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Toxohormones responsible for cancer cachexia syndrome in nude mice bearing human cancer cell lines

Abstract Toxohormones are tumor-derived factors that induce cancer cachexia syndrome in tumor-bearing animals. Nude mice bearing tumors induced by eight human cancer cell lines with this activity were studied for cytokine production and expression of a newly identified gene, *ob*, which has the ability to control body weight. A melanoma cell line, SEKI, and a neuroepithelioma cell line, NAGAI, produced a large amount of the cytokine, leukemia-inhibitory factor (LIF). A uterine carcinoma cell line, Yumoto, produced a large amount of interleukin 6 (IL-6), and an oral cavity carcinoma cell line, OCC-1C, concomitantly produced LIF, IL-6, and IL-11. Reverse transcription polymerase chain reaction studies revealed that *ob* gene mRNA was not expressed in any of these cell lines, suggesting that the gene does not have a role as a tumor product responsible for cancer cachexia in this model. These findings suggest that in four of eight animal models in which cancer cachexia syndrome developed, LIF, IL-6, or possibly IL-11 produced by cancer cells may be toxohormones, but in the remaining four cancer cell lines the mechanism responsible for cachexia syndrome remains unknown.

Key words Cachexia · LIF · IL-6 · IL-11 · *ob* gene

Introduction

Progressive weight loss, or cachexia, is a common clinical feature in cancer patients. In most cases, weight loss can be explained in part by anorexia or decreased food intake as a result of drug toxicity, chronic pain, a depressive state, gastrointestinal tract obstruction, hypercalcemia, uremia, and/or other factors. It is well recognized that weight loss stops on tumor resection. This clinical observation fits into the humoral syndrome, and the hypothetical factor responsible for this syndrome, originally designated toxohormone [14], has long been sought.

Our recent experimental studies have demonstrated that two cytokines, leukemia-inhibitory factor (LIF) [8, 13, 21] and interleukin 6 (IL-6) [20, 21], are the factors most likely to be responsible for this morbidity, at least in animal experiments, on the basis of the following observations: (1) LIF and IL-6 inhibit lipoprotein lipase expressed in 3T3-L1 adipocytes, suggesting that these cytokines inhibit adipocyte differentiation; (2) exogenous administration of both cytokines induces body weight loss in experimental animals; and (3) there is a close relationship between LIF and IL-6 production and cancer cachexia development. However, our study of five human cancer cell lines associated with cancer cachexia syndrome demonstrated that LIF and IL-6 production could explain this morbidity in some but not all experimental models tested [8]. In the present study we examined nude mice bearing tumors induced by eight human cancer cell lines to determine whether cytokines that show lipoprotein lipase (LPL)-suppressing activity are involved in causing cachexia and whether the newly identified *ob* gene product, leptin, plays a role in this morbidity [23].

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Materials and methods

Cell lines

The human cancer cell lines examined were SEKI, a melanoma cell line established at the National Cancer Center, Tokyo, Japan [18]; NAGAI [9], a human neuroepithelioma cell line, and MKN-1 [6], an adenosquamous gastric carcinoma cell line, both kindly provided by the First Department of Pathology, Niigata University, Niigata, Japan; LS-180 [22], established from a well-differentiated colon adenocarcinoma and purchased from the American Type Culture Collection

(Rockville, Md., USA); LX-1 [3], established from an undifferentiated lung carcinoma and provided by Banyu Tsukuba Research Institute, Tsukuba, Japan; Yumoto, a uterine cervical-carcinoma cell line of the well-differentiated keratotic epidermoid type, provided by Nippon Roche Research Center, Kamakura, Japan; LC-1, established from a moderately differentiated lung squamous carcinoma and provided by the Japanese Cancer Research Resources Bank, Tokyo, Japan; and OCC-1C, a novel cancer cell line established from a tumor xenograft of oral cavity carcinoma, OCC-1 [21]. All cell lines were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (Mitsubishi Kasei, Tokyo, Japan) at 37 °C under an atmosphere containing 5% CO_2. All cell lines were confirmed to be free of mycoplasma as demonstrated by staining with Hoechst 33258 (Wako Pure Chemical Industries, Osaka, Japan).

Nude mice

Female BALB/c nu-nu mice were purchased at the age of 5 weeks (Charles River Japan, Atsugi, Japan). They were kept at 23 ± 2 °C under a 12-h on:off lighting cycle and had access to a breeding diet (CMF; Oriental Yeast, Tokyo, Japan) and water ad libitum.

Tumor xenografts

Cell suspensions (1×10^7 cells) of SEKI, NAGAI, Yumoto, MKN-1, LS-180, and LX-1 were inoculated subcutaneously into the right flank of 6-week-old nude mice. Studies using the OCC-1 or LC-1 cell lines have been reported elsewhere [21]. The body weight of the mice and the length (a) and width (b) of the xenograft tumors were measured three times each week using automatic calipers. Tumor weight was estimated by calculating the tumor volume ($ab^2/2$). When mice had become emaciated, blood was sampled by cardiac puncture, and the tumor tissue was removed and immediately stored in liquid nitrogen until mRNA extraction. Carcass body weight was calculated as the difference between the weight of the whole body and that of the tumor tissue.

LPL activity assay

3T3-L1 preadipocytes, provided by the Japanese Cancer Research Resources Bank, Tokyo, Japan, were cultured and differentiated into adipocytes as previously described [8]. Conditioned culture medium from SEKI, NAGAI, OCC-1C, MKN-1, LS180, or LX-1 cells cultured for 5 days was added to the differentiated 3T3-L1 cells. After exposure to the media for 16 h the cells were washed twice with Dulbecco's phosphate-buffered saline and then incubated for a further 60 min at 37 °C with fresh medium containing sodium heparin at 0.05 mg/ml. Heparin-releasable LPL activity was measured using a method described elsewhere [10].

mRNA extraction

Total RNA was extracted from cells and resected tumors by the acid guanidinium-phenol-chloroform method [1]. Poly(A)$^+$ RNA was isolated using Oligotex dT-30 affinity chromatography [15].

Northern-blot analysis

Northern-blot hybridizations were performed as previously reported [7]. To detect human LIF and tumor necrosis factor α (TNFα) mRNA, oligodeoxynucleotides were synthesized and used as probes. These consisted of the antisense sequence for nucleotides 292–345 of the LIF gene [13] and that for nucleotides 552–587 of the TNFα gene [17]. IL-6 mRNA expression was examined using a cDNA probe corresponding to nucleotides 174–708 of human IL-6 [5]. The RNA samples were also examined for β-actin mRNA to determine their integrity; the method used has been described elsewhere [7].

Reverse transcription-polymerase chain reaction

Complementary DNA was synthesized using 100 ng mRNA, Moloney murine leukemia-virus reverse transcriptase, and an oligo(dT) primer (Stratagene, La Jolla, Calif., USA) in a volume of 50 µl containing (final concentrations) 50 m*M* TRIS-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, and 1 m*M* of each dNTP. For polymerase chain reaction (PCR) amplification, the reverse transcriptase (RT) reaction mixture (1.3 µl) was used in a reaction mixture (25 µl) containing 12.5 pmol of each primer, which encompassed the coding sequence corresponding to nucleotides 20–428 of the human *ob* gene [2, 23]; 5 nmol of each dNTP; 0.63 units of Taq DNA poIymerase (Takara, Otsu, Japan); and (final concentrations) 10 m*M* TRIS-HCl (pH 8.3), 50 m*M* KCl, and 1.5 m*M* $MgCl_2$. Reaction mixtures were subjected to 35 successive cycles of PCR, consisting of 45 s of denaturation at 94 °C, 45 s of annealing (at 55 °C for human *ob* and 65 °C for β-actin), and 3 min of primer extension at 72 °C. An additional 10-min extension step at 72 °C was added after the 35 cycles. PCR was performed in a temperature cycler (RoboCycler 40; Stratagene). PCR products were size-fractionated in 3% Nusieve 3:1 agarose gels (FMC BioProducts, Rockland, Me., USA).

Enzyme-linked immunosorbent assay of cytokines

Cytokine levels were evaluated in conditioned media (5-day culture) from cancer cells using the Quantikine enzyme-linked immunosorbent assay (ELISA) kit for human TNFα, LIF, IL-6, IL-11, and oncostatin M (OSM). (R & D Systems, Minneapolis, Minn., USA).

Statistical analysis

Differences in cytokine levels measured in the conditioned culture media of tumor cells were analyzed using Student's t-test. Differences of $P < 0.05$ were considered to be significant.

Table 1 Cancer cachexia syndrome: causative factors (*ND* Not determined)

Cell line	Body weight (% of control)[a]	Causative factor					
		TNFα	LIF	IL-6	IL-11	OSM	Leptin
SEKI (melanoma)	59	–	+++	–	–	–	–
NAGAI (neuroepithelioma)	63	–	+++	–	–	–	–
OCC-1 (oral cavity carcinoma)	57	–	+++	+++	++	–	–
Yumoto (uterine carcinoma)	83	–	–	+++	+	ND	–
MKN-1 (gastric carcinoma)	61	–	–	+	+	–	–
LC-1 (lung carcinoma)	59	–	–	–	–	–	–
LX-1 (lung carcinoma)	58	–	–	–	–	–	–
LS180 (colon carcinoma)	59	–	–	–	–	–	–

[a] Mean values (n = 10)

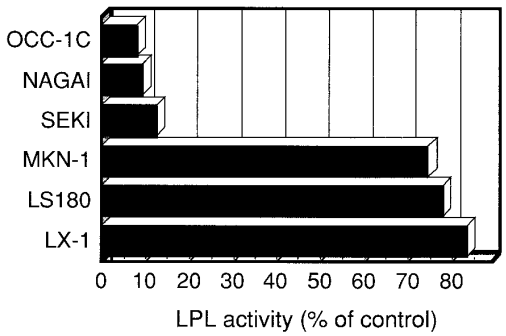

Fig. 1 Inhibition of LPL activity by conditioned media from cultured cancer cells associated with cachexia. LPL activity in control 3T3-L1 adipocytes was taken to be 100%

Results

Experimental cancer cachexia

All nude mice bearing cancer cell xenografts developed cancer cachexia syndrome. Tumor-bearing mice showed decreased body weight, lack of appetite, decreased activity, skin dryness, and low temperature. Their body weight decreased to about 60% of that of control mice, except in mice bearing the Yumoto xenografts, in which body weight decreased to 83% of that of controls (Table 1).

Inhibition of LPL activity by culture media

Conditioned media from OCC-1C, NAGAI, SEKI, MKN-1, LS180, and LX-1 cells had a significant inhibitory effect on LPL activity as shown in Fig. 1. It is noteworthy that media from the OCC-1C, NAGAI, and SEKI cells showed particularly strong LPL-inhibitory activity.

Northern-blot analysis

A band of 3.8 kb showing strong hybridization to the human LIF mRNA probe was detected in SEKI and NAGAI xenografts. A moderately hybridizing band was detected in OCC-1C xenografts (Fig. 2). For human IL-6 mRNA, a strongly hybridizing band (1.3 kb) was detected in OCC-1C and Yumoto xenografts and a moderately hybridizing band was detected in MKN-l xenografts (Fig. 2). There was no detectable human TNFα mRNA band in any xenograft examined. The 2.0-kb human β-actin band showed integrity in all RNA samples.

ELISA for cytokines

Human TNFα, LIF, IL-6, IL-11, and OSM levels in conditioned media from SEKI, NAGAI, OCC-1C, Yumoto, MKN-1, LC-1, LX-1, and LS180 cell lines were determined by ELISA. LIF levels measured in conditioned media from

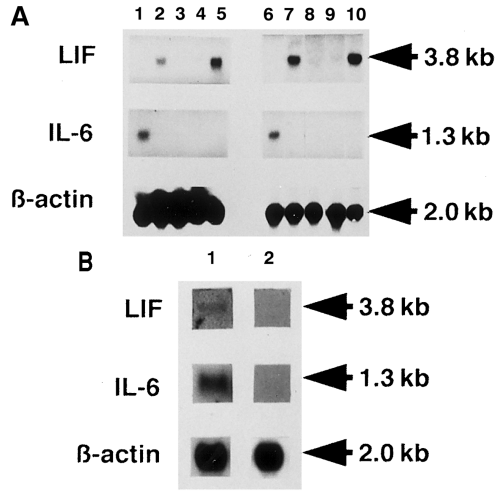

Fig. 2 **A** Expression of LIF and IL-6 mRNA in cultured cancer cell lines (*lanes 1–5*) and in xenografts (*lanes 6–10*). (*Lanes 1, 6* MKN-1, *lanes 2, 7* SEKI, *lanes 3, 8*, LX-1, *lanes 4, 9* LS180, *lanes 5, 10* NAGAI) **B** Expression of LIF and IL-6 mRNA in human OCC-1C (*lane 1*) and LC-1 (*lane 2*) cancer xenografts

the SEKI, NAGAI, and OCC-1C cell lines were 0.71, 2.6, and 2.2 ng/ml, respectively. In the remaining cell lines, LIF was not detected.

IL-6 levels measured in conditioned media from the OCC-1C and Yumoto cell lines were 330 and 26 ng/ml, respectively. In conditioned media from MKN-1 cells the IL-6 level was 0.14 ng/ml; however, in those from the other five cell lines, IL-6 was not detectable. An appreciable amount of IL-11 (1.3 ng/ml) was detected in conditioned culture media from OCC-1C cells, and 0.042 and 0.030 ng/ml was detected in those from Yumoto and MKN-1 cells, respectively. None of these cell lines produced TNFα or OSM.

RT-PCR analysis for the *ob* gene

No detectable band corresponding to human *ob* mRNA was present in any cell line or xenograft studied. In contrast, β-actin mRNA, run in the RT-PCR as a positive control, was expressed in all samples tested.

Discussion

In 1949, Nakahara and Fukuoka [14] first proposed that a tumor-derived factor or toxohormone induced cachexia in mice bearing cancers. However, all subsequent attempts to identify the factor have been unsuccessful. Kawakami and Cerami [11] found that a cachexia factor obtained from infectious macrophages was TNFα; however, to date there has been no evidence to demonstrate that TNFα is a toxohormone produced by cancer cells.

In 1989 we first proposed that LIF is a toxohormone in a cancer cachexia model developed in nude mice bearing the

human melanoma cell line SEKI and in a similar model bearing another human melanoma cell line, G-361 [12, 13]. Later, Strassmann et al. [19] reported the development of cachexia in animals bearing IL-6-producing cancer cell lines. In a recent study carried out to identify the toxohormone produced in nude mice bearing five cancer cell lines, including SEKI, we confirmed that LIF was a causative factor in the cachexia shown by mice bearing two of the cell lines and that in the mice bearing the remaining three cell lines, neither LIF nor IL-6 was a causative factor in this morbidity [8]. The aim of the present study was to analyze an additional three cultured cell lines, Yumoto, OCC-1C, and LC-1, that induce cancer cachexia syndrome in tumor-bearing mice and to examine whether in these eight cell lines a newly discovered protein, the *ob* gene product, or leptin, which decreases body weight in mice, plays a role in the cancer cachexia syndrome.

Among the three additional human cancer cell lines examined, results recorded for the Yumoto cell line have previously been published [20] and showed that IL-6 is the causative factor in this model. We also reported that nude mice bearing OCC-1 or LC-1 xenografts developed cancer cachexia syndrome and that the OCC-1 xenograft produced large amounts of LIF and IL-6; LIF and IL-6 were not detectable in LC-1 xenografts [21]. We subsequently established a cultured OCC-1 cell line, termed OCC-1C, and examined this cell line in this study (Kajimura et al., unpublished results).

In the eight cell lines studied, we confirmed our previous observations that a large amount of LIF was produced by the SEKI and NAGAI cell lines and that a large amount of IL-6 was produced by the Yumoto cell line (Table 1). Furthermore, we found that culture media conditioned by OCC-1C, the newly established cell line, exhibited potent LPL-inhibitory activity, and Northern-blot analysis and an ELISA for cytokines revealed that OCC-1C produced large amounts of LIF and IL-6. Northern-blot analysis also revealed that none of the cell lines expressed TNFα mRNA. The ELISA for IL-11 showed that OCC-1C, Yumoto, and MKN-1 cells produced IL-11 (Table 1), with OCC-1C producing high levels, suggesting that IL-11 may play a role in the development of cancer cachexia syndrome in OCC-1C-bearing nude mice. OSM was not detected in any cell line examined.

On the basis of these observations, we concluded that in four of the eight cancer cell lines, LIF, IL-6, or concomitant production of both of these cytokines and IL-11 was a causative factor in cancer cachexia syndrome and that in the remaining four cell lines the cytokines LIF, IL-6, TNFα, IL-11, and OSM did not play a role in the development of the syndrome.

Body weight is controlled by the combination of food intake and energy expenditure. A novel cloned gene, *ob*, is one of the genes that regulate body weight. In C57BL/6J *ob*/*ob* mice, overexpression of *ob* gene mRNA and a mutation in codon 105 of the protein have been observed [23]. Another *ob* gene mutation was found in SM/Ckc+Dac *ob* 2J/*ob* 2J mice, which express no *ob* mRNA [23]. The *ob* gene product is now termed leptin. Administration of leptin to C57BL/6J *ob*/*ob* mice, in which mature leptin cannot be produced, decreases their body weight, reducing their body fat, food intake, and serum glucose and insulin levels [4, 16]. These findings prompted us to investigate whether leptin is a toxohormone in our cancer cachexia model. However, RT-PCR analysis revealed that none of the eight cancer cell lines expressed leptin mRNA, suggesting that leptin is not likely to be a factor responsible for the cancer cachexia syndrome in terms of the toxohormone concept. Further studies are required to elucidate the mechanisms responsible for the cancer cachexia syndrome in mice bearing cancer cell lines MKN-1, LC-1, LX-1, and LS180 that do not produce large amounts of the cytokines that induce cachexia.

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