², Phe (4-CI)⁶, Ala⁸, Har², Tyr (Me)¹⁰, His¹¹, Nle¹², D-Arg¹³, Har¹⁴] human GHRH (1–29)NH₂, MZ-5-156 [PhAc-Tyr¹, D-Arg², Phe (4-CI)⁶, Abu¹⁵, Nle²⁷] hGHRH (1–28)Agm and MZ-4-71 [Ibu-Tyr¹, D-Arg², Phe (4-CI)⁶, Abu¹⁵, Nle²⁷] hGHRH (1–28)Agm were synthesised in our laboratory by solid phase methods (1, 19). Growth hormone-releasing hormone (1–44)NH₂ and 30% with human GHRH (1–29)NH₂.

Cell culture

The cell lines (LNCaP, MCF-7, MDA-MB-468, MDA-MB-435s, T47D, and NCI-H838) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C in a humidified 95% air/5% CO₂ atmosphere. Breast cancer cell lines MCF-7, MDA-MB-468, MDA-MB-435s and T47D were cultured in humidified 95% air/5% CO₂ atmosphere. Breast cancer cell lines, LNCaP prostate cancer cell line and NCI H838 non-SCLC cell line growing for 0, 24, 48 and 72 h were assayed for GHRH immunoreactivity.

Protein isolation and western blot assay

The expression of GHRH and SV1 receptor was assayed by western blot to T47D, MDA-MB-435s, MDA-MB-468 breast cancer cell lines, LNCaP prostate cancer cell line and NCI H838 non-SCLC cell line growing for 0, 24, 48 and 72 h were assayed for GHRH immunoreactivity.

Radioimmunoassay of GHRH

Tumour cells (3 × 10⁵ cells) were seeded in 48-well petri dishes and allowed to attach for 24 h when the media were replaced by serum-free medium (SFM). Aliquots of 0.1 ml medium from MCF-7, T47D, MDA-MB-435s, MDA-MB-468 breast cancer cell lines, LNCaP prostate cancer cell line and NCI H838 non-SCLC cell line growing for 0, 24, 48 and 72 h were assayed for GHRH immunoactivity.

Transfection

Small interfering (si) RNA designed specially for the inhibition of the human GHRH was used to knock down the GHRH gene expression of the T47D, MDA-MB-435s, MDA-MB-468 breast cancer cell lines, LNCaP prostate cancer cell line and NCI H838 non-SCLC cell line. A pool of three oligonucleotides especially designed for the inhibition of the human GHRH (sc39519, Santa Cruz Biotechnology) and cationic liposomes (Lipofectamine, Invitrogen, Carlsbad, CA, USA) were used to knock down the gene expression of the GHRH. The sequence of the sense strand (b) was CCAGUUAAUCCUCU CAUUUTT and its mRNA location is 434. RNA that does not lead to any specific degradation of any known cellular mRNA (control siRNA, sc57007, sc57007, sc57007).
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Santa Cruz Biotechnology) was used as control. The sequence of the sense strand was UUCUCGGAACUGUCACGU. One day before the transfection, the cells were plated in six-well plates in 2 ml of growth media without antibiotics to be 40% confluent on the day of transfection. For the transfection 40–60 nM siRNA was diluted in 0.25 ml Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA) without serum and 5 μl lipofectamine 2000 were diluted in an equal volume of Opti-MEM I. After incubation for 5 min at room temperature, the diluted oligomers were combined with the lipofectamine 2000 and incubated for 30 min also in room temperature. The oligomer-lipofectamine complexes were added to each well, which contained 2 ml of medium without antibiotics. The medium was changed 8 h after transfection. The cells were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air for 48–72 h after transfection and then assayed by western blot for gene knockdown.

Cell proliferation rate assay
The rate of the cell proliferation was calculated by seeding 10 000 cells in six-well plates and after an incubation for 4 days counting them under light microscope using the trypan blue assay.

Statistical analysis
These data are expressed as the mean ± s.e.m. Statistical evaluation of the results was performed by the Student’s t-test (two-tailed). P-values shown are against the control group.

RESULTS
Expression of growth hormone releasing hormone and splice variant 1 of the GHRH receptor in breast and prostate cancer and non-SCLC cell lines
A band of 45 KDa which reflects the production of pre-pro GHRH (Othman et al., 2001) was detected in all the cancer cell lines examined. The results are shown in Figure 1. Pre-pro GHRH protein expression was the highest in T47D (R.I:0.464), followed by MDA-MB-435s (R.I:0.449), NCI H838 (R.I:0.437), MDA-MB-468 (R.I:0.312) and LNCaP (R.I:0.193). A band at 39.5 KDa which is consistent with the size of the SV1 receptor (Havt et al., 2005) was also detected in all the cancer cell lines examined. The results are shown in Figure 2. The levels of the SV1 GHRH-R protein were the highest in T47D (R.I:0.696), followed by NCI H838 (R.I:0.376), MDA-MB-435s (R.I:0.316), LNCaP (R.I:0.169), MDA-MB-468 (R.I:0.160).

Detection of the secretion of growth hormone-releasing hormone in the conditioned medium of the cancer cell lines by radioimmunoassay
The concentration of the GHRH in samples from culture medium was measured by RIA. Significant amounts of GHRH were detected in the medium from T47D (1.518, 2.083, 0.266 ng ml⁻¹), NCI H838 (0.303, 0.442, 0.689 ng ml⁻¹), MDA-MB-435s (0.675, 1.036, 1.442 ng ml⁻¹), LNCaP (0.202, 0.218, 0.208 ng ml⁻¹), and MDA-MB-468 (0.637, 0.816, 0.649) cell lines after 24, 48 and 72 h respectively as shown in Table 1. Growth hormone-releasing hormone was not detected either in the cultured medium without cells or in the conditioned medium of the MCF7 breast cancer cell line.

Inhibition of the GHRH gene expression in breast, prostate and non-SCLC cancer cell lines and its effect on proliferation
The effective inhibition of the GHRH gene expression was confirmed by western blot. The results are seen in Figure 3. The GHRH expression in the transfected NCI H838, LNCaP, T47D cell lines was suppressed by 85, 85 and 92% respectively. The decreased proliferation rate of the MDA-MB-468 and MDA-MB-435s cell lines did not allow us to isolate protein from those cells during early cell passages. Since the silencing of the gene expression for GHRH lasts only for limited cell passages, the western blots of the proteins obtained in late cell passages show

Table 1  Production of GHRH in culture medium from human breast cancer (MCF-7, MDA-MB-468, MDA-MB-435s, T47D), lung cancer (NCI-H838) and prostate cancer (LNCaP) cell lines. MCF-7 breast cancer cell line was used as negative control. Signal intensity of the 45 kDa precursor GHRH protein is indicated by the symbol ++. Protein levels were normalised to β-actin signal.

| Time (hour) | MCF-7 | T47D | MDA-MB-468 | MDA-MB-435s | LNCaP | NCI H838 |
|------------|-------|------|------------|-------------|-------|---------|
| 0          | ND    | ND   | ND         | ND          | ND    | ND      |
| 24         | ND    | 1.518| 0.637      | 0.675       | 0.202 | 0.303   |
| 48         | ND    | 2.083| 0.816      | 1.036       | 0.218 | 0.442   |
| 72         | ND    | 0.266| 0.649      | 1.442       | 0.208 | 0.689   |

ND = not detectable. Aliquots of medium were subjected to RIA for the detection of GHRH at the indicated periods of time.
that the silencing of the GHRH gene expression was less effective for the two breast cancer cell lines in these passages (28 and 64% respectively). After the knocking down of GHRH expression, the proliferation rate of the T47D, MDA-MB-435s, MDA-MB-458, LNCaP, NCI H838 human cancer cell lines was decreased by 28.3, 32, 29, 143, 112, 134, 140% respectively. MZ-4-71 did not affect the proliferation of MCF-7 breast cancer cell line, which was also transfected with siRNA for GHRH. The results are shown in Figure 5.

**Effect of GHRH(1–29)NH2 and GHRH antagonists on the proliferation of cancer cell lines in vitro**

When knocked down T47D, MDA-MB-486, MDA-MB-435s breast, LNCaP prostate and NCIH-838 lung cancer cell lines were exposed to 0.1 μM. Growth hormone-releasing hormone (1–29)NH2 the proliferation rate of the T47D, MDA-MB-468, MDA-MB-435s, MDA-MB-435s, LNCaP and NCI H838 lung cancer cell lines were exposed to 0.1 μM concentrations. The results are shown in Figure 6.

**Effect of GHRH(1–29)NH2 and GHRH antagonists on the proliferation of cancer cell lines in vitro**

**Effect of GHRH(1–29)NH2 on the proliferation of the knocked down cancer cell lines in vitro**

When knocked down T47D, MDA-MB-486, MDA-MB-435s breast, LNCaP prostate and NCIH-838 lung cancer cell lines cultured in vitro were exposed to two concentrations of GHRH(1–29)NH2 and GHRH antagonists MZ-5-156, JMR-132 and MZ-4-71. At the dose of 1 μM GHRH(1–29)NH2 did not appreciably influence the proliferation rate of the cells, producing a change only of 1–5%. However GHRH(1–29)NH2 at 0.1 μM concentration stimulated of the proliferation rate of T47D, MDA-MB-468, MDA-MB-435s, LNCaP and NCI H838 cells by 15, 17, 14, 14, 16% respectively. The proliferation of MCF-7 cells (negative control) was not affected by GHRH at 0.1 and 1 μM concentrations. The results are shown in Figure 6.

Growth hormone-releasing hormone antagonist MZ-4-71 at the dose of 0.1 μM decreased the proliferation of T47D, MDA-MB-486, MDA-MB-435s, LNCaP and NCI H838 cancer cell lines by 39, 35, 36, 35 and 33% respectively. MZ-4-71 did not affect the proliferation of MCF-7 cells (negative control) (Figure 7). Similar inhibitory effects on proliferation were obtained with the other two antagonists. Thus GHRH antagonist MZ-5-156 at doses of 0.1 and 1 μM reduced the proliferation of T47D, MDA-MB-486, MDA-MB-435s, LNCaP and NCI H838 cancer cell lines by 30–35,
The proliferation of T47Ds, MDA-MB-468, MDA-MB-435, LNCaP and NCI H838 cancer cell line was also inhibited by 31–37%, 26–31%, 31–38%, 34–41%, 37–42% respectively after exposure to GHRH antagonist JMR-132 at doses of 0.1 and 1 μM. Higher doses of JMR-132 produced a greater inhibition, indicated by the second set of numbers. The results are shown in Figure 9.

DISCUSSION

Growth hormone-releasing hormone and the major SV1 of the full length GHRH receptor are expressed in surgical specimens of diverse human cancers as well as in a various human cancer cell lines (reviewed in Schally and Varga, 2006). These findings led to the concept that GHRH may function as an autocrine growth factor in many human malignancies.

The precise role of the production of GHRH in the process of tumorigenesis and tumour progression has not been investigated previously. One way to elucidate the role of GHRH in the pathogenesis of cancer is to inhibit its gene expression. In the present study, we first examined the expression of pre-pro GHRH by western blot in human cancer cell lines. The band that reflects the production of pre-pro GHRH appeared to have a molecular weight of 45 kDa on the blots. The facts that this band disappeared in the knocked down cells and could not be detected in the negative control (MCF-7), together with the stimulation of the knocked down cells by exogenous GHRH, indicate that this band represents a precursor of GHRH (Othman et al., 2001). We also tried to...
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identify bands corresponding to GHRH (1 – 44)NH₂ by doing western blots for synthetic GHRH (1 – 44) NH₂ but no corresponding signal was detected. Biologically active GHRH was detected by RIA in the conditioned medium of all the cancer cell lines, except for MCF-7.

We then evaluated the expression of the SV1 of GHRH receptor by western blot. The expression of the GHRH and its receptor SV1 in the breast, prostate and non-SCLC cancer cell lines examined suggested the presence of a stimulatory loop in those cells based on GHRH and its receptors. This raised the issue of establishing the role of GHRH in these cancer lines.

Small interfering siRNA for GHRH was used to elucidate the exact function of GHRH. The siRNA for GHRH is a pool of specifically designed RNAs for knocking down the expression of human GHRH. After the transfection of the siRNA for GHRH, the proliferation rate of the MDA-MB-468 and MDA-MB-435s cancer lines was dramatically decreased by 85.1 and 85.9% respectively. The decreased proliferation, in combination with the fact that the silencing of the gene expression for GHRH lasts only for limited cell passages, did not allow us to isolate protein from these cells for western blot analysis during early cell passages. The isolation of the proteins obtained in late cell passages showed that the silencing of the GHRH gene expression was less effective in these passages.

In the case of the T47D cells the proliferation rate was decreased by 28.3%. The T47D cell line has the highest expression of SV-1 of the GHRH receptor with its ligand-dependent and independent activity. The inhibition of the gene expression for GHRH in T47D breast cancer cell line did not have the potent antiproliferative effect found in the other two breast cancer cell lines (MDA-MB-468 and MDA-MB-435S), possibly because the ligand independent activity of the SV1 continued to enhance the proliferation rate of the T47D cells (Kiaris et al, 2003; Barabutis et al, 2007).

Prostate cancer cell line LNCaP and non-SCLC cell line NCI H838 showed decreases in proliferation rates of 51.8 and 48.4% respectively after the inhibition of the GHRH gene expression. Both these cancer cell lines express SV-1 and because of its ligand-independent activity their proliferation rate continued to be stimulated in the absence of the intrinsic production of the GHRH.

The possible presence of GHRH and other related peptides such as VIP in the media of the cells that were tested cannot be excluded. Thus GHRH could keep acting as a growth factor and the proliferation rate of all the cancer cell lines that were assayed may not reflect the conditions of a total absence of GHRH.

MCF-7 breast cancer cells were also transfected with siRNA for knocking down the GHRH gene. This was done to test for any possible toxic effects, which could be related to the presence of the siRNA because MCF-7 line does not produce GHRH, its behaviour after the transfection had to remain the same. The transfection of the siRNA for GHRH did not influence the proliferation rate of the MCF-7 cells. Consequently the decreased proliferation rate of the breast, prostate and non-SCLC after the transfection was not due to toxic effects. The transfection of control siRNA did not lead to any changes in the proliferation of these cells.

In addition, we exposed the knocked down MDA-MB-468, MDA-MB-435s, T47D, LNCaP and NCI-H838 cancer cell lines to two different concentrations of GHRH (1 – 29)NH₂. The proliferation rate of the cells was strongly stimulated by the addition of exogenous GHRH, not only at the concentration of 0.1 μM but also at a concentration of 1 μM. The silenced cancer cell lines, lacking intrinsic GHRH, were still able to respond to exogenous GHRH, confirming the absence of possible toxic effects related to the transfections. MCF-7 cells transfected with siRNA for GHRH were also exposed to GHRH. The proliferation rate of the transfected MCF-7 cells was not influenced by the addition of the exogenous GHRH.

We also investigated the effect of GHRH and GHRH antagonists at two concentrations in MCF-7, MDA-MB-468, T47D, MDA-MB-435s, LNCaP and NCI-H838 cancer cell lines. The proliferation rate of the MCF-7 breast cancer cell line was not influenced by the presence of the GHRH or its antagonists, since MCF-7 does not express specific receptors for GHRH. At the dose of 0.1 μM GHRH stimulated the proliferation rate of the cancer cell lines by 15 – 17%. However, at a dose of 1 μM GHRH did not influence significantly the proliferation rate of cell lines. Thus, because of the possible presence of GHRH or other related peptides in the medium, as well as of GHRH secreted by the cells, it is likely that the corresponding signalling pathways were saturated after exposure to 1 μM GHRH.

These breast, lung and prostate cancer cell lines were also exposed in vitro to two concentrations of GHRH antagonists MZ-4-71, MZ-5-156 and JMR-132. At concentrations of 0.1 and 1 μM, the proliferation rate of these cells was decreased by 26 – 37% and 31 – 42% respectively.

The present study demonstrates the tumorigenic effect of GHRH in the human experimental tumour cell lines representative of leading cancers. Our work supports the concept that GHRH functions as growth factor in human cancers.

ACKNOWLEDGEMENTS

The work described in this paper was supported by the Medical Research Service of the Veterans Affairs Department and a grant from Zentaris AG, Frankfurt on Main, Germany to South Florida Veterans Affairs Foundation for Research and Education and University of Miami Miller School of Medicine Departments of Pathology and Medicine Division of Heratology/Oncology (all to AVS). NB is a recipient of a fellowship from the Alexander S Onassis Public Benefit Foundation. This paper is dedicated to Eleni, Asteria and Aristides Barabutis for inspiring one of us (NB).
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