Expression of cancer cachexia-related factors in human cancer xenografts: an immunohistochemical analysis

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ABSTRACT
We immunohistochemically evaluated the involvement of five cancer cachexia-related factors, including leukemia-inhibitory factor (LIF), zinc-α2-glycoprotein (ZAG), interleukin 6 (IL-6), proteolysis-inducing factor (PIF) and tumor necrosis factor α (TNF α) in causing cancer cachexia. Twenty-six xenografts implanted into mice were examined for the expression of the cancer cachexia-related factors, in relation to the body weight loss of the hosts. Five xenografts were categorized in the cachectic group, and the remaining 21 xenografts belonged to the non-cachectic group. LIF was extensively expressed in both the cachectic and non-cachectic groups. ZAG and IL-6 were expressed in one of the cachectic and some non-cachectic xenografts. PIF and TNF α were detected in one and two non-cachectic xenografts, respectively, but in none of the cachectic ones. Any of five factors examined were not conclusive for causing cancer cachexia in the murine xenograft model. Further analysis is needed in order to elucidate the mechanisms responsible for cancer cachexia.

Cancer cachexia is one of the most serious conditions associated with advanced malignant disease, and has a substantial impact on the quality of life and survival of affected patients; 10 to 20% of cancer patients die from the consequences of cachexia rather than tumor burden (15, 42). Cachexia is characterized by weight loss and anorexia, resulting in marked depletion of skeletal muscle and adipose tissue. Although extensive research has characterized the clinical and metabolic alterations in cancer cachexia, the cellular mechanisms remain poorly understood, and therapeutic strategies to alleviate the condition are not yet available.

Most research effort has focused on the rule of cytokines as mediators of the process of cachexia, particularly tumor necrosis factor α (TNF α), a 17-kDa protein, and interleukin 6 (IL-6), a 26-kDa protein. Kawakami and Cerami purified a lipoprotein lipase-inhibitory factor from macrophages in cases of infectious disease, and called it cachectin (20). The amino acid sequence of cachectin was reportedly identical to that of TNF α (3). Further studies revealed that exogenous administration of human TNF α induced cachexia in mice (29, 33). TNF α was hence suspected to be a factor responsible for cancer cachexia. However, to date there has been no evidence that TNF α is tumor-derived, and most clinical studies have failed to correlate TNF α levels with the cachectic state (1, 34). Strassmann et al. described that IL-6 was a key substance in developing cachexia in a murine colonic cancer model (36). Tamura et al. demonstrated that human IL-6 was an
Leukemia-inhibitory factor (LIF), a pleiotropic glycoprotein belonging to the IL-6 family of cytokines, regulates the growth promotion and differentiation of target cells, cachexia and inflammation (24). Mori et al. (26, 27) proposed that LIF played a role in cancer cachexia syndrome in melanoma-bearing nude mice. When recombinant murine LIF was injected into mice, significant weight loss occurred (23).

Zinc-alpha2-glycoprotein (ZAG), a 43-kDa protein originally isolated from human plasma, is one putative mediator of a substantial loss of body fat (41). It has been shown that a lipid-mobilizing factor isolated from a cachexia-inducing murine adenocarcinoma (MAC16) and from the urine of patients with cancer cachexia is identical to ZAG (41). Murine and human ZAGs share up to 100% identity in specific regions thought to be important in lipid metabolism (31). Both human and murine ZAGs stimulate lipolysis in the adipocytes, resulting in glycerol release and increased lipid utilization (14). Reportedly, serum levels of ZAG in cancer patients were proportional to the extent of weight loss (12), and reduced in patients’ sera after response to chemotherapy (2).

Proteolysis-inducing factor (PIF) has been identified as a possible tumor-derived cachectic factor. PIF was initially isolated from the murine MAC16, and later identified in the urine of weight-losing human cancer patients, but absent from the urine of normal subjects or patients with weight loss due to trauma or sepsis (7, 22, 38–40). PIF from both murine and human sources is a 24-kDa sulfated glycoprotein (38, 40). When injected into non-tumor-bearing mice, PIF caused rapid weight loss principally due to skeletal muscle proteolysis, without a reduction in food and water intake and a loss of visceral protein (21, 38). An in vivo study using mice showed that the murine monoclonal antibody against PIF could attenuate weight loss induced by human PIF (7).

In the present study, we immunohistochemically investigated the expression of a total of five cancer cachexia-related factors in murine xenografts of human cancers, in order to determine whether factors are really involved in causing cancer cachexia.

MATERIALS AND METHODS

Animals. Four-weeks-old-male BALB/cA nude mice were purchased from Clea Japan, Co. Ltd (Tokyo, Japan). The animals were housed under specific-pathogen-free conditions and given access to food and water ad libitum.

Human cancer xenografts. One oral cavity cancer cell line (OCC-1), six pulmonary cancer cell lines (LC-6, LC-11, Lu-99, Lu-130, Lu-134 and LX-1), five gastric cancer cell lines (SC-2, SC-4, ST-40, 4-1ST and AZ-521), three colonic cancer cell lines (Co-3, COL-1 and HCT-15), five pancreatic cancer cell lines (PAN-3, PAN-4, PAN-12, BxPC-3 and MIAPaCa-2), five mammary cancer cell lines (MC-2, MC-5, MX-1, MDA-MB-435S and MDA-MB-231), and one uterine cancer cell line (SW756) were used in the present study. Lu-134 was obtained from RIKEN (Saitama, Japan). AZ-521 was purchased from JCRB (Osaka, Japan). HCT-15, BxPC-3, MIA-PaCa-2 and MDA-MB-435S were obtained from ATCC (VA, USA). Other cell lines were established at the Central Institute for Experimental Animals (Kawasaki, Japan). The tumor was removed from the nude mice about one month after implantation, and tumor pieces (about 2 mm in size) were prepared. One piece was subcutaneously implanted into the right flank. Total body weight and tumor size were measured two to four times a week. The tumor volume was calculated by use of the following equation; tumor volume (mm$^3$) = width of tumor$^2$ × length of tumor/2. When the tumor volume has reached 100 mm$^3$, it was designated as day 0. The body weight change of the mice was calculated by using the following equation; the body weight change (%) = (body weight on day A - body weight on day 0)/body weight on day 0 × 100. When the xenograft-bearing mice showed body weight loss more than 10% at day 14, it was defined as cachectic. The OCC-1 model, in which the mice died before day 10 due to marked progression of body weight loss and tumor growth, was also regarded as cachectic. The tumors were sampled on day 7 for the OCC-1 xenograft and on day 14 for the others. Two subjects in the respective cell lines were processed for measuring tumor volume and body weight change, and for immunostaining cancer cachexia-related factors. No drugs were administered in any mice. The animal experiments were carried out in conformity with the guidelines of ethical review commissions.
out in accordance with the Guidelines for the Welfare of Animals in Experimental Neoplasia.

Immunohistochemistry. Immunostaining was performed using formalin-fixed, paraffin-embedded tissue sections. Endogenous peroxidase was inactivated by 0.03% hydrogen peroxide in methanol for 30 min. Heat-induced antigen retrieval was applied on a pressure cooker (Delicio 6L; T-FAL, Rumily, France) for 10 min. Optimal soaking solutions were selected for the respective markers determined by preliminary experiments: 10 mM citrate buffer, pH 6.0 for PIF, LIF, IL-6 and TNF α, and 1 mM ethylenediaminetetraacetic acid solution, pH 8.0 for ZAG. After pressure cooking, the sections were left at room temperature for cooling in the soaking solution for 30 min.

The primary antibodies used react specifically with a synthetic PIF peptide (YSPEAASAPGSHEA) (rabbit polyclonal, 1:100 dilution; Immunobiological Laboratories, LIF (goat polyclonal, 1:1000 dilution; Santa Cruz Biotechnology, CA, USA), ZAG (rabbit polyclonal, 1:400 dilution; Santa Cruz Biotechnology), IL-6 (goat polyclonal, 1:1000 dilution; Dako, Kyoto, Japan) and TNF α (goat polyclonal, 1:2000 dilution; R & D Systems, MN, USA). The sections were incubated with the antibody overnight at room temperature. For localizing PIF and ZAG, Histofine® Simple Stain MAX-PO (Nichirei, Tokyo, Japan), employing the universal immuno-peroxidase polymer method, was utilized as a second-layer reagent. Immunostaining for LIF, IL-6 and TNF α was performed using a biotin-free catalyzed amplification system (Dako). The reaction products were visualized in 50 mg/dL 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide. Negative control studies were performed without applying the primary antibodies. Sections known to be stained positively were included in each run as a positive control.

The specificity of immunostaining was checked by the preabsorption experiment. Prior to immunostaining, the diluted antibody was admixed with the synthetic peptide or recombinant antigens at a final concentration of 0.01, 0.1, 1.0, or 10 μg/mL at 37°C for 1 h. Sources of the peptide fragments or recombinant antigens were as follows: Sigma Genosys, Hokkaido, Japan for the PIF peptide, Santa Cruz Biotechnology for LIF and ZAG, and R & D Systems for IL-6 and TNF α.

Evaluation of the immunostained sections. The immunostained sections were independently reviewed by two investigators (S. K. and M. S.). The staining results were classified into four groups based on the proportion of positively stained cancer cells in the xenograft tumor: −, no tumor cells are positive; (+), only a few tumor cells are positive; +, less than one-third of tumor cells are positive; ++, more than one-third of tumor cells are positive.

Statistical analysis. The Fisher’s exact probability test was employed for determining the statistical significance of correlation between marker expression in the xenografts and weight loss of the mice.

RESULTS

Intracellular localization and staining specificity

Immunoreactivities of all factors examined were consistently localized in the cytoplasm of the cells, showing a clearly brown-colored reaction (Fig. 1). They were completely abolished by admixing the antibody with the peptide or recombinant antigen at the concentration of 0.01 to 1.0 μg/mL, confirming the specificity of immunostaining.

Expression of cancer cachexia-related factors in xenograft models

Table 1 shows the relationship between the expression of cancer cachexia-related factors in xenografts and the body weight change of the mice. Cachectic weight loss was seen in the mice bearing five kinds of xenograft; OCC-1 (−12.4%, −20.5%; mean, −16.5%), LX-1 (−13.0%, −15.2%; mean, −14.1%), Co-3 (−10.1%, −13.6%; mean, −11.9%), COL-1 (−15.0%, −18.9%; mean, −17.0%), and SW756 (−11.8%, −8.1%; mean, −10.0%). The mice became cachectic in appearance, accompanying a decrease of activity, skin dryness and loss of appetite. In contrast, the tumor growth progressed in all xenograft models examined.

PIF expression was detected in none of the five cachectic xenografts and one of the 21 non-cachectic xenografts. LIF was expressed in three (60%, OCC-1, Co-3 and SW756) of the cachectic xenografts and 17 (81%) of the non-cachectic xenografts. Only a few tumor cells of one cachectic xenograft (COL-1) were positive for ZAG, while a considerable number of ZAG-positive cells were observed in three non-cachectic, mammary cancer xenografts. IL-6 expression was found in one cachectic xenograft (OCC-1), and three (14%) of the non-cachectic xenografts. Only a few TNF α-positive cells were seen in two non-cachectic, mammary cancer xenografts, while all the cachectic xenografts lacked...
TNF α immunoreactivity. There was no significant difference in the marker expression between the cachectic and the non-cachectic groups. No apparent difference in the marker expression was noted between two subjects in the respective cell lines.

DISCUSSION
Since cachexia is an important cause of mortality in cancer patients, effective therapies for cachexia not only should improve the quality of life of cancer patients, but also may extend the survival time. Knowledge of the mechanism by which tumors induce a cachectic state is essential to the development of effective inhibitors. Data from various sources, including animal and human studies, have suggested the involvement of some cytokines and cancer-derived glycoproteins such as ZAG and PIF in cancer cachexia. We thus evaluated the expression of a panel of cancer cachexia-related factors in

**Fig. 1** Expression of LIF (A, B), PIF (C), ZAG (D), IL-6 (E), and TNF α (F) in human cancer xenografts. (A) Cachectic, colon cancer xenograft Co-3. (B) Non-cachectic, pancreatic cancer xenograft PAN-3. LIF immunoreactivity is observed in scattered tumor cells. (C) Non-cachectic, pancreatic cancer xenograft PAN-4. A few PIF-positive cells are noted. (D) Non-cachectic, mammary cancer xenograft MC-5. A large number of tumor cells are immunoreactive for ZAG. (E) Non-cachectic, pulmonary cancer xenograft LC-11. A number of tumor cells are positive for IL-6. (F) Non-cachectic, mammary cancer xenograft MC-2. TNF α immunoreactivity is observed in one tumor cell (arrow). All the factors are expressed in the cytoplasm of the cancer cells.
human cancer xenografts in relation to weight loss of the host mice, by means of the immunohistochemical technique.

The present analysis revealed that TNF α was hardly expressed in any xenografts, suggesting that TNF α is not likely to be a tumor-derived factor responsible for the cancer cachexia. Similarly, most clinical studies have failed to correlate TNF α levels with the cachectic state (1, 34).

The reported evidence of a role of IL-6 in clinical cancer cachexia is variegated. Some investigators have found that higher IL-6 levels in patients with non-small cell lung, pancreatic, and prostatic cancers are associated with weight loss (10, 28, 30, 32). However, other data have raised doubts as to whether IL-6 is an independent cachetic factor (11, 35). In the present study, IL-6 was expressed in 14% of the non-cachetic xenografts as well as in 20% of the cachetic xenografts.

LIF was frequently expressed in various types of xenograft. Kamohara et al. (19) showed that LIF mRNA was detected in all 24 cancer cell lines of the lung, breast, stomach, colon, liver, gallbladder, pancreas and melanocytes in origin. Dhingra et al. (8) observed LIF immunoexpression in 78% of breast cancers. These findings are consistent with ours.

Iseki et al. (16) and Kajimura et al. (18) described that IL-6 and/or LIF produced by tumor cells were the factors most likely to be responsible for cancer cachexia in four of eight xenograft models, including OCC-1 (examined also in our study), but in the other four cancer cell lines the causative factors remained unknown. However, they did not examine the marker expression in the non-cachetic xenograft models.

Only a few tumor cells of one colonic cachectic xenograft were positive for ZAG, while a considerable number of ZAG-positive cells were observed in

### Table 1

<table>
<thead>
<tr>
<th>Xenograft (origin)</th>
<th>Body weight changea</th>
<th>Expression of marker</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>PIF</td>
</tr>
<tr>
<td>OCC-1 (oral cavity cancer)</td>
<td>-16.5b</td>
<td>+</td>
</tr>
<tr>
<td>LC-6 (pulmonary cancer)</td>
<td>3.8</td>
<td>+</td>
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<tr>
<td>LC-11 (pulmonary cancer)</td>
<td>2.5</td>
<td>+</td>
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<tr>
<td>Lu-99 (pulmonary cancer)</td>
<td>15.5</td>
<td>+</td>
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<tr>
<td>Lu-130 (pulmonary cancer)</td>
<td>8.4</td>
<td>−</td>
</tr>
<tr>
<td>Lu-134 (pulmonary cancer)</td>
<td>4.1</td>
<td>−</td>
</tr>
<tr>
<td>LX-1 (pulmonary cancer)</td>
<td>-14.1b</td>
<td>−</td>
</tr>
<tr>
<td>SC-2 (gastric cancer)</td>
<td>-4.1</td>
<td>+</td>
</tr>
<tr>
<td>SC-4 (gastric cancer)</td>
<td>-4.1</td>
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</tr>
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<td>ST-40 (gastric cancer)</td>
<td>6.3</td>
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</tr>
<tr>
<td>4-1ST (gastric cancer)</td>
<td>8.9</td>
<td>(+)</td>
</tr>
<tr>
<td>AZ-521 (gastric cancer)</td>
<td>10.4</td>
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</tr>
<tr>
<td>Co-3 (colonic cancer)</td>
<td>-11.9b</td>
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</tr>
<tr>
<td>COL-1 (colonic cancer)</td>
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<td>HCT-15 (colonic cancer)</td>
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<td>PAN-3 (pancreatic cancer)</td>
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<td>PAN-4 (pancreatic cancer)</td>
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<td>PAN-12 (pancreatic cancer)</td>
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<tr>
<td>BxPC-3 (pancreatic cancer)</td>
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<tr>
<td>MIA PaCa-2 (pancreatic cancer)</td>
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<tr>
<td>MC-2 (mammary cancer)</td>
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</tr>
<tr>
<td>MC-5 (mammary cancer)</td>
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</tr>
<tr>
<td>MX-1 (mammary cancer)</td>
<td>12.3</td>
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</tr>
<tr>
<td>MDA-MB-435S (mammary cancer)</td>
<td>2.8</td>
<td>(+)</td>
</tr>
<tr>
<td>MDA-MB-231 (mammary cancer)</td>
<td>6.5</td>
<td>(+)</td>
</tr>
<tr>
<td>SW756 (uterine cervical cancer)</td>
<td>-10.0b</td>
<td>+</td>
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*Mean values from two experiments, measured on day 7 for OCC-1 or day 14 for other xenografts; bRegarded as the cachectic xenograft; ++, More than one-third of tumor cells are positive; +, Less than one-third of tumor cells are positive; (+), Only a few tumor cells are positive; −, No tumor cells are positive.
three non-cachectic mammary xenografts. Overexpression of ZAG occurs in several types of malignant tumor (5, 9, 13). As for the breast cancer, it has been reported that ZAG expression correlates with tumor differentiation but does not affect the prognosis (9). In addition, it has been found that human and murine adipose tissues express ZAG (4). The role of ZAG in cancer cachexia must be elucidated by further studies.

PIF, a sulfated glycoprotein, was detected in two non-cachectic, pancreatic cancer xenografts, but undetectable in any of the cachectic xenografts examined in the present study. In a previous study employing 47 patients with cancer of the lung, colorectum, pancreas, liver, ovary or breast, PIF was detected in the urine of all weight-losing patients, but absent in that of weight-stable patients with the same type of tumor (7). An immunohistochemical study demonstrated that tumor cells in metastatic sites of human gastrointestinal cancers expressed PIF, and that PIF expression correlated with weight loss of the patients (6). These reports suggest that PIF may play an important role in cancer cachexia other than the other factors. However, Monitto et al. reported that PIF peptide lacks a putative glycosylation site, and that forced expression of PIF does not induce cachexia de novo in a murine xenograft model (25). Recently, Jatoi et al. described that there were no significant correlations between the presence of PIF in the urine of patients with metastatic gastric/esophageal cancer, and weight loss, tumor chemosensitivity or patient survival (17).

In conclusion, we did not confirm that any of five markers examined here were causative for cancer cachexia in murine xenograft models. The results suggest complex mechanisms involved in the development of cachexia. Further investigation is needed in order to elucidate the mechanisms responsible for cancer cachexia.

REFERENCES


