Involvement of Human Interleukin 6 in Experimental Cachexia Induced by a Human Uterine Cervical Carcinoma Xenograft

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ABSTRACT

A human tumor xenograft model for cancer cachexia was established by growing a uterine cervical carcinoma, Yumoto, in nude mice. The tumor transplanted into the mice induced severe body weight loss (30% of body weight) when the tumor weight was only 1 g. In addition, other indicators for cachexia, such as adipose tissue and muscle wasting and hypoglycemia, were observed in the tumor-bearing mice, suggesting that this is a proper model for experimental cachexia induced by a human tumor. We then examined the association of this model with various cytokines, such as tumor necrosis factor α, interleukin (IL)-1α, IL-1β, IFN-γ, IL-6, and leukemia inhibitory factor, and identified human IL-6, which was produced by the tumor cells, as a mediator of cachexia. A neutralizing antibody against hIL-6 administered to the mice after the development of cachexia symptoms significantly improved body weight loss, adipose tissue wasting, hypoglycemia, acute phase reaction, and leukocytosis, although it did not suppress the tumor growth. These results demonstrate that the hIL-6 produced by the tumor cells is an essential mediator of the cachexia induction in this model.

INTRODUCTION

Cancer cachexia, which includes disorders of homeostasis such as progressive wasting, weakness, anorexia, and anemia, is commonly seen in cancer patients (1, 2). Cachexia is associated not only with deterioration of the quality of life but also with shorter survival (1) and poor response to chemotherapy (3). Elucidation of the mechanism of cachexia induction will help in the search for potential therapeutic interventions for advanced cancer.

In studies on the mechanism of cachexia induction, several cytokines, such as TNFα (4, 5), IL-1 (6), IFN-γ (7, 8), IL-6 (9–12), LIF (13), and transforming growth factor β (14), have been proposed as cachexia inducers in different cachexia models. In cachexia models of murine tumors, however, it is rather difficult to identify whether the tumor cells or the host cells are producing these cytokines. In contrast, in nude mouse models bearing human tumor xenografts, tumor cell-derived cytokines should be easily distinguishable from host cell-derived cytokines. Therefore, experimental models for cachexia induced by human tumor xenografts should be useful for the identification of tumor cell-derived cachexia factors. Indeed, one of the authors has reported a close relationship between the expression of hLIF mRNA and the development of cachexia syndrome in human melanoma xenograft models (13). The participation of hIL-6 in the hypercalcemia, and cachexia induced by a human head and neck squamous cell carcinoma was also reported (11).

In this report, we described a cachexia model established by growing a human uterine cervical carcinoma, the Yumoto line, in nude mice. We also showed that hIL-6 produced by the tumor cells is a mediator of cachexia. This action was demonstrated by an experiment in which a neutralizing antibody against hIL-6 was administered to the mouse model. The roles of IL-6 in the induction of cachexia were also discussed.

MATERIALS AND METHODS

Mice. Female BALB/c- nu/nu mice (Charles River Japan Inc., Atsugi, Japan) were used at the age of 6–7 weeks. They were kept at 22 ± 2°C with a 12 h on and off lighting cycle and given breeding diet F1 (Funabashi Farm, Funabashi, Japan) containing 21.3% protein, 57.1% carbohydrates, 5.6% fat, 3.3% fiber, 5.7% ash, and 7.0% moisture (metabolic calories, 3.76 kcal g−1) and water ad libitum. All studies were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals in Nippon Roche Research Center.

Tumors. Tumor tissue of the human uterine cervical carcinoma Yumoto, a well-differentiated keratotic epidermoid type, was transplanted s.c. into a nude mouse and established as a transplantable tumor line, as described previously (15). In the present study, this in vivo tumor line was adapted for in vitro tissue culture using the following procedure. The tumor was excised, minced into small fragments, and then plated onto a 25-cm² Primaria flask (Falcon 3813; Becton Dickinson, Lincoln Park, NJ) with MEM supplemented with 10% FCS to suppress the growth of contaminating fibroblasts. After three passages in the Primaria flasks, the growing adherent tumor cells were maintained in ordinary tissue culture flasks (Falcon 3028; Becton Dickinson) at 37°C, 5% CO₂, in RPMI 1640 medium supplemented with 10% FCS. Compared with observations in

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; G-CSF, Granulocyte-colony stimulating factor; LIF, leukemia inhibitory factor; hLIF, human LIF; IL-1, human IL-1; IAP, immunosuppressive acidic protein; hG-CSF, human G-CSF; hIFN, human IFN; mIL, murine IL; hTNF, human TNF.
the mice bearing the in vivo tumor line (15), cells from this established tissue culture line transplanted (s.c.) into nude mice showed similar histological characteristics and caused a similar extent of weight loss in the tumor-bearing mice. This tissue culture line is, however, still a mixture of various clones. The ability of such a line to induce cachexia might be unstable, since individual clones may have different abilities to induce cachexia and the proportions of various clones could be changeable in a long-term tissue culture. Therefore, we have established a single-cell derived clone which retains the stable ability to induce cachexia using a limiting dilution method. Clone 17 was isolated as a cell line free of Mycoplasma by testing with 6-methylpurine deoxyribose (Boehringer Mannheim, Mannheim, Germany).

Measurement of Body Wasting and Other Cachexia Parameters. A single-cell suspension of tumor cells (10⁶ cells/mouse) was inoculated s.c. into the right flank of the nude mice. The body weight and the length (a) and width (b) of the tumors were measured two or three times a week. For time course experiments, the tumor weight was estimated by calculating the tumor volume (ab²/2) and multiplying this by a correction factor. The correction factor was determined by comparing actual tumor volumes and tumor weights in separate experiments. When the mice were sacrificed, the actual tumor weight was measured. Carcass weight was then calculated as the difference in weight between the whole body and the tumor tissue. To determine the extent of tissue wasting, we measured the weights of the left perivascular white adipose tissues and the gastrocnemius muscles of the left hind legs. The concentrations of substances in serum or plasma were measured in blood samples collected from the heart by using the following methods and reagents: an enzyme reaction method with mutarotase and glucose oxidase for measuring glucose (Glucose CII test; Wako Pure Chemical Ind. Ltd., Osaka, Japan); an immunodiffusion assay with antiserum to TNF-α antibody for measuring murine TNF-α (Genzyme); a radioimmunoassay for thyroxine (T-3 RIA Beads; Dynabot, Tokyo, Japan); an enzyme reaction method with thrombin using COBAS FIBRO (Nippon Roche, Tokyo, Japan) for measuring fibrinogen in citrate plasma; a color-chelate reaction method with o-cresol phthaeline complexone for measuring calcium (Calcium C-Test; Wako); and a radioimmunoassay for thyroxin (T-3 RIA Beads; Dynabot, Tokyo, Japan).

Cytokine Assays and Neutralizing Antibodies. The levels of cytokines secreted by the tumor cells in vitro were determined by assaying the supernatants from 18-h cultures of tumor cells (10⁶ cells/ml). Circulating levels of cytokines in these samples were determined by assaying the supernatants from 18-h cultures of tumor cells (10⁶ cells/ml). The cytokine concentrations in serum or plasma were measured in blood samples collected from the heart by using the following methods and reagents: an enzyme reaction method with mutarotase and glucose oxidase for measuring glucose (Glucose CII test; Wako Pure Chemical Ind. Ltd., Osaka, Japan); an immunodiffusion assay with antiserum to TNF-α antibody for measuring murine TNF-α (Genzyme); a radioimmunoassay for thyroxine (T-3 RIA Beads; Dynabot, Tokyo, Japan).

RESULTS

Experimental Cancer Cachexia Induced in Nude Mice by Human Cervical Cancer Yumoto. A xenograft model established by growing a human cervical carcinoma, Yumoto line, in nude mice was capable of inducing marked body weight loss in the early stage of tumor growth. The tumor-bearing mice started losing weight by day 35, when the tumor weight was only 1 g (4% of total body weight), and continued to lose weight until day 45, when their calculated carcass weight was 10 g less than that of age- and sex-matched controls. As Table 1 shows, these mice also showed significant loss of adipose tissue and muscle weight, as well as hypoglycemia, indicating the presence of disorders in energy and nitrogen homeostases. Other changes observed in these mice included elevated levels of the acute phase proteins (fibrinogen and IAP), reduced level of thyroxin, splenomegaly, hypercalcemia, and leukocytosis. These abnor-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Changes in parameters associated with cancer cachexia in mice bearing a human cervical carcinoma, Yumoto line</th>
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<tbody>
<tr>
<td>Parameters</td>
<td>Tumor-bearing mice</td>
</tr>
<tr>
<td>Tumor wt (g)</td>
<td>0.72 ± 0.46</td>
</tr>
<tr>
<td>Carcass wt (g)</td>
<td>18.4 ± 2.2b</td>
</tr>
<tr>
<td>Adipose tissue wt (mg)</td>
<td>8 ± 11b</td>
</tr>
<tr>
<td>Muscle wt (mg)</td>
<td>74 ± 19b</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>121 ± 24b</td>
</tr>
<tr>
<td>Plasma fibrinogen (mg/dl)</td>
<td>21.9 ± 7.6b</td>
</tr>
<tr>
<td>Serum IAP (µg/ml)</td>
<td>491 ± 119</td>
</tr>
<tr>
<td>Serum calcium (mg/dl)</td>
<td>12.7 ± 4.0</td>
</tr>
<tr>
<td>Serum thyroxin (µg/dl)</td>
<td>0.17 ± 0.36b</td>
</tr>
<tr>
<td>WBC count (×10⁶ cells/ml)</td>
<td>416 ± 293b</td>
</tr>
</tbody>
</table>

*The mice were inoculated s.c. with tissue culture cells of the Yumoto line (10⁶ cells/mouse). Tumor-bearing mice and age- and sex-matched normal mice were sacrificed on day 66. Data are expressed as the mean ± SD from six mice per group.

Statistical Analysis. Statistical analysis of data was performed using SAS software. Differences in tumor size, carcass weight, tissue weight, and concentrations of substances were analyzed using the Mann-Whitney U test. Differences in the time courses of carcass weight changes were analyzed by ANOVA. Differences in IL-6 levels in the culture supernatant of tumor cells were analyzed using Student’s t test. Differences were considered to be significant when the P was < 0.05.
caused a rapid and significant recovery of their body weight. In mice and control mice were sacrificed on day 50, 3 h after the final antibody injection, and their carcass weights, tumor weights, and various cachexia parameters were compared (Table 4). Anti-hIL-6 antibody treatment caused a significant increase in the carcass weight, although it did not suppress tumor growth. Loss of adipose tissue weight and hypoglycemia were also significantly improved. The serum level of IAP and WBC counts were reduced. The biological activity of hIL-6 in the serum and tumor tissue, which was determined by hybridoma B9 cell proliferation assay, was indeed reduced by the antibody. These results strongly suggest that hIL-6 produced by tumor cells is one of the essential inducers of cachexia in the Yumoto model. It is worth noting that tumor tissue levels of mIL-6 and serum levels of hG-CSF were also reduced by anti-hIL-6 antibody.

Stimulation of IL-6 Production by Cytokines in Vitro. We detected other cytokines produced by the tumor cells, such as hIL-1α, in the tumor tissues. Since IL-1 is known to induce IL-6 production in various types of cells (20), we examined the possibility that hIL-1α is an autocrine stimulator for hIL-6 production in clone 17 of the Yumoto line. Fig. 2 shows in vitro hIL-6 production by Yumoto clone 17 cells treated with anti-hIL-1α antibody. The neutralization of IL-1 activity significantly reduced hIL-6 production by the tumor cells. Thus, it is highly possible that the hIL-1 detected in the tumor tissues could be acting as an autocrine stimulator for hIL-6 production by the tumor cells in the tumor tissues.

DISCUSSION

Appropriate animal models are indispensable to examine the induction mechanism of cancer cachexia. Although several animal models have been reported as models for cancer cachexia (21–24), not many of them were established from human tumor xenografts. It was reported in the previous article that a human uterine cervical carcinoma, the Yumoto line, induced remarkable weight loss in tumor-bearing mice (15). In the present study, we established a tissue culture line of the Yumoto line and demonstrated that the in vitro line induced the typical cachexia syndrome after transplantation (s.c.) into nude mice even when the tumor burden was small. Severe adipose tissue and muscle wasting and hypoglycemia as well as decreased carcass weight were observed in the mice bearing less than 1 g of tumor. These findings suggest that there are tumor-induced disorders in the host that involve energy and nitrogen homeostasis. These mice also exhibited elevated levels of acute phase proteins, hypercalcemia, and leukocytosis, abnormalities that are often observed in advanced cancer patients (19). Thus, the mice bearing the human cervical carcinoma Yumoto line can be considered an appropriate model to investigate mechanisms of human tumor-induced cachexia and disorders in homeostasis of the host.

Host cell-derived and tumor cell-derived cytokines have been suggested as mediators of the metabolic changes associated with cachexia (25, 26). In the murine carcinoma colon 26 model, mIL-6 has been proposed as a cachexia factor, because a mAb to mIL-6 (but not anti-TNF antibody) was able to suppress the development of key parameters of cachexia in tumor-bearing mice (10, 12). In the colon 26 model, however, it
Table 3  In vivo cytokine productions in mice bearing clone 17 of the Yumoto line

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Serum-type cytokines</th>
<th>Murine-type cytokines</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(ng/ml serum or ng/g tumor tissue)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human-type cytokines</td>
<td>Murine-type cytokines</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>Tumor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>&lt;0.05</td>
<td>0.12 ± 0.03b</td>
</tr>
<tr>
<td>IL-1α</td>
<td>&lt;0.01</td>
<td>34.9 ± 5.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>&lt;0.01</td>
<td>21.4 ± 5.6</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.38 ± 0.19</td>
<td>15.2 ± 7.6</td>
</tr>
<tr>
<td>LIF</td>
<td>&lt;0.05</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>&lt;0.02</td>
<td>0.17 ± 0.18</td>
</tr>
<tr>
<td>G-CSF</td>
<td>4.82 ± 2.36</td>
<td>188 ± 71</td>
</tr>
</tbody>
</table>

* Cytokine levels in the sera of tumor-bearing mice and in the tumor tissues were determined by corresponding ELISA systems. Samples were obtained from mice bearing clone 17 of the Yumoto line on days 50 for hIL-6, mIL-6, and hG-CSF, and on day 48 for the other cytokines. None of these cytokines was detected in the sera of nontumor-bearing mice. The lower detection limits for hIL-6 and mIL-6 in the serum were 0.01 and 0.05 ng/ml, respectively.

a Data are expressed as the mean ± SD for samples from six animals.

Fig. 1 Improvement of cachexia by anti-hIL-6 antibody. Cells (10^7 cells) of the Yumoto line clone 17 were inoculated into nude mice on day 0 (1). Anti-hIL-6 antibody, B-E8 (2), or control IgG (3) was administered i.p. to the tumor-bearing mice (six mice per group) at a dose of 50 μg/mouse/day for 7 days from day 44. Calculated carcass weight and tumor volume were determined periodically. The control nontumor-bearing nude mice (4) were matched for age and sex. The difference in carcass weight changes between the anti-hIL-6 antibody group and the control IgG group was statistically significant (ANOVA, P < 0.05). Points and bars, mean ± SD from six mice per group.

Table 4  Improvement of cachexia by anti-hIL-6 antibody in mice bearing clone 17 of the Yumoto line

<table>
<thead>
<tr>
<th>Administration*</th>
<th>Carcass wt (g)</th>
<th>Tumor wt (g)</th>
<th>WBC count (×10^5/ml)</th>
<th>Adipose tissue wt (mg)</th>
<th>Muscle wt (mg)</th>
<th>Serum glucose (mg/dl)</th>
<th>Serum calcium (mg/dl)</th>
<th>Serum IAP (μg/ml)</th>
<th>Serum hIL-6 (ng/ml)*</th>
<th>Tumor hIL-6 (ng/g)*</th>
<th>Tumor mIL-6 (ng/g)*</th>
<th>Serum hG-CSF (ng/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>17.8 ± 2.1</td>
<td>20.7 ± 1.3a</td>
<td>181 ± 113</td>
<td>15 ± 8</td>
<td>89 ± 12</td>
<td>126.0 ± 78.8</td>
<td>11.9 ± 1.7</td>
<td>1142 ± 294</td>
<td>0.38 ± 0.19</td>
<td>15.2 ± 7.63</td>
<td>3.13 ± 2.23</td>
<td>4.82 ± 2.36</td>
</tr>
<tr>
<td>Anti-hIL-6 Ab</td>
<td>18.2 ± 1.9</td>
<td>22.0 ± 1.9a</td>
<td>201 ± 133</td>
<td>17 ± 9</td>
<td>91 ± 12</td>
<td>126.4 ± 80.4</td>
<td>10.5 ± 1.7</td>
<td>1294 ± 304</td>
<td>0.38 ± 0.19</td>
<td>15.2 ± 7.63</td>
<td>3.13 ± 2.23</td>
<td>4.82 ± 2.36</td>
</tr>
<tr>
<td>Nontumor-bearing mice</td>
<td>21.4 ± 1.3</td>
<td>24.0 ± 1.9a</td>
<td>181 ± 113</td>
<td>15 ± 8</td>
<td>77 ± 76</td>
<td>246.4 ± 85.6</td>
<td>8.6 ± 0.7</td>
<td>183 ± 120</td>
<td>0.38 ± 0.19</td>
<td>15.2 ± 7.63</td>
<td>3.13 ± 2.23</td>
<td>4.82 ± 2.36</td>
</tr>
</tbody>
</table>

* Anti-hIL-6 mAb (B-E8) or control IgG was administered i.p. to the mice bearing clone 17 of the Yumoto line at a dose of 50 μg/mouse/day for 7 days from day 44. Parameters were assayed on day 50. Data are expressed as the mean ± SD from six mice per group.

b Statistically different from the control IgG group using the Mann-Whitney U test (P < 0.05).

c Statistically different from the control IgG group using the Mann-Whitney U test (P < 0.01).

d Determined by bioassay (B9 cell proliferation) in the presence of anti-mIL-6 antibody.

The association of murine or human IL-6 with cachexia induction was recently reported in several animal models. Chinese hamster ovarian cells transfected with the mIL-6 gene induced body weight loss (27). Murine Lewis lung carcinoma cells transfected with the hIL-6 gene induced cachexia (28). Anti-mIL-6-neutralizing antibody partially prevented the induction of cachexia in a colon 26 model (10, 12). Anti-hIL-6-neutralizing antibody improved some cachexia parameters in a human head and neck squamous cell carcinoma model, although the reversal of body weight loss was not statistically significant (11). Anti-mIL-6 antibody partially prevented body weight loss.
induced by acute inflammation (29). It is also reported that IL-6 has an ability to suppress the expression in adipocytes of lipoprotein lipase, one of the key enzymes regulating lipid metabolism in cachexia (30). It is noteworthy that IL-6 and LIF, the latter of which was proposed as a cachexia factor in human melanoma xenograft models by one of the authors (13), share a common subunit for their receptor, glycoprotein 130 (31). Thus, IL-6 and LIF might play similar biological roles in the induction of cachexia.

In contrast, body weight loss was not reported in mice given injections of recombinant hIL-6 (32, 33), or in hIL-6 transgenic mice (34). Thus, the role of IL-6 in cachexia induction is still controversial. There are several possible explanations for these apparent discrepancies. First, although IL-6 is an essential factor for the induction of cachexia in certain models, it might not be the sole inducer of cachexia. Systemic symptoms of cachexia such as body weight loss may only occur in the presence of some additional factors. Indeed, we detected hIL-1α, hIL-1β, and mIL-1α in the tissue tumors of the Yumoto model. These cytokines might also be involved in the induction of cachexia, although we have not yet examined their roles in combination with IL-6. The other possibility is that IL-6 is not a direct inducer of cachexia, but is able to make tumor cells produce some other unidentified cachexia factor(s). If the production of such a factor is dependent on the cell type, then the IL-6 production would not always be associated with the cachexia induction in some cell types. Thus, further studies are still required to clarify the role of IL-6 in cachexia induction. However, the result of the anti-hIL-6 antibody treatment, we reported here, gives clear-cut evidence for the participation of tumor cell-derived hIL-6 in experimental cachexia models.

Besides typical disorders involving a host metabolism in cancer cachexia, we also observed other disorders in this model, such as leukocytosis and hypercalcemia. The leukocytosis was improved by the anti-hIL-6 antibody treatment. We have detected a high level of hG-CSF by ELISA in the serum of tumor-bearing mice, which is decreased by the anti-hIL-6 antibody treatment. Therefore, the suppression of leukocytosis could be mediated by the reduction of the serum levels of hG-CSF. The mechanism for in vivo suppression of hG-CSF production by anti-hIL-6 antibody is still unclear, since in vitro production of hG-CSF by the tumor cells was not affected by a treatment with either hIL-6 or anti-hIL-6 antibody (data not shown). On the other hand, the anti-hIL-6 antibody treatment did not significantly reduce serum levels of calcium, indicating that hIL-6 is not the sole inducer of hypercalcemia in this model. One possibility is suggested by the finding that the human parathyroid hormone-relating protein was detected in tissue cultures of the Yumoto line.3 Involvement of hG-CSF or human parathyroid hormone-relating protein in the body weight loss or tissue wasting, however, is still unclear. These factors might play auxiliary roles in cachexia induction, although this has to be confirmed.

Thus, our data indicate that the hIL-6 produced by the tumor cells is an essential mediator for the cachexia induction in this human cervical carcinoma xenograft model. Whether or not IL-6 is a mediator common to clinical cancer cachexia or just to certain types of cachexia has yet to be established.

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