—**Original**—

Growth Hormone Enhances Natural Killer Cell Activity Against Glioma

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Abstract

Although it is now known that pituitary hormones activate the immune system, there are still numerous questions to be answered with regard to the mechanisms involved. In this study, which focuses on growth hormone (GH) and natural killer (NK) cells, the latter's antitumor effects on glioma were investigated. Using fluorescein isothiocyanate-labeled rat 9L glioma and NK-receptive YAC-1 cells as target cells and rat splenocytes as effector cells, a cytotoxicity assay was carried out with the fluorescence-activated cell sorter method, which stains dead cells with propidium iodide. The effector cells were pretreated 48 hours in advance with various concentrations of GH. A similar experiment was also carried out in the presence of anti-asialo-GM1 antibodies. When the GH concentration of 9L was 10 to 40 μg/m*l*, cytotoxicity was confirmed to have been enhanced 17% to 39%. This enhancement disappeared in the presence of anti-asialo-GM1 antibodies. A similar increase in cytotoxic activity was also confirmed in YAC-1 cells. In this experiment we observed GH enhancement of natural killer activity against glioma.

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Key words: growth hormone, 9L glioma, natural killer cell activity

Introduction

It has been known since the 1930s that removal of the pituitary gland in rats results in atrophy of the thymus gland[1], and it has also been reported in mice that administration of mouse anti-pituitary antibodies leads to atrophy of both the spleen and the thymus[2,3], suggesting that pituitary hormones have effects on the immune system.

While transplant rejection, decreased ability to produce antibodies, and a reduced mixed lymphocyte test reaction have been observed after removal of the pituitary gland in rats, improvement in symptoms after administration of growth hormone (GH)[4,5] has led to attention being focused on the involvement of GH in the immune system.

In humans, although it has been reported that natural killer (NK) activity in patients with acromegaly is equal to that in healthy adults[6], it has also been reported that GH administration in healthy adults leads to increased NK activity[7].

Furthermore, while it has been reported that no abnormality exists within the immune system in pituitary dwarfism[8], NK activity is also reduced[9], thus, a consensus on GH replacement therapy and how it is related to immune function has not been achieved.

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However, GH is believed to affect immune function in some way therefore in the present study, which focuses on GH and NK cells, we examined the effect of GH on NK cells ***in vitro*** by studying their antitumor effects on glioma cells.

Materials and Methods

Rats

Sixteen female Fisher rats aged 5 to 6 weeks were used.

Drugs

Recombinant human GH was purchased from Sigma-Aldrich (St. Louis, MO) and used to boost NK cell activity, and rabbit anti-asiolo-GM1 antibody (Wako Pure Chemical, Osaka) was used to block NK activity.

Target Cell Line

Rat 9L glioma cells[10] and YAC-1 NK-sensitive cells were cultured in RPMI 1640 (Mediatech, Washington, DC), supplemented with 10% fetal calf serum (FCS), 200 U/*l* penicillin, 200 μg/*l* streptomycin, and 600 mg/*l* L-glutamine. These cells were cultured in fresh tissue culture medium for 48 h before being harvested for assay.

Effector Cells

After the rats were asphyxiated with CO_2, the spleen was removed and minced by being passed through a sieve. The splenocytes, including the NK fraction, were washed twice in the medium (10% FCS in RPMI 1640). The cells were then cultured in RPMI 1640 supplemented with rh-GH (40, 20, and 10 μg/m*l*, which were determined by our preliminary experiment) for 48 h in 5% CO_2.

NK Cell Assay

Both the 9L glioma cells and YAC-1 cells were washed twice in RPMI 1640, then covered in a 1 : 30 dilution of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat monoclonal antibodies for 10 min. The cells were then washed twice and suspended at a final density of 2×10^7 cells/m*l*.

Effector splenocytes were centrifuged at 1,500 rpm for 5 min at 4℃ (GH was washed out) and suspended at a final density of 2×10^5 cells/m*l*.

The effector cells were mixed with the target cells at ratios of 100 : 1, 50 : 1, and 25 : 1 (effector [E] : target [T] ratio) in 24-well flat-bottomed plates. The plates were incubated for 20 h in a humidified, 37℃, 5% CO_2 incubator. The dead cells were identified by staining with propidium iodide, and a two-color analysis was carried out using a fluorescence-activated cell sorter (FACS Scan, Becton Dickinson, New Jersey). A similar experiment was carried out in the presence of 50 μg/m*l* anti-asialo-GM1 antibodies (rabbit IgG was used as negative control).

Statistical analysis of the data was carried out with Student's ***t***-test.

Results

The cytotoxic effects on YAC-1 and 9L glioma of naive splenocytes that were not pretreated with GH were quantified by changing the E : T ratio. As demonstrated in **Fig. 1**, cytotoxicity was enhanced in relation to the E : T ratio.

We then used effector cells that were pretreated with GH and quantified NK activity against YAC-1. The concentration of GH was adjusted to various levels (10, 20, and 40 μg/m*l*). Within a single concentration of GH, the E : T ratio was also adjusted to various levels (25 : 1, 50 : 1, and 100 : 1). A comparison with data from the untreated group, showed marked enhancement of NK activity at all concentrations in the treated group (**Fig. 2**). NK activity was enhanced in relation to GH concentrations, but, no correlation with the E : T ratio was seen. The data are expressed as relative NK activity, which was calculated after defining the result from the group not treated with GH as 100%.

We used the GH-pretreated effector cells and quantified their cytotoxicity against 9L glioma. In this experiment, marked enhancement of cytotoxicity was again recognized at all concentrations as compared with data from the untreated group. The NK activity was enhanced in relation to GH concentrations, but to no correlation with the E : T ratio was seen (**Fig. 3**).

To block NK activity, we added anti-asialo-GM1

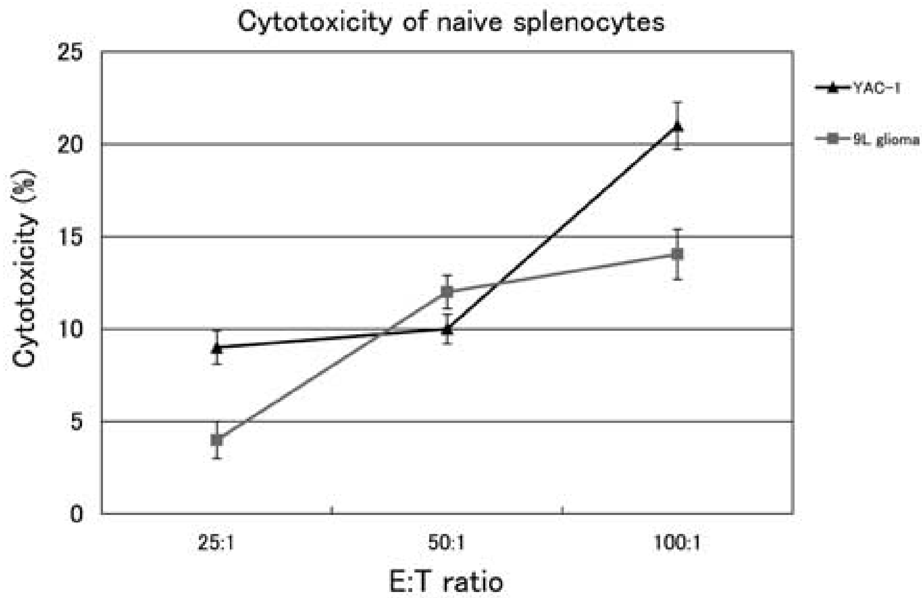

Fig. 1 Cytotoxicity of naive splenocytes against YAC-1 and 9L glioma cells at various E : T ratios (25 : 1, 50 : 1, and 100 : 1)
Cytotoxicity increased in proportion to E : T ratio.
Data are presented as means ± SD. (n = 3)

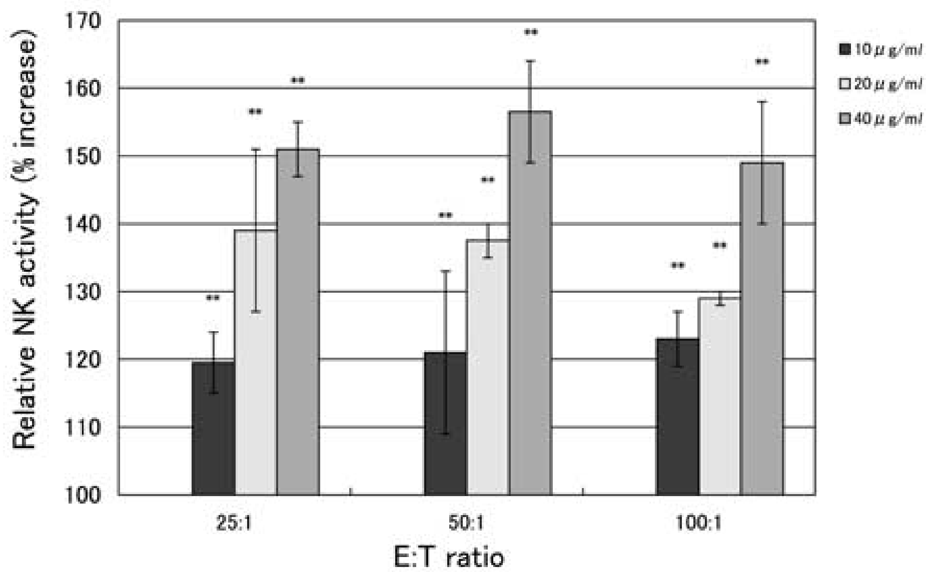

Fig. 2 Changes in the relative NK activity of GH-pretreated effector cells against YAC-1 cells were assessed on the basis of results from the untreated group being defined as 100%.
For measurement, GH concentration was adjusted to 10, 20, and 40 μg/m*l*, and the E : T ratio was adjusted to 25 : 1, 50 : 1, and 100 : 1. Cytotoxicity was enhanced at all concentrations in the treated group when compared with results in the untreated group.
Data are presented as means ± SD. (n = 5) **p<0.01 (vs. untreated group)

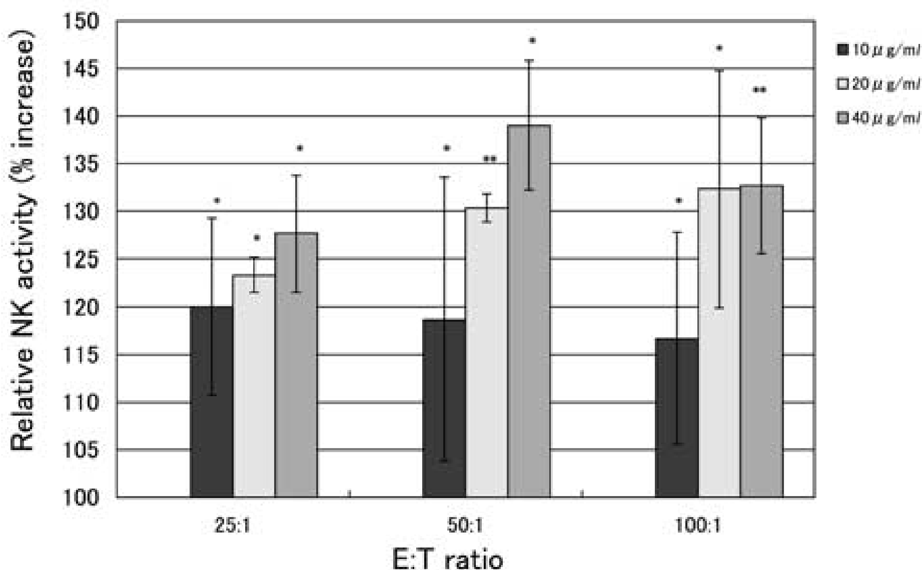

Fig. 3 Changes in the relative NK activity of GH-pretreated effector cells against 9L glioma cells were assessed on the basis of results from the untreated group being defined as 100%.
For measurement, the GH concentration was adjusted to 10, 20, and 40 μg/m*l*, and the E : T ratio was adjusted to 25 : 1, 50 : 1, and 100 : 1. Cytotoxicity was enhanced at all concentrations in the treated group when compared with results in the untreated group.
Data are presented as means ± SD. (n = 5) $^{*}p<0.05$, $^{**}p<0.01$ (vs. untreated group)

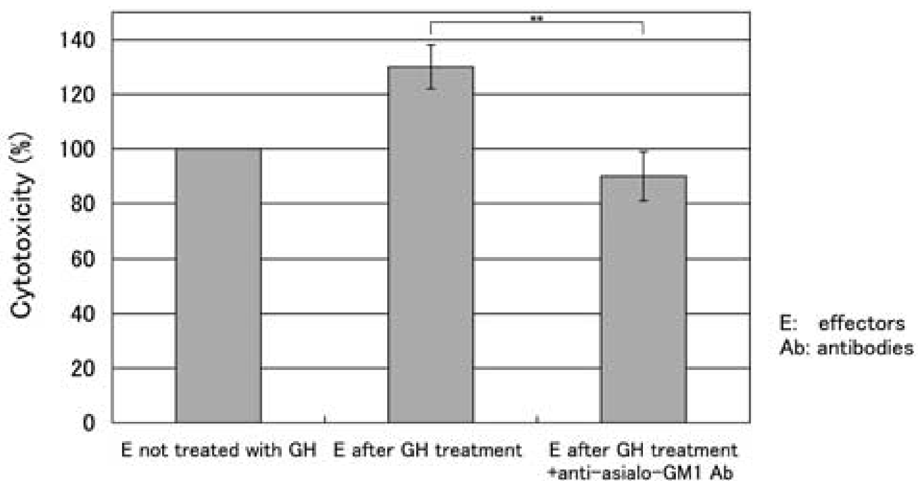

Fig. 4 Changes in cytotoxicity were measured when NK cell activity was blocked by anti-asialo-GM1 antibodies. Anti-asialo-GM1 antibodies inhibited cytotoxicity that was once enhanced by GH pretreatment.
Data are presented as means ± SD. (n = 3) $^{**}p<0.01$ (vs. E after GH treatment)

antibodies. After this treatment, enhanced cytotoxicity resulting from GH pretreatment was markedly inhibited (**Fig. 4**).

These results demonstrate that GH had a considerable effect in enhancing NK activity and was also effective in treating 9L glioma in the form of malignant brain tumor.

Discussion

The existence of GH receptors was first confirmed in cultured human lymphocytes in 1973[11]. Recently, using two-color flow cytometric analysis, the presence of GH receptors has been proven in human T-lymphocytes and in NK cells[12]. In this experiment, when NK activity was evaluated with YAC-1 NK-sensitive cells, pretreatment with GH enhanced cytotoxicity relative to GH density, consequently, it is assumed that GH receptors are present in NK cells and that the number of GH receptors was enough to react with the amount of GH in our experiment.

On the other hand, anti-tumor cytokines, such as interleukin-2, -12, -18 and interferon γ, enhance NK activity. Our study also suggests that T-cells stimulated by GH produce these cytokines, further enhancing NK activity.

GH is species-specific, because GH in phylogenetically higher animals is effective in lower animals[13]. Therefore, recombinant human GH was used in this experiment.

NK cells display cytotoxic activity in certain virus-infected cells and tumor cells, but if these cells have not been previously immunized by target cells, they still show strong cytotoxic activity and can be considered to have natural immunity. However, T-cell antigen receptors, like those occurring by clonal DNA arrangement in killer T-cells, are not expressed by NK cells, and the type of receptors used by NK cells to damage target cells is unclear.

In this experiment, 9L glioma malignant brain tumor cells were used as target cells. As was the case with YAC-1 cells, cytotoxicity was enhanced by naive effector cells in proportion to the E : T ratio. Pretreatment with GH similarly further enhanced the cytotoxic activity in relation to GH concentration. Furthermore, it was confirmed that this enhancement effect was suppressed when NK activity was blocked by administration of anti-asialo-GM1 antibodies. Thus, it appears that NK cells play a significant role as effector cells against 9L glioma.

In healthy adults, GH concentrations are less than 3 ng/m*l*. In patients with acromegaly, GH concentrations are assumed to be high for a prolonged period but data such as serum immunoglobulin concentrations and lymphocyte cell surface marker distribution are within the normal ranges. Furthermore, NK cell counts and NK activity in patients with acromegaly are not significantly different from those in healthy volunteers[14]. According to one report, however, treatment of healthy adults with supplemental GH for 6 weeks increased NK cell activity[7]. Therefore, NK cell kinetics ***in vivo*** cannot be explained by the direct effects of GH alone.

Untreated acromegaly carries high rates of morbidity and mortality due to a higher incidence of cardiovascular disease and cancer[15]. In patients with acromegaly, however, immunological variables are within normal limits and defenses against infectious diseases and autoimmunity are normal. Patients with acromegaly show increased levels of insulin-like growth factor (IGF)-I as well as GH. IGF-I plays a key role in the proliferation of many types of tumour cells; this is less often the case with GH. Therefore, the higher cancer rates in patient with acromegaly[16] are probably not due to GH but to the positive effects of IGF-I on tumor growth.

This study focused on GH; however, hormones, such as ACTH and β-endorphin, enhance NK activity, and by a combination of these hormones, it is possible that an even greater effect could be obtained. We intend to investigate this matter next.

Finally, in our present research, the ***ex vivo*** activation of NK cells, even in brain tumors, demonstrates that hormone-activated NK treatment has the potential for clinical applications.

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