The effects of recombinant human GH on promoting tumor growth depend on the expression of GH receptor in vivo

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Abstract

Cancer-related malnutrition is a mortal threat to gastric carcinoma patients. However, conventional nutrition treatment is not effective for recovery. Recombinant human GH (rhGH) is widely accepted clinically to treat severe malnutrition caused by non-malignant diseases, but not approved to treat malignant diseases due to the safety concern. To explore the safety of rhGH on gastric cancer, we assessed the effect of rhGH on two tumor-bearing mice models in vivo established by human gastric adenoma cell lines of SGC–7901 and MKN–45. VEGF expression in tumor tissues was detected using immunohistochemistry. The expression of GH receptor (Ghr), Jak-2, Stat3, Vegf, Hif-1α, Fgf, and MMP-2 was measured by RT-PCR and protein expression of STAT3, phosphorylated STAT3, VEGF, HIF-1α, and MMP-2 was measured by western blotting. The immunocytochemistry results showed that the GHR expression of SGC–7901 was strongly positive (GHR+++), while GHR expression of MKN–45 was regarded as negative (GHR−−). After 14 days of rhGH treatment in SGC–7901 (GHR+++), tumor-bearing mice, we found that the tumor growth was significantly increased, and the expressions of downstream factors and VEGF were increased. However, in MKN–45 (GHR−−) tumor-bearing mice, tumor growth was not significantly increased by rhGH, but tumor-free body weight was increased especially in high-dose rhGH-treated group (P<0.05). These findings suggest that the level of GHR expression is a key target that influences the effectiveness of rhGH on promoting the growth of gastric cancer and angiogenesis. rhGH may promote the activation of tumor angiogenesis factors through the Jak-2–STAT3 pathway.

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Introduction

Cancer-related malnutrition is a mortal threat to gastric carcinoma patients. In China, 160 000 people die of gastric cancer every year with a 5-year survival rate not exceeding 10% (Muscaritoli et al. 2006). Fifty percent of gastric cancer death was related with severe malnutrition caused by negative nitrogen balance. However, conventional nutrition treatment is not efficient to recover from malnutrition and may bring about some side effects, such as low tolerability of chemotherapy and aggravation of cancer-related symptoms. It is therefore crucial to find a new way to overcome the malnutrition to extend life span and improve life quality of gastric cancer patients.

GH, composed of 191 amino acids, is a kind of non-glycosylated proteohormone secreted by antehypophysis. Recombinant human GH (rhGH), with the same molecular constitution and characteristics as GH, is reported to promote protein synthesis, to potently correct negative nitrogen balance, and to improve nutrition effectively (Bettany et al. 1998, Barry et al. 1999, Zhu et al. 2004). rhGH is widely accepted clinically to treat severe malnutrition caused by non-malignant diseases (Blake 1995, Waters et al. 1996, Zhu et al. 2004). It is, therefore, important to understand the role of rhGH in metabolism regulation when searching for new methods to improve gastric cancer-related malnutrition. The tumor-related biological effect and mechanism of rhGH are still not well understood. rhGH contributes to protein synthesis and cell proliferation (Yoshida et al. 1992, Gu et al. 2008), which might also promote the malignant biological behavior of tumor cells. So, it is necessary to evaluate the safety of rhGH on tumor treatment and explore the mechanism of rhGH in treating cancer-related malnutrition.

The biological effect of GH is associated with the GH–GH receptor (GHR)–insulin-like growth factor 1 (IGF1) axis (Yash et al. 2011). First, when rhGH binds to GHR, a series of biochemical reactions are triggered and transcription of target genes is initiated. Among the reactions, IGF1 is a key factor that acts in an autocrine manner in local tissues. The site of action through which GH exerts its effects is mainly concordant with the distribution of GHR-positive cells (GHR+) in vivo. The action of rhGH on tumor cells in vivo is similar as above, involving two possible pathways (Cai et al. 2008):
1) GH can stimulate hepatic cells and other tissues to produce and release IGFs, which promote differentiation, proliferation, and growth of target cells, i.e. the functions of GH via the GH–IGFs axis, and 2) GH may combine with GHR on target cells to hasten their growth directly.

Research on postoperative gastric cancer tissues proved that GHR protein expression was not found in tissues from all patients (Zhang & Sun 2006). Dagnaes-Hansen et al. (2004) found that after treating COLO 205 nude mice with GHR antagonist, the serum IGFI1 and insulin-like growth factor-binding protein 3 (IGFBP3) levels were decreased, apoptosis was increased, tumor volumes were diminished by 39% \((P<0.05)\), and weight was diminished by 44% \((P<0.01)\). Zhang et al. (2007) established the mice genetic model, and TAg/GHR\(^{-/-}\) group (GH signal conduction blocked) and TAg/GHR\(^{+/+}\) group (GH signals normally transferred) were set. The result displayed that the latency period of mammary cancer in TAg/GHR\(^{-/-}\) groups was significantly prolonged by disrupting GH signaling. We speculate that the expression level of GHR may be a key factor to determine whether GH/rhGH can be used in patients with malignant cancer.

In our previous study (Li et al. 2010), we found that rhGH could dramatically stimulate proliferation of GHR\(^{+}\) human hepatoma carcinoma cells, induce VEGF secretion, participate in tumor angiogenesis, and increase GHR density on the surface of tumor cell membrane. However, on GHR\(^{-}\) human hepatoma carcinoma cells, rhGH could not induce the proliferation of GHR\(^{-}\) human hepatoma carcinoma cells or co-cultured vascular endothelial cells (VECs), nor increase the density of GHR on the cell surface.

Clinical research (Tacke et al. 2000) manifested that short-term rhGH treatment was safe for postoperative patients that experienced major gastrointestinal surgery for malignancy, and it did not bring about high risk of tumor recurrence. No significant influence on median-term or long-term disease-free survival rate was observed when combining parenteral alimentation and rhGH to treat postoperative patients that experienced hepatectomy for hepatocellular carcinoma (Cao et al. 2007). However, the application of rhGH on tumor-bearing patients was never reported. We think that it is possible to use rhGH clinically for gastric cancer treatment after considering the safety of the individual patient carefully.

In this study, we assessed the effect of rhGH on two gastric cancer models established by human gastric adenoma cell lines of SGC-7901 and MKN-45 and explored the gene expression and protein level of VEGF and tumor angiogenesis factors to clarify the relationship between the level of GHR expression in gastric cancer cells and the biological effect of rhGH on tumors.

Materials and Methods

Materials

Human gastric adenoma cells SGC-7901 and MKN-45 were purchased from Shanghai Institute of Cell Biology (Shanghai, China). BALB-c/nunu male nude mice (4–5 weeks old, 14–16 g) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences.

rhGH was obtained from Serono Pharm Co., (Geneva, Switzerland); RPMI medium 1640 was purchased from Gibco Co.; rabbit anti-human GHR polyclonal antibody, rabbit anti-human VEGF monoclonal antibody, and HRP-labeled goat anti-rabbit secondary antibody were purchased from Boster Biological Company of China (Wuhan, China); signal transducers and activators of transcription 3 (STAT3), phosphorylated STAT3 (pSTAT3), hypoxia-inducible factor 1, alpha subunit (HIF-\(\alpha\)), matrix metalloproteinase 2 (MMP-2), and other antibodies were purchased from Santa Cruz Co., (Santa Cruz, CA, USA). All the primers were synthesized by Shanghai Invitrogen Company.

Methods

Immunocytochemistry for determining GHR density on the membrane of tumor cells SGC-7901 and MKN-45 cells were cultured in RPMI medium 1640. After being blocked by goat serum, staining for GHR was performed on sections using rabbit anti-human GHR polyclonal antibody, biotin-labeled goat anti-rabbit secondary antibody, and HRP-labeled streptavidin; visualized with diaminobenzidine (DAB) chromogen; and observed with a microscope. Positive cells were stained brown in membrane and cytoplasm. Three randomly selected visual fields were observed with high microscope \((200\times\) original magnification, Olympus, Japan). The percentage of GHR-positive cells was calculated, ‘-’ represented no expression of GHR, ‘+’ represented low expression of GHR, ‘++’ represented medium expression of GHR (percentage of positive cells between 25 and 50%), and ‘+++’ represented high expression of GHR (percentage of positive cells over 50%).

The effect of rhGH in vivo Tumor implantation. BALB-c/nunu male nude mice were implanted SGC-7901/MKN-45 s.c. at the armpit of right anterior limb with 1\(\times\)10\(^7\) SGC-7901/MKN-45 cells in 0·1 ml PBS. When the diameter of the tumor reached 1·5–2·0 cm, the tumor-bearing mice were killed and tumor tissue was collected and triturated to tissue homogenate. The cell pellets were re-suspended in PBS and adjusted to a concentration of 1\(\times\)10\(^7\) cells/ml.

SGC-7901/MKN-45 tumor-bearing mice treatment. Fourteen days after tumor implantation, 24 SGC-7901/MKN-45 tumor-bearing mice were randomly divided into three groups with eight mice per group: low-dose group (rhGH\(_L\)) received 0·5 IU/day (=2·1 \(\mu\)g/kg per day), 0·2 ml/day rhGH; high-dose group (rhGH\(_H\)) received 2·5 IU/day (=10·5 \(\mu\)g/kg per day), 0·2 ml/day rhGH; and control group (group C) received normal saline, 0·2 ml/day. The mice were treated s.c. once daily for 14 days. The action and food consumption of mice were recorded. Tumors were measured individually with a vernier caliper every 3 days. Volumes were determined using the formula: tumor
Table 1  Forward (For) and reverse (Rev) primer sequences (5′ → 3′) and RT-PCR product length of the investigated factors

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHR</td>
<td>For: atggatctgtgctggctgtg</td>
<td>1064</td>
</tr>
<tr>
<td>JAK-2</td>
<td>For: atggaaagctgctggctctctctgtg</td>
<td>319</td>
</tr>
<tr>
<td>STAT3</td>
<td>For: aagctgtgctggctgtg</td>
<td>722</td>
</tr>
<tr>
<td>VEGF</td>
<td>For: gcacctgctggctgtg</td>
<td>498</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>For: atggctgctggctgtg</td>
<td>591</td>
</tr>
<tr>
<td>MMP-2</td>
<td>For: ctctgctggctgtg</td>
<td>474</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>For: ggaatctgtgctggctgtg</td>
<td>1027</td>
</tr>
</tbody>
</table>

Western blotting  Protein expressions of STAT3, pSTAT3, VEGF, HIF-1α, and MMP-2 were measured by western blotting. Tumor tissues were weighed, diced into very small pieces using a clean razor blade, transferred to microcentrifuge tubes, and then centrifuged at 10,000 g for 10 min at 4 °C. The supernatants were removed and centrifuged again. The supernatant fluid was the total cell lysate. Protein levels in the extracts were quantified using the Bio-Rad DC protein assay. Whole cell extracts (50–100 μg/lane) were separated on an SDS–PAGE gel. After SDS–PAGE electrophoresis, proteins were further identified by western blot analysis with nitrocellulose western blotting membranes. The staining was performed using specific monoclonal antibodies of high titer and specificity and HRP-labeled secondary antibody, visualized with DAB chromogen.

Statistical analysis  Data were shown as mean ± s.d. and were then analyzed by one-way ANOVA using SPSS Software (version 13.0; Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

GHR expression of the tumor cells

The immunocytochemical stain is shown in Fig. 1. SGC-7901 cells were stained claybank, mainly on cellular membrane and cytoplasm. The percentage of GHR-positive cells was about 83%. The expression of GHR was regarded as strongly positive (GHR(+++); Fig. 1A). No obvious stain of cytoplasm was observed in MKN-45 cells (Fig. 1B). The percentage of GHR-positive cells was <5%. The expression of GHR in MKN-45 cells was regarded as negative (GHR(−)).

In vivo activity of rhGH

After tumor cell implantation for 10 days, tumorlet can be found s.c. Tumor volumes and mice weight were recorded every 3 days from the 12th day. rhGH treatment was started on the 15th day after transplantation. Three days before treatment (day 12), mice weight and tumor volumes between the three groups were not statistically significant, both in SGC-7901 and in MKN-45 tumor-bearing mice (P>0.05).

Figure 1  Immunocytochemical stain for detecting the expression of GHR on two cell lines: SGC-7901 (A) and MKN-45 (B; 200× original magnifications). Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-11-0100.
After rhGH treatment for a week, the active state and food consumption of mice in rhGH-treated group were found to be improved than control group.

On the sixth day of rhGH treatment (day 21), in the SGC-7901 group, the tumor volumes in the two rhGH-treated groups were significantly increased ($P<0.05$) compared with control; the tumor volumes between the two rhGH-treated groups were statistically significantly different ($P<0.05$); differences in mice weight among the three groups was not statistically significant ($P>0.05$).

On the third day of rhGH treatment (day 18), in the MKN-45 group, the tumor volume between the three groups was not statistically significantly different ($P>0.05$); compared with control, body weight in the two rhGH-treated groups was significantly higher ($P<0.05$), especially in rhGHH-treated group ($P<0.05$ vs rhGHH-treated group).

The speed of body weight gain was slower in the last 7 days than the first 7 days in both the SGC-7901 and MKN-45 groups (Fig. 2).

The tumor-free body weight is shown in Fig. 2E. In the SGC-7901 group, a minor decrease in tumor-free body weight was found in the rhGHH-treated group ($P>0.05$). In MKN-45 group, tumor-free body weight gain was found after rhGH treatment, especially in rhGHH-treated group ($P<0.05$).

After the mice were killed, tumor tissues (liver, spleen, lung, and kidney) were collected for HE staining, and no metastatic lesion was found in any of these tissues.

The expression of VEGF in tumor tissues

The expression of VEGF in tumor tissues was examined by immunohistochemistry (Fig. 3). Positive VEGF cells were stained brown diffusely in cytoplasm. The grade of VEGF expression was determined by calculating the percentage of VEGF-positive cells.

In the SGC-7901 tumor tissues (Fig. 3A–C), the percentage of VEGF-positive cells in control, rhGHH-treated groups was 47.0, 87.5, and 95.3% respectively. Therefore, VEGF expression in control group was medium positive, and VEGF expression in the two rhGH-treated groups was strongly positive.

In the MKN-45 tumor tissues (Fig. 3D–F), the percentage of VEGF-positive cells in control, rhGHH-treated groups was 48.5, 51.2, and 51.0% respectively. Therefore, VEGF expression in the three groups was medium to strongly positive.

The mRNA expressions of angiogenesis-related factors in tumor tissues

RT-PCR analyses of Ghr, Jak–2, Stat3, Vegf, Hif-1α, Fgf, and Mmp–2 expression in tumor tissues are shown in Fig. 4.
**SGC-7901 group.** The expressions of all these target genes in rhGH$_H$-treated group were up-regulated compared with control ($P<0.05$; Fig. 4B). The expressions of these target genes in rhGH$_L$-treated group were down-regulated compared with control ($P<0.05$), except Mmp-2. The expressions in rhGH$_L$-treated group were down-regulated compared with rhGH$_L$ group and were statistically significant ($P<0.05$) except Stat3 and Vegf.

**MKN-45 group.** Jak-2, Vegf, and Hif-1α were detected to be expressed in MKN-45 tumor tissues, and no significant differences were found between control and rhGH-treated groups (Fig. 4C). No expression of Ghr, Stat3, Fgf, and Mmp-2 was found in MKN-45 tumor tissues.

**The expressions of angiogenesis-related protein in tumor tissues**

Western blot analysis of STAT3, pSTAT3, VEGF, HIF-1α, and MMP-2 expression in tumor tissues is shown in Fig. 5.

**SGC-7901 group.** The protein expression of all these target genes in rhGH$_H$-treated group was up-regulated compared with control ($P<0.05$; Fig. 5B). The protein expression of these target genes in rhGH$_L$-treated group was up-regulated compared with rhGH$_L$ group and showed statistically significant ($P<0.05$); STAT3 was up-regulated and pSTAT3 was down-regulated compared with rhGH$_L$-treated group, but this was not statistically significant ($P>0.05$).

**MKN-45 group.** The protein expressions of all target genes in rhGH-treated groups were not statistically significant compared with control ($P>0.05$; Fig. 5C).

**Discussion**

In this study, the immunocytochemical method was used to classify the level of GHR expression in two human gastric adenoma cell lines of SGC-7901 and MKN-45. The expression of GHR in SGC-7901 cells was regarded as strongly positive (GHR$^++$), while the expression of GHR in MKN-45 cells was regarded as negative (GHR$^-$). Then, two gastric cancer models were induced in nude mice using subcutaneous transplantation of SGC-7901 and MKN-45 tumor cells to evaluate the effect of rhGH on tumor growth, expression of related factors, and mice nutrition. After rhGH therapy for a week, the active state and food consumption of animals in rhGH-treated group were better than control animals, which demonstrated that rhGH could improve body metabolism condition in some way.

The effect of rhGH on tumor growth and mice nutrition appeared to be different in the two animal models. In SGC-7901 tumor-bearing mice (GHR$^++$), tumor volumes appeared to be different in two rhGH-treated groups. The tumor growth rate was significantly higher in two rhGH groups compared with control, especially in the rhGH$_H$ group. No significant difference in body weight was observed among the three groups. In the late stage of treatment, the speed of body weight gain gradually slowed down while the tumor volumes gradually increased. Eventually, both body weight and tumor-free body weight in the rhGH$_H$-treated group were even lower than that in the rhGH$_L$-treated group. We speculated that this might be related to body consumption. In MKN-45 tumor-bearing mice (GHR$^-$), the body
weight after rhGH therapy was significantly increased ($P<0.05$), but tumor volumes were not significantly different among the three groups throughout the entire treatment period. Tumor-free body weight was increased after rhGH treatment, especially in the rhGH$_L$-treated group ($P<0.05$).

Low-dose GH administration was reported to mobilize endothelial progenitor cells in healthy adults (Devin et al. 2008), and the activity of rhGH in angiogenesis was explored (Lincoln et al. 2007); rhGH can activate downstream factors IGF1 and VEGF mainly through the P44/42 MAPK pathway and is synergetic with VEGF, which is a major local angiogenesis factor. Angiogenesis is required for tumor growth and metastasis and constitutes an important point in the control of cancer progression (Folkman 2002). Multiple cytokines secreted by tumor cells can promote the migration of VEC to tumor tissues, promote proliferation, inhibit apoptosis, and contribute to tumor angiogenesis and maturation eventually.

Therefore, rhGH plays a role in tumor angiogenesis by regulating the tumor cell proliferation and apoptosis. In vitro, rhGH can promote the proliferation of GHR$^+$ human hepatoma carcinoma cell lines, but cannot promote the proliferation of GHR$^-$ human hepatoma carcinoma cell lines (Li et al. 2010). In vivo, tumor growth was inhibited when GH of human colon carcinoma cells was inhibited by GHR antagonist in tumor-bearing nude mice (Dagnaes-Hansen et al. 2004). Clinical research demonstrated that rhGH treatment might induce proliferation and malignization of GHR$^+$ endothelial cells in benign/malignant hemangioma (Lincoln et al. 2007). Research on angiogenesis showed that the expression of VEGF at mRNA/protein level in prostatic carcinoma cell lines can be down-regulated by lowering the GH level. So, tumor angiogenesis might be promoted by rhGH via GHR$^+$ on the surface of tumor cellular membrane and excess secretion of VEGF (Zhang et al. 2007). rhGH can be regarded as a systemic angiogenesis factor, while VEGF acts locally.

However, VEGF is important in tumor infiltration and metastasis. VEGF can promote the expression of fibrin and interstitial collagenase, accelerate the degradation of basal membrane, and promote tumor metastasis; it can also inhibit the maturation of dendritic cells and lower the effective immune response to cancer cells (Johnson et al. 2007). Clinical research found that the expression of VEGF in serum and tumor tissue of gastric cancer patients was extremely high, positively correlated with the depth of infiltration and tumor tissue of gastric cancer patients was extremely high, positively correlated with the extent of cell differentiation (Liu et al. 2000, Karayiannakis et al. 2002). In this study, VEGF expression in the two animal models after rhGH therapy showed similar results during tumor growth. In SGC-7901 tumor-bearing mice (GHR$^+$), protein VEGF was significantly increased in rhGH$_H$-treated mice than in rhGH$_L$-treated mice. However, in MKN-45 tumor-bearing mice (GHR$^-$), protein VEGF was expressed in both rhGH-treated groups and the control group but not in a dose-dependent manner. The expression of VEGF was concordant with the tumor growth in vivo.

The activation of STAT3 has been linked with the proliferation of a variety of human cancer cells, including multiple myeloma. Agents that can suppress STAT3 activation have potential for prevention and treatment of cancer (Pathak et al. 2007). rhGH exerts its effect when rhGH binds to GHR on the tumor cell surface, and then
JAK-2 is activated, which leads to serial signal cascade reaction and cell biological reactions including proliferation, differentiate, migration, inhibition of apoptosis, remodeling of cytoskeleton, and regulation of metabolic pathways. Chen et al. (2008) found that pSTAT3 was a biomarker of endothelial activation that reported VEGF–VEGFR2 activity and also a key activating transcription factor on angiogenesis.

The Jak–2–STAT3 pathway is one of the three classical STAT3 activating pathways, which accomplish signal transduction from extracellular to intracellular immediately. The main procedures are as follows: receptor identification by cytokine, self and receptor activation by JAK kinase, phosphorylation of STAT3, dimerization, and nuclear translocation of pSTAT3. After the Jak–2–STAT3 pathway is activated, the expression of downstream target genes may be up-regulated or down-regulated. These genes are related to tumor angiogenesis including Vgf, Hif-1α, bFGf, Mmp-2, Mmp-7, Mmp-9, and so on (Xie et al. 2004, Jung et al. 2007, Ardi et al. 2009, Behera et al. 2010, Ferrara 2010). It can be inferred that the activation of Jak–2–STAT3 can promote tumor angiogenesis.

In this study, we detected the expression of molecules involved in Jak–2–STAT3 pathway. Major differentiated expression of these molecules was found in GHR+ gastric tumor-bearing mice and GHR− gastric tumor-bearing mice. After rhGH treatment in GHR+ tumor-bearing mice, the expressions of related molecules were generally increased: in the rhGHtreated group, the mRNA expression level of Ghr, Jak-2, Stat3, Vgf, Hif-1α, Fgf, and Mmp-2 was increased and was statistically significant compared with control ($P<0.05$), while the protein expression level of STAT3, pSTAT3, VEGF, HIF-1α, and MMP-2 was increased and was statistically significant compared with control ($P<0.05$); in the rhGHtreated group, the expressions of these molecules at mRNA and protein level were all increased, excluding STAT3, and were statistically significant compared with control ($P<0.05$).

However, after rhGH treatment in GHR− tumor-bearing mice, the mRNA or protein expressions of these molecules were not statistically significant compared with control ($P>0.05$), both in rhGHtreated- and rhGHtreated-groups. The difference between GHR+ and GHR− tumor-bearing mice on the expression of these molecules demonstrated that the expression level of GHR might be a critical target, which influenced the expression of tumor angiogenesis-related factors. It also showed that activation of tumor angiogenesis factors by rhGH might be through Jak–2–STAT3 pathway.

Owing to the gene homology of human and mice, most research in vivo was found not to be influenced by species specificity of GH (Takahashi et al. 1997). In this study, the intrinsic bionomics difference of human gastric cancer cell lines with different GHR expression was not excluded and will be further evaluated in the next paper.

In conclusion, the level of GHR expression may be a critical target, which influences the effect of rhGH on promoting gastric tumor growth and angiogenesis. rhGH can activate tumor angiogenesis factors mainly through the Jak–2–STAT3 signaling pathway. GHR may be a key factor to determine whether GH/rhGH can be used in patients with gastric cancer.

**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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**Effects of rhGH depend on GHR expression**

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