ORIGINAL ARTICLE

Growth Hormone-Releasing Hormone stimulates the secretion of interleukin 17 from human peripheral blood mononuclear cells *in vitro*

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Abstract

OBJECTIVES: Growth hormone-releasing hormone (GHRH) plays a crucial role in the secretion of GH from the pituitary, acts as a growth factor in variety of cancer cells and possesses immunomodulatory activity. Interleukin(IL)-17 apart from its pro-inflammatory role has been also shown to play a role in carcinogenesis. The effect of GHRH on the IL-17 has not been studied so far.
AIM: To evaluate the effect of GHRH on the secretion of IL-17 from human peripheral blood mononuclear cells (PBMC) *in vitro*.
MATERIALS AND METHODS: The concentrations of IL-17 in supernatants from PBMC cultured for 24hrs were assessed using ELISA kit.
RESULTS: We show for the first time that GHRH can stimulate the secretion of IL-17 from human PBMC in 24hrs culture, and that GHRH antagonist counteracts this effect.
CONCLUSION: Our study further elucidates the immunomodulatory role of GHRH.

INTRODUCTION

Numerous studies indicate the bidirectional communication between the neuroendocrine and immune systems (Besedovsky & del Rey 1996). This interaction is supported by the presence of many hypothalamic peptides and their receptors in the immune system (Besedovsky & del Rey 1996). Immunoreactive GHRH and its mRNA as well as GHRH-binding sites have been found in rodent lymphocytes (Guarcello *et al.* 1991; Weigent & Blalock 1990; Weigent *et al.* 1991). Also human leukocytes were shown to secrete a biologically active GHRH (Khorram *et al.* 2001).

The physiological role of immune-derived GHRH is not well understood. It has been shown that supraphysiological concentrations of GHRH inhibited the chemotactic response of human peripheral lymphocytes (Zelazowski *et al.* 1989) and natural killer cell activity *in vitro* (Sirianni *et al.* 1992). GHRH also stimulated mitogen-induced secretion of interleukin (IL)-2, soluble receptor for

IL-2 alpha (sIL-2alpha)(Siejka *et al.* 2005), and interferon gamma (Siejka *et al.* 2004) from human peripheral blood mononuclear cells cultured *in vitro*. In studies with elderly men and women, GHRH analog treatment induced an increase in the number of monocytes and percentage of B lymphocytes with no effect on the percentage of NK cells and total T lymphocytes and the rise in serum immunoglobulins and sIL-2R (Khorram *et al.* 1997). Taken together, studies in humans demonstrated that the administration of GHRH analog in humans has stimulatory effects on the immune system *in vivo*.

Recent studies indicate that extrahypothalamic GHRH acts as a locally active growth factor in various cancers (Barabutis & Schally 2008; Schally *et al.* 2008). Interestingly, levels of GHRH expression in hematologic tumor cell lines were higher than in the corresponding nonmalignant cells (Khorram *et al.* 2001). In addition, GHRH antagonists, which mechanism of action involves also inhibition of the peripheral action of locally produced GHRH (Schally *et al.* 2008), decreased the growth of human non-Hodgkin's lymphoma cell lines RL and HT *in vitro* and *in vivo* (Keller *et al.* 2005). In animal studies, the growth-promoting activity of GHRH on the immune system was shown in transgenic mice, in which expression of human GHRH resulted in the induction of thymic hyperplasia (Botteri *et al.* 1987) and increased splenic progenitor cell colony formation and DNA synthesis (Blazar *et al.* 1995).

Interleukin (IL)-17 is a family of cytokines produced mainly by T helper 17 (T_H17) cell subset, with IL-17A being the most abundant in humans (Gaffen 2009). Beside the important role of this cytokine in protecting the host against extracellular pathogens, it has been also shown to promote inflammatory processes in autoimmune disease (Gaffen 2009). Moreover, the activation of T_H17 T cell responses was shown to promote colon tumorigenesis (Wu *et al.* 2009). IL-17 is also involved in the angiogenesis and tumor growth (Murugaiyan & Saha 2009) and the increased number of IL-17 positive or T_H17 infiltrating cells has been shown in variety of cancers (Murugaiyan & Saha 2009).

The effect of GHRH on the secretion of IL-17 has not been studied so far. In this report we evaluated if GHRH can modulate the secretion of IL-17 from mitogen-stimulated PBMC. We demonstrate that the levels of IL-17 in the supernatants of cultured PBMC are increased after the treatment with GHRH and that GHRH antagonist inhibits the GHRH-evoked secretion of this cytokine.

MATERIALS AND METHODS

Peptides

GHRH(1-44)NH_2 was purchased from Sigma (Sigma, USA) and JV-1-36, synthetic antagonist of GHRH, [PhAc-Tyr1, D-Arg2, Phe(4-Cl)6, Arg9, Abu15, Nle27, D-Arg28, Har29]hGH-RH(1-29)NH2] where PhAc – phenylacetic acid, Phe(4Cl) – 4chlorophenylalanine, Abu – α-aminobutyric acid, Har – homoarginine, was obtained from Bachem AG (Bubendorf, Switzerland). GHRH was dissolved in DMSO and JV-1-36 in acetic acid and then diluted in culture media. The final concentration of DMSO and acetic acid did not exceed 0.1%.

Subjects

Eight healthy volunteers (blood donors, men, age 24–28 yrs) were examined in every experiment. Subjects gave oral and written consent. Blood samples (40 ml) were taken between 08.00–09.00 am., with the subjects having fasted overnight. The project was approved by the Ethical Committe for Scientific Studies at Medical University of Lodz.

Cell culture preparation

Peripheral blood mononuclear cells (PBMC) were isolated as described previously (Siejka *et al.* 2004). The cells were suspended in RPMI-1640 medium with 10% heat inactivated FCS and distributed in 700 µl aliquots per disposable 24 well tissue culture plates (Nunclon Multidish 24 wells, NUNC) at the final concentration of 10^6 cells/ml. The cells cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 were stimulated by suboptimal dose (10 µg/ml) of phytohemaglutinin (PHA, Murex, UK) and after 2 h of preincubation peptides listed below were added to appropriate wells at various doses:

1. Growth hormone-releasing hormone (GHRH(1-44) NH_2, Sigma, USA) at the final concentrations of 10^{-10} and 10^{-8} M.
2. GHRH(1–44)NH_2 and GHRH antagonist JV-1-36 (Bachem AG, Switzerland) added together at the final concentrations of 10^{-8} M to study the interactions (JV-1-36 was added one hour before GHRH).

An equal volume of culture medium was added to the appropriate wells (in order to obtain the final volume of 1 ml in each culture well).

The incubation was stopped 24 h after the addition of the test peptides. The medium was collected and centrifuged at 1 250 g for 10 min at 20 °C. The supernatants were removed and stored at –80 °C until IL-17 determination.

Human IL-17 ELISA (Cat No: D1700; R&D Systems, USA) was used to measure IL-17 concentrations according to the manufacturer's recommendations (sensitivity: <15 pg/ml; intra-assay precision <7.3%; inter-assay precision <8.6%). The absorbance was measured on a microplate reader (METERTECH R960, USA) at 450 nm wavelengths.

Statistical analysis

The results present the representative data from two separate cell cultures (4 wells for each concentration in a single experiment) with similar results. All results

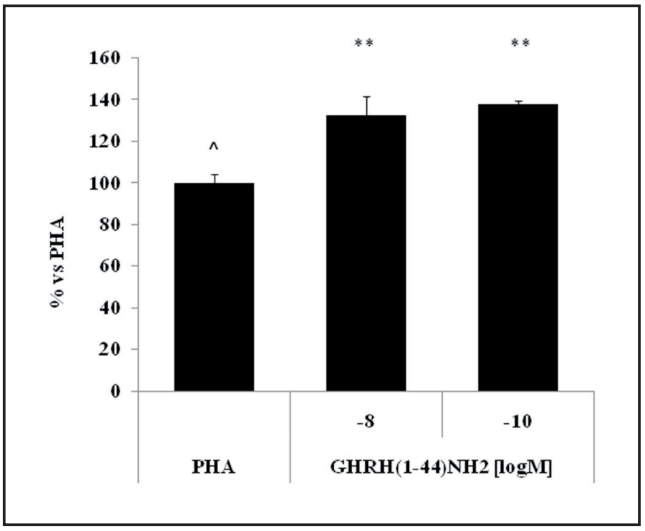

Fig. 1. Effect of human GHRH(1–44)NH_2 at 10^{-10} and 10^{-8} M concentrations on the secretion of IL-17 into the supernatants of PBMC cultured *in vitro* (C, control; PHA, phytohemaglutinin). Graphs represent the % vs PHA-stimulated cells. Data are shown as pooled mean values ± SE. ^ 0.001 vs control (unstimulated cells); ** 0.01 vs PHA.

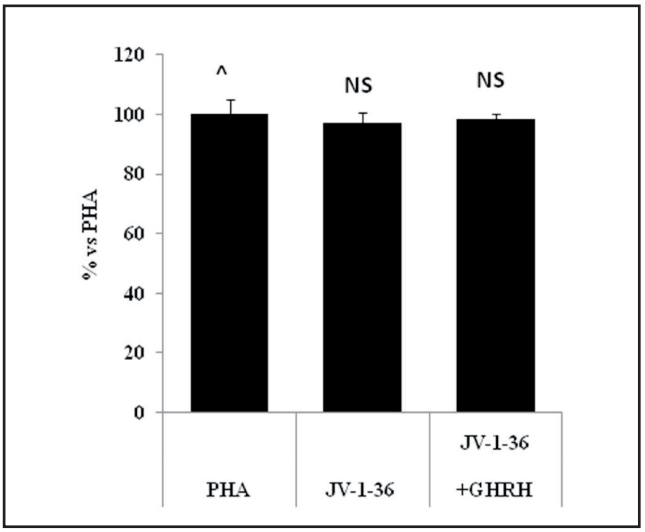

Fig. 2. Effect of GHRH antagonist JV-1-36 (10^{-6} M) given separately or together with GHRH (10^{-8} M) on IL-17 concentrations levels in supernatants of PBMC cultured *in vitro* (C, control; PHA, phytohemaglutinin). Graphs represent the % vs PHA-stimulated cells. Data are shown as pooled mean values ± SE. ^ 0.001 vs control (unstimulated cells); NS – not significant vs PHA-stimulated cells.

are expressed as percent of PHA-stimulated cells (means±SE). Comparisons between tested groups were made by one-way ANOVA followed by the least statistical difference (LSD) test. The differences were considered significant if $p<0.05$.

RESULTS

Cell viability in the presence of GHRH and JV-1-36 was not significantly different from that observed in control cultures (estimated by trypan blue dye uptake after 24 h of cell cultures incubation). Suboptimal dose of PHA significantly stimulated IL-17 production ($p<0.001$ vs control) (Figures 1 and 2). GHRH(1-44)NH_2 at 10^{-10} and 10^{-8} M concentrations, significantly ($p < 0.01$ for both concentrations) increased IL-17 concentration in supernatants of cultured cells by 38.01±1.25 and 32.6±8.85% respectively, as compared to the PHA-stimulated cells (Figure 1). JV-1-36 (GHRH antagonist) did not influence the release of IL-17 when given alone, but at 10^{-8}M inhibited GHRH-evoked secretion of IL-17 to the levels corresponding to PHA-stimulated cells (Figure 2).

DISCUSSION

Interactions between the neuroendocrine and the immune systems are now well recognized (Besedovsky & del Rey 1996; Komorowski *et al.* 2000). The expression of hypothalamic hormones and their respective receptors is common in the cells of immune origin (Besedovsky & del Rey 1996; Khorram *et al.* 1997). However their physiological role is not completely known.

Growth hormone-releasing hormone, apart from its physiological role as a hypothalamic neuropeptide, acts as a growth factor in variety of cancer cells (Schally *et al.* 2008). It binds the GHRH receptors (GHRH-Rs) (Mayo *et al.* 2000) and activates intracellular signals such as the phosphorylation of mitogen activated protein kinases (MAPK) (Barabutis *et al.* 2009; Pombo *et al.* 2000; Siriwardana *et al.* 2006; Zeitler & Siriwardana 2000) and Janus kinase (JAK) 2/Signal transductor and activator of transcription (STAT) 3 (Siejka *et al.* 2009). These pathways are linked with increased cellular proliferation rate (Mayo *et al.* 2000). STAT3 is crucial for the differentiation of T cells into IL-17 secreting subset (Gaffen 2009). In the present study we demonstrate that GHRH increases the secretion of IL-17 from mitogen-stimulated human PBMCs. Current report supports previous findings on the immunomodulatory effects of GHRH (Guarcello *et al.* 1991; Khorram *et al.* 2001; Khorram *et al.* 1997; Siejka *et al.* 2004; Siejka *et al.* 2005; Weigent & Blalock 1990; Weigent *et al.* 1991).

GHRH antagonists have been shown to be effective peptides with antitumor activity (Schally *et al.* 2008). In present study GHRH antagonist JV-1-36 inhibited the GHRH-evoked secretion of IL-17 into the supernatants of PBMCs cultured *in vitro*. In previous reports from our laboratory GHRH antagonists inhibited IFN-gamma secretion from human PBMCs (Siejka *et al.* 2004), VEGF secretion from murine endothelial cell line HECa10 (Siejka *et al.* 2003) and human neuroendocrine tumor cell line NCI-H727 (Sacewicz *et al.* 2008).

Many cytokines are part of the tumor-promoting communication between malignant cells and the immune system. Our results demonstrating increased secretion of IL-17 after treatment with GHRH are also interesting due to the link between this cytokine and cancer (Murugaiyan & Saha 2009). IL-17 has been shown to promote tumor growth of different malignant tumors, including colon (Wu *et al.* 2009), ovarian (Charles *et al.* 2009), melanoma and bladder (Wang *et al.* 2009) acting either directly on tumor cells and tumor-associated stromal cells, or through the induction of IL-6-STAT3 pathway with upregulation of pro-survival and proangiogenic genes (Mumm & Oft 2008; Wang *et al.* 2009). IL-17 is also associated with poor prognosis and/or reduced survival of human patients (Mumm & Oft 2008). It has been suggested that it can be a target for cancer immunotherapy, as is the transcription factor STAT3 (Kortylewski *et al.* 2009; Kortylewski & Yu 2008).

In conclusion, we show for the first time that GHRH stimulates the secretion of IL-17 from human peripheral blood mononuclear cells *in vitro*. We demonstrate also that this effect is inhibited by the pretreatment with GHRH antagonist. Collectively, our studies support the immunomodulatory effect of GHRH.

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