Combined treatment with GH, insulin, and indomethacin alleviates cancer cachexia in a mouse model

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Abstract

Cancer-induced cachexia involves weight loss, catabolic activity, and inflammation. We have evaluated the effects of various treatments (GH, insulin (INS), indomethacin (IND), and all possible combinations) on cancer cachexia in a mouse model. BALB/c mice that were implanted with colon-26 adenocarcinoma developed cachexia in 9 days. Body weight, tumor volume, tumor-free weight, inflammatory cytokines (tumor necrosis factor- α (TNF- α), interleukin 6 (IL6)), and nutritional markers (glucose (Glu), albumin, triglycerides (TGs)) of treatment and control groups were monitored. In the cachexia group,

there was a significant decrease in tumor-free bodyweight by day 11. Treatment with GH+INS+IND significantly alleviated tumor-free bodyweight reduction and cachexia-induced changes in nutritional markers and cytokines, and prolonged survival time. GH+INS+IND treatment was more effective than other treatment combinations in elevating Glu and TGs, reducing TNF- α and IL6 levels, and prolonging survival time. In conclusion, GH+INS+IND alleviated cachexia symptoms in a murine model of cancer cachexia.

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Introduction

Cachexia accounts for about 25% of cancer-related mortalities (Lelbach *et al.* 2007). The main manifestations of cancer-induced cachexia are weight loss, malnutrition, lean muscle wasting, anorexia, and metabolic disorders, and these lead to progressive glyconeogenesis, high muscle albuminolysis, and increased lipolysis (Saini *et al.* 2006). Cancer-associated weight loss of more than 6% is associated with decreased survival time for patients with metastatic breast cancer, colon cancer, non-small cell lung cancer, and prostate cancer, but not pancreatic cancer (Jatoi 2008).

The pathogenesis of cancer-associated cachexia is multifactorial, and the causes have been extensively studied. Lean muscle wasting is due to reduced protein synthesis and increased protein degradation (Tisdale 2009). The systemic inflammatory response associated with cancer cachexia is characterized by elevated levels of tumor necrosis factor- α (TNF- α), interleukin 6 (IL6), TWEAK (a member of the TNF family), IL1 receptor antagonist, and C-reactive protein (McMillan 2009). At the physiological level, elevation of these cytokines leads to increased energy expenditure, suppression of appetite, catabolic activity, and muscle wasting (Argilés *et al.* 2009). Elevation of TNF- α and IL6 also promotes lipolysis, although adipose tissue is not the major source (Agustsson *et al.* 2007).

There are currently no standard practice guidelines for treatment of cancer cachexia (Mantovani & Madeddu 2010),

but five therapeutic strategies are under investigation: i) steroids, ii) nonsteroid anti-inflammatory drugs and biological modifiers specific for TNF- α , iii) progesterone derivatives, iv) omega-3 fatty acids with anti-inflammatory activity and essential nutritional value (Lelbach *et al.* 2007), and v) anabolic agents that enhance protein synthesis, including GH (Berman *et al.* 1999) and insulin (INS; Lundholm *et al.* 2007). The use of progesterone derivatives has been criticized because these agents induce tumor weight gain in animal models (Argilés *et al.* 2004) and the weight gain in megestrol-treated cancer patients is mainly due to water retention and increased fat tissue (Lelbach *et al.* 2007).

A previous clinical study showed that daily administration of s.c. INS to cachectic cancer patients improved micro-nutrient absorption and fat metabolism (Lundholm *et al.* 2007). INS has anabolic activity and can antagonize IL6 signaling in adipose tissue (Andersson *et al.* 2007). Conversely, INS-dependent activation of RAC- β serine/threonine-protein kinase-2 (AKT2), which is important in the mediation of its downstream metabolic actions, is markedly inhibited by IL6 (Senn *et al.* 2002). TNF- α impedes some functions of INS, such as receptor engagement (O'Connor *et al.* 2008).

A clinical study of patients with cancer cachexia indicated that indomethacin (IND) suppressed cyclooxygenase (COX), TNF- α , and IL6, improved energy homeostasis, reduced the amount of weight loss, and reduced chronic inflammation (Lundholm *et al.* 2004). An animal study showed that IND

decreased the activation of ubiquitin-dependent proteases involved in protein degradation, reduced nuclear factor κ B (NF- κ B) activation, reduced the levels of TNF- α and IL6, and alleviated muscle wasting (Zhou *et al.* 2003).

GH has well-known anabolic affects, including promotion of nutrient assimilation, protein synthesis, and fatty acid oxidation (Carli *et al.* 1997). Importantly, a study of tumor-bearing mice indicated that stimulation of the GH-insulin-like growth factor (IGF) axis did not promote tumor growth (Khan *et al.* 2005). A clinical study of cancer patients indicated that administration of GH alone or in combination with INS led to bodyweight gain and increased upper arm muscle mass and strength (Berman *et al.* 1999).

Thus, INS, IND, and GH can each alleviate some manifestations of cachexia, but the efficacy of these treatments needs to be improved. Moreover, because of its multifactorial pathogenesis, cachexia may be more responsive to combination therapy (Mantovani & Madeddu 2010). The presence of multiple interactions in the INS and proinflammatory cytokine pathways suggests that there may be benefits for using multiple agents for treatment of cachexia.

In this study, we examined the effect of GH (to stimulate anabolic activity), INS (to stimulate anabolic and anti-inflammatory activity), and IND (to block inflammation) alone and in all possible combinations in a murine model of cancer cachexia.

Materials and Methods

Reagents, cells, and animals

GH and INS were purchased from Shanghai United Cell Biotechnology Co., Ltd (Shanghai, People's Republic of China) and Novo Nordisk, Inc. (Copenhagen S, Denmark)

Figure 1 Effect of GH (0.5 mg/kg per day), insulin (INS, 0.5 μ g/kg per day), and indomethacin (IND, 0.5 mg/kg per day) on tumor volume (cm³) of mice with cancer cachexia. The cachexia group received PBS injections. The bars indicate means $\pm$ s.d.s, $n=10$. For each day, there were no significant differences across all the treatment groups or between the pairs of treatment groups.

Figure 2 Effect of GH (0.5 mg/kg per day), insulin (INS, 0.5 μ g/kg per day), and indomethacin (IND, 0.5 mg/kg per day) on tumor-free bodyweight (g) of mice with cancer cachexia. The cachexia group and healthy group received PBS injections. The bars indicate means $\pm$ s.d.s, $n=10$. There were significant differences across all the treatment groups on day 9 ($P<0.0333$) and on days 11, 13, and 16 ($P<0.0001$). Pair-wise comparisons with Bonferroni adjustment indicated that (i) each of the eight groups was significantly different ($P<0.05$) from the healthy group on days 11, 13, and 16; (ii) the GH group (day 16), INS group (days 13 and 16), IND group (day 16), GH+INS group (day 16), GH+IND group (days 13 and 16), and INS+IND group (day 13) were significantly different ($P<0.05$) from the GH+INS+IND group; and (iii) the INS+IND group (day 16) and the GH+INS+IND group (days 13 and 16) were significantly different ($P<0.05$) from the cachexia group.

respectively and were diluted in sterile 0.9% NaCl prior to use. IND was purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and a stock solution (10 mg/ml in 100% ethanol) was stored in the dark and diluted with sterile PBS (pH 7.4) on the day of use.

BALB/c male mice (6–8 weeks old, 20–24 g bodyweight) were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, People's Republic of China; security processing facility (SPF) certificate numbers: SCXK (Hu) 2007-0005). SPF habitation was provided by the Animal Center of Fujian Institute of Medical Sciences and included free access to standard laboratory chow and tap water in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) on a 12 h light:12 h darkness cycle. All procedures were approved by the Institutional Animal Care Committee of the Fujian Medical University.

A colon-26 (C-26) adenocarcinoma specimen was purchased from the Institute Materia Medica, Chinese Academy of Medical Sciences (Beijing, People's Republic of China). Tumors were implanted under the left armpit of mice following the third or fourth passage.

Cancer cachexia models

All mice were initially acclimated for 7 days. A total of 128 mice were implanted with tumor cells. A homogenate of 50 mg ($2\text{--}3\text{ mm}^3$) minced solid murine C-26 adenocarcinoma in

0.1 ml of sterile 0.9% NaCl was injected s.c. into the armpits (day 0). A group of 10 mice with no tumor cell implantation was used as 'healthy control'; a group of 10 mice with tumor cell implantation but no treatment was used as 'cachexia control'. The physical activity, fur condition, bodyweight, general health, and tumor inoculation site were monitored every 2 days. Food intake was measured at 1000 h daily.

The tumor was considered to have ellipsoid or prolate spheroid geometry. We measured tumor volume (V , cm^3) as described by Zhou *et al.* (2003). In particular, V was estimated as $(a \times b^2)/2$, where a is length and b is width. Tumor mass (M) was estimated by the formula ($M = V \times \rho$), where ρ is the tumor density at the time of killing or expiration. When the tumor-free weights of tumor-bearing mice and healthy mice (Agustsson *et al.* 2007) were significantly different, and cachexia symptoms (poor physical activity, asthenia, piloerection, shedding, and nonglossy fur) became obvious, cachectic mice were randomly divided into an untreated group ($N=16$; i.p. injection with PBS) and seven treatment groups ($N=16$). The treatment groups were GH, INS, IND, GH + INS, GH + IND, INS + IND, and GH + INS + IND. I.p. administration of GH (0.5 mg/kg per day), INS (0.5 $\mu\text{g/kg}$ per day), and/or IND (0.5 mg/kg per day) began on day 9 (at the onset of cachexia) and continued daily, according to mouse weight and the dosage table.

Ten mice from each group were killed on day 16, with blood samples collected from orbital veins prior to killing. There was significant mortality of the experimental animals at 16 days, so we terminated the experiment at this point. An average of 0.6 ml blood was withdrawn from each mouse, and 20–40 μl were used for each measurement. Duplicate samples were not possible because of the limited amount of serum recovery from each mouse. Tumors were removed and tumors and tumor-free weights were determined. Survival times for the remaining mice in each group (six mice for each group) were recorded and analyzed.

Biochemical and serological markers

On day 16, blood samples were collected from orbital veins and stored at -70°C . Levels of TNF- α and IL6 were determined by ELISA kits purchased from R&D Systems, Inc. Serum glucose (Glu), triglyceride (TG), and albumin were measured by an automated analyzer (BA-88 MINDRAY Biochemistry Analyzer, Shenzhen, People's Republic of China).

Figure 3 Box-plots showing the effect of GH (0.5 mg/kg per day), insulin (INS, 0.5 $\mu\text{g/kg}$ per day), and indomethacin (IND, 0.5 mg/kg per day) alone and in various combinations on serum glucose (A), albumin (B), triglycerides (C), TNF- α (D), and IL6 (E) on day 16 after tumor implantation. The cachexia group and healthy group received PBS injections. Horizontal bars indicate lower and upper quartiles, and median values, $n=10$. †Significant difference in the healthy group after Bonferroni adjustment; ‡significant difference in the cachexia group after Bonferroni adjustment; §significant difference in the GH + INS + IND group after Bonferroni adjustment.

Statistical analysis

Means and S.D.s were calculated for each group. Comparisons were performed using a mixed model considering repeated measure effect with *post hoc* comparison adjusted by the Bonferroni method. The model was two-way factorial with variance components covariance structure and the degree of freedom method was between-within (subject) method. The log-rank test was used for survival analysis. Data were analyzed using SAS 9.0 (SAS Institute, Inc., Cary, NC, USA). A *P* value < 0.05 was considered statistically significant.

Results

Tumor growth and cachexia

The mice showed symptoms of cachexia on day 9 (cachexia onset) after the tumor transplantation. These mice exhibited delayed responsiveness, poor physical activity, scruffy fur, piloerection, and darkening of dorsal hairs. A two-way repeated measure ANOVA indicated that all the groups had significant weight change (data not shown) and increase in tumor volume over time (Fig. 1). For the cachexia control group, the tumor volume increased from $1.49 \pm 0.25 \text{ cm}^3$ on day 9 to $4.73 \pm 0.71 \text{ cm}^3$ on day 16. On days 13 and 16, there were significant differences in total bodyweight across all treatment groups (data not shown) but the tumor volume was not significantly affected by any of the treatments (Fig. 1).

Tumor-free bodyweight

In the cachexia control group, there was a significant reduction in tumor-free bodyweight (total body weight minus tumor mass) beginning on day 11 (Fig. 2). Relative to the cachexia control group, mice given GH + INS + IND had greater tumor-free bodyweights on days 13 and 16 (see Fig. 2). Pair-wise comparisons indicated additional statistically significant differences in tumor-free bodyweight between several of the treatment groups on days 11, 13, and 16 (see Fig. 2). However, none of the treatments restored tumor-free bodyweight to that of the healthy controls.

Nutritional markers and cachexia-related cytokines

The cachexia control group experienced a significant reduction in tumor-free bodyweight on day 11 (Fig. 2). Relative to healthy controls, this group also experienced significant reductions in three nutritional markers (Fig. 3A–C) and significant elevations in two cachexia-related cytokines (Fig. 3D and E). Treatment with GH + INS + IND significantly alleviated the cachexia-induced changes in Glu, TG, TNF- α , and IL6 (Fig. 3). However, the cachexia markers in the GH + INS + IND group did not return to those of healthy controls (Fig. 3). Tumor-bearing mice treated with GH + INS + IND had significantly higher levels of Glu

Figure 4 The Kaplan–Meier survival curve of mice in each of the seven treatment groups and the untreated (cachexia) group, *n* = 6. The survival curves were significantly different among various treatments (log-rank test, *P* < 0.0001). The GH + INS + IND treatment group had a significantly longer survival time than the cachexia group and the other treatment groups (*P* < 0.05 for all comparisons).

(Fig. 3A) and lower levels of IL6 (Fig. 3E) than those given other treatments. Tumor-bearing mice treated with GH + INS + IND also had significantly higher levels of TG (Fig. 3C) and lower levels of TNF- α (Fig. 3D) than mice treated with GH, INS, or GH + INS.

Survival time

Finally, we examined the effect of our seven treatments on survival time of mice that were not killed (six mice per group; Fig. 4). Log-rank tests indicated that the survival curves were significantly different among various treatments (*P* < 0.0001) and that the GH + INS + IND treatment group had a significantly longer survival time than the cachexia control group and the other treatment groups (*P* < 0.05 for all comparisons). All mice in the healthy control group survived for 16 days.

Discussion

Cancer cachexia is a common condition that complicates cancer treatment strategies and reduces survival time (Jatoi 2008). This study demonstrated that a multimodal treatment strategy that targets three different pathogenic mechanisms of cachexia ameliorates cancer cachexia in a mouse model. Although anorexia is a common feature of cancer-induced cachexia in humans (Lelbach *et al.* 2007), we found no effect of cachexia on food intake in our mouse model, consistent with a previous report (Zhou *et al.* 2003).

On days 13 and 16, there were significant differences among the treatment groups. Although there was no statistical difference between the healthy control and cachexia control groups in total body weight (data not shown), the tumor-free body weight of the cachexia control group was significantly

lower than that of the healthy control group (Fig. 2). This is simply because the cachexia control group had a large tumor mass (Fig. 1). In addition, total body weight and tumor-free body weight (Fig. 2) of mice treated with GH+INS+IND were significantly higher than those of the cachexia control group. This suggests that GH+INS+IND treatment partially reverses the weight loss induced by cachexia.

INS can induce anabolic and anti-inflammatory activities in humans with cancer (Dandona *et al.* 2001). Consistent with this clinical finding, our results indicate that INS alone or in combination with other treatments (i.e. IND) increased tumor-free bodyweight of cachectic mice and led to significantly longer survival times. Some research has shown that INS antagonizes IL6 signaling in adipose tissue via the methyl ethyl ketone/mitogen-activated protein kinase pathways (Andersson *et al.* 2007), but other research suggests that INS stimulates IL6 production (LaPensee *et al.* 2008). In our cachectic mouse model, we found that administration of INS alone, INS+IND, or INS+IND+GH significantly reduced serum levels of IL6. IL6 is a potential prognostic marker for survival time of patients with cancer cachexia (Ebrahimi *et al.* 2004). Thus, our observations suggest that INS may be partially responsible for the alleviation of cachexia symptoms and prolongation of survival time.

In our mice, GH did not promote growth of the implanted C-26 adenocarcinomas. Similarly, GH has been reported to have no effect on the growth of human bronchoalveolar carcinoma and human breast adenocarcinoma (Khan *et al.* 2005). GH increases anabolic activity, and some of its effects may also be mediated by increasing the immune response (Redelman *et al.* 2008). However, we observed no effect of GH on the slowing of tumor growth during the course of our relatively short (25–40 days) study. GH did not significantly reduce inflammatory cytokines nor elevate nutritional markers relative to untreated cachectic control mice, and GH treatment alone did significantly increase survival time. One possibility is that the modest (but statistically insignificant) GH-induced modulation of nutritional markers was insufficient to increase survival time.

IND inhibits COX and suppresses the production of proinflammatory cytokines. A previous clinical study reported that malnourished cancer patients treated with IND experienced improved nutritional status, longer survival time, and better quality-of-life (Lundholm *et al.* 2004). Our IND groups had significantly higher tumor-free bodyweight, Glu, and TG, longer survival times, and reduced levels of proinflammatory cytokines. This suggests that proinflammatory cytokines may affect the nutritional status of patients with cancer cachexia (Argilés *et al.* 2009, Tisdale 2009).

Mice treated with GH+INS+IND had significantly better survival times than the cachexia control group and all other treatment groups. Moreover, mice given this triple treatment had improved nutritional and inflammatory markers. Crosstalk and feedback loops between the INS and proinflammatory cytokine pathways may be responsible for these effects. In agreement with a recent review article

(Mantovani & Madeddu 2010), our results suggest that combination therapy (GH+INS+IND) may be an effective strategy for the alleviation of cancer cachexia.

GH, INS, and IND all have extensive clinical histories and are inexpensive. We suggest that future studies examine the pharmacokinetics of these three agents when given together, before they are studied in clinical trials. All animal models are limited in that they do not fully mimic responses in a heterogeneous human population with different types of cancer. Moreover, our mice were not INS resistant, whereas cancer cachexia patients usually are INS resistant (Lelbach *et al.* 2007). In addition, our doses of INS, GH, and IND were based on previous reports and were not optimized by titration; this may be why none of our treatments reduced cachexia-associated abnormalities relative to those of the healthy control group. Finally, an adverse effect of the IND and INS treatment was not an objective in this study, but it is possible that a side effect, such as hypertrophy of some body parts, may be a confounding factor.

In summary, our study demonstrated that treatment of a murine cancer cachexia model with GH+INS+IND alleviated cachexia-associated symptoms, increased tumor-free bodyweight, increased Glu and TG, reduced TNF- α and IL6, and increased survival time. However, none of our treatment regimens were able to fully reverse all the symptoms associated with cancer cachexia. These three agents have long clinical histories and are inexpensive, so it is feasible to evaluate similar combination therapies in clinical trials of cancer cachexia.

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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