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Cytokine Production in Five Tumor Cell Lines with Activity to Induce Cancer Cachexia Syndrome in Nude Mice

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To identify the so-called toxohormone, which is a tumor-derived factor with activity to induce cancer cachexia syndrome in tumor-bearing animals, 5 human cancer cell lines with this activity were studied for cytokine production. Tumor cell products with activity to inhibit lipoprotein lipase (LPL) were shown to play an important role in the development of the cancer cachexia syndrome. All culture media conditioned by the 5 cell lines possessed LPL-inhibitory activity. However, the activity differed with the cell line. In order to characterize the activity, we examined whether the cultured cells produced cytokines with activity to inhibit LPL. A melanoma cell line, SEKI, and a neuroepithelioma cell line, NAGAI, were found to express a large amount of leukemia inhibitory factor (LIF) mRNA. Furthermore, both of these cell lines were demonstrated to produce a large amount of LIF protein, and plasma levels of LIF were extremely elevated in SEKI- and NAGAI-bearing nude mice, indicating that LIF produced by the tumor cells induced cancer cachexia syndrome in the animals. Thus, LIF fulfills the requirements for a toxohormone, except for suppressive activity on liver catalase. In contrast, the mechanisms responsible for cachexia in the MKN-1-, LX-1- and LS180-bearing mice remain unknown. These findings suggest that various types of bioactive substances produced by cancer cells could be toxohormones.

Key words: Cachexia — LIF — IL-6 — Cytokine — Cancer

Cancer cachexia is a morbidity that develops in cancer patients or experimental animals bearing tumors. Although the mechanisms responsible for this morbidity are multifactorial, humoral factors produced by cancer cells could play a predominant role. In 1949, Nakahara and Fukuoka first proposed the concept that a tumor-derived factor, so-called toxohormone, induced cachexia in mice bearing cancers.[1)] However, attempts to purify the factor were unsuccessful. In 1981, Kawakami and Cerami succeeded in purifying a lipoprotein lipase (LPL)-inhibitory factor from macrophages in cases of infectious disease, and called it cachectin.[2)] A part of the amino acid sequence of cachectin was revealed to be identical with that of tumor necrosis factor-α (TNF-α).[3)] Further studies revealed that exogenous administration of cachectin/TNF induced cachexia in mice.[4)] Therefore, cachectin/TNF is suspected to be a factor responsible for cachexia in infectious diseases, but to date no evidence has been presented that cachectin/TNF is a toxohormone produced by cancer cells.

We reported that the leukemia inhibitory factor (LIF) could be a possible toxohormone in the cancer cachexia model in nude mice bearing the human melanoma cell lines SEKI and G361.[5,6)] Later, Strassmann *et al.* reported the development of cachexia in animals bearing interleukin (IL)-6-producing cancer cell lines.[7)] Although data indicating that IL-6 is not a cachexia-causing factor have also been reported,[8,9)] LIF and IL-6 are now thought to be toxohormones in terms of activity to induce cancer cachexia syndrome. It is not yet clear whether these cytokines possess an activity to suppress liver catalase activity, which is another important property of toxohormone according to Nakahara and Fukuoka.[1)]

In the present study, we investigated 5 human cancer cell lines with the ability to develop cancer cachexia syndrome, and analyzed whether they produced cytokines which might be associated with cancer cachexia syndrome.

MATERIALS AND METHODS

Cell lines Five human cancer cell lines, SEKI, NAGAI, MKN-1, LS180 and LX-1, were studied. SEKI, a melanoma cell line, was established at the National Cancer Center, Tokyo.[10)] NAGAI[11)] and MKN-1[12)] were established from human neuroepithelioma and adenosquamous gastric carcinoma tissues, respectively, and kindly provided by the First Department of Pathology, Niigata University, Niigata. LS180[13)] and LX-1[14)] were established from human well-differentiated colon adenocarcinoma and undifferentiated lung carcinoma; LS180 was

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purchased from the American Type Culture Collection (Rockville, MD, USA) and LX-1 was kindly provided by Banyu Tsukuba Research Institute (Tsukuba, Ibaraki). All these cell lines were maintained at 37°C under 5% CO_2/95% air in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Mitsubishi Kasei Co., Tokyo), L-glutamine (0.3 mg/ml) (Nissui Pharmaceutical Co., Ltd.), penicillin (100 units/ml) (GIBCO BRL, Grand Island, NY, USA), and streptomycin (100 μg/ml) (GIBCO BRL), routinely passed at confluency.

LPL activity 3T3-L1 pre-adipocytes, provided by the Japanese Cancer Research Resources Bank (Tokyo) were grown and converted into differentiated adipocytes as previously described.[5] The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Ltd.) with heat-inactivated calf serum (GIBCO BRL). When they were grown to confluence, differentiation was induced by changing the medium to DMEM supplemented with 10% FCS, 10 μg/ml of insulin (Sigma Chemical Co., St. Louis, MO, USA), 1 μM dexamethasone (Sigma Chemical Co.) and 0.5 mM methylisobutylxanthine (Aldrich Chemical Co. Inc., Milwaukee, WI, USA). After a 48-h incubation, the medium was changed to DMEM containing insulin at a reduced concentration of 50 ng/ml and 10% FCS, and then the differentiated 3T3-L1 cells were used for assay of LPL activity as described below. Culture media conditioned by SEKI, NAGAI, MKN-1, LS180 and LX-1 cells for 5 days were diluted with RPMI medium 2, 10 or 50 times and added to the culture of differentiated 3T3-L1 adipocytes 3 days after the induction. After exposure for 24 h, adipocytes were washed twice with Dulbecco's PBS(−) (Nissui Pharmaceutical Co., Ltd.) and incubated for 1 h with 0.5 ml of DMEM containing 0.05 mg of sodium heparin (Sigma Chemical Co.). Heparin-releasable LPL activities were measured by the method described previously.[15]

Animal experiments Female BALB/cAnNCrj-nu/nu mice at 5 weeks of age were purchased from Charles River Japan Inc. (Atsugi, Kanagawa). The cultured cells were collected at a subconfluent state and 5 mice were inoculated subcutaneously with 1×10^7 cells into their flank. Total body weight and tumor size were measured twice a week and the tumor weight was calculated by use of the following equation[16]; tumor weight (g) = {major axis of tumor (cm) × (minor axis of tumor (cm))^2}/2. The body weight of hosts was compared with that of the control mice by use of the following equation; % of control mice weight = (total body weight − tumor weight)/(control mice weight) × 100. SEKI was used as a representative cachexia model,[17] for comparison with the data of the other 4 cell lines. The animals were killed when they had become emaciated. At that time, blood was drawn by cardiac puncture and the plasma was obtained after the removal of particulates by centrifugation of the blood. Tumor tissues were removed and stored in liquid nitrogen until the experiments were performed. Plasma samples were used for measurement of glucose, immunosuppressive acidic protein (IAP), LIF and IL-6, and the resected tumors were used as sources of poly(A)$^+$ RNA for use in Northern blot studies.

Northern blot analyses Total RNA was extracted from the collected cells or the resected tumors by means of the acid guanidinium-phenol-chloroform (AGPC) method.[18] Poly(A)$^+$ RNAs were collected by Oligotex dT-30 affinity chromatography, and 5 μg of each sample was electrophoresed, then transferred from the gel to nitrocellulose filters as described previously.[18] The expressions of mRNA of LIF,[19] IL-1α, IL-1β,[20] TNF-α[21] and interferon (IFN)-γ[22] were examined, using synthetic oligodeoxyribonucleotide probes. These probes were complementary to the coding sequences of nucleotide numbers 292–345 of human LIF mRNA, 339–398 of human IL-1α mRNA, 351–410 of human IL-1β mRNA, 411–470 of human TNF-α mRNA and 552–587 of human IFN-γ mRNA. The expression of IL-6 mRNA[23] was examined by using a complementary DNA probe corresponding to nucleotide numbers 174–708 of human IL-6. To estimate the intensity of each mRNA band, the expression of human β-actin mRNA was also examined.

Quantification of cytokine levels The concentrations of LIF, IL-6, TNF-α, oncostatin M (OSM) and IL-11 in the culture media of the cell lines and the concentrations of LIF and IL-6 in the plasma of the tumor-bearing nude mice were measured by using enzyme-linked immunosorbent assay (ELISA) systems, i.e., Quantikine Immunoassay systems (R&D Systems, Inc., Minneapolis, MN, USA) for human LIF, human IL-6, human TNF-α, human OSM and human IL-11. The supernatant of the medium of each cell line was obtained after 5 days of incubation.

Quantification of plasma glucose and IAP Plasma glucose concentrations were measured by using the TIDE/TIDEX system (Miles Inc., Elkhart, IN, USA) and plasma IAP levels were determined using a mouse IAP plate (Sanko Junyaku Co., Ltd., Tokyo).

RESULTS

Cachexia in tumor-bearing mice All the tumor-bearing nude mice showed a loss in body weight, as shown in Table I. In addition, they became cachectic in appearance, exhibiting decrease of activity, skin dryness, loss of appetite and low body temperature. The body weight of the mice bearing SEKI, LS180 or LX-1 decreased rapidly, falling below 65% of the control at the end of the third week (Table I). They were killed on the 21st

Table I. Body Weight Loss in Nude Mice Bearing 5 Tumor Cell Lines with Activity to Induce Cancer Cachexia Syndrome

Cell line	Period after the tumor transplantation (weeks)						
	0	1	2	3	4	5	6
SEKI	102.0±2.0[a)]	96.5±4.8	76.6±6.8	59.1±6.1			
LS180	98.9±6.4	93.2±9.7	68.8±5.7	62.7±7.5			
LX-1	103.0±4.4	90.0±6.8	69.0±11.0	57.5±5.3			
NAGAI	96.7±2.4	95.9±3.6	99.2±5.3	81.8±10.4	66.8±11.5	59.1±8.4	
MKN-1	96.6±8.8	98.7±12.5	96.2±11.2	87.5±13.1	80.8±16.3	67.6±15.7	60.9±12.3

a) Mean body weight±standard deviation. Data are expressed as % of the contol.

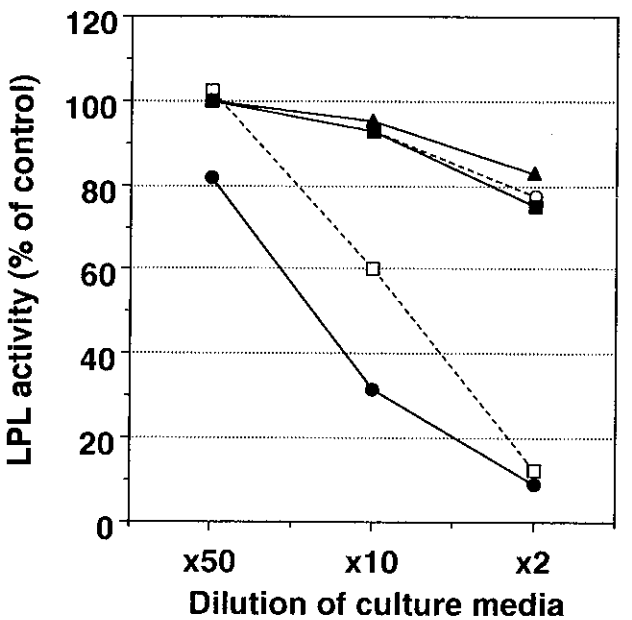

Fig. 1. Inhibition of LPL activity by culture media conditioned by cancer cell lines. Percentages of LPL activity of the control 3T3-L1 adipocytes are shown at every dilution of the culture media. ●, NAGAI; □, SEKI; ■, MKN-1; ○, LS180; ▲, LX-1.

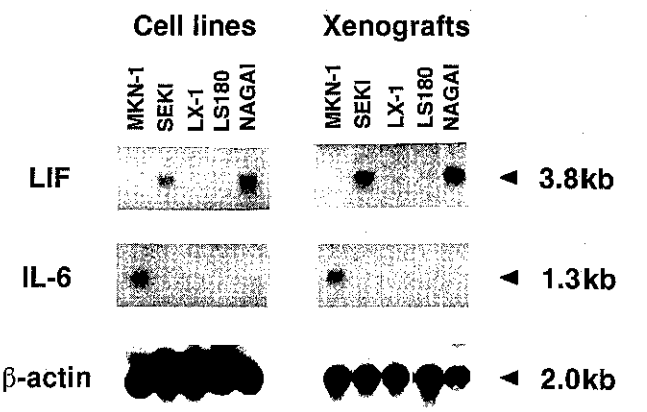

Fig. 2. Expression of mRNA for LIF, IL-6 and β-actin in the 5 cancer cell lines and their xenografts in nude mice.

day after tumor inoculation. By contrast, the NAGAI- and MKN-1-bearing mice showed a gradual loss in body weight; it took 6 weeks for their body weight to fall below 65% of the control. The NAGAI-bearing mice were killed at the 35th day and the MKN-1-bearing mice were killed at the 42nd day.

Inhibitory effects of the culture media on LPL activity As described in Fig. 1, all the culture media conditioned by the NAGAI, SEKI, MKN-1, LS180 and LX-1 cell lines inhibited the LPL activity. The media from the NAGAI and SEKI cells had especially strong inhibitory activity on LPL. The other 3 cell lines showed weak but significant activity.

Northern blot analyses Fig. 2 shows typical autoradiographs obtained by Northern blot analyses for LIF and IL-6. There were hybridizable bands of LIF mRNA with the molecular size of 3.8 kilobases (kb) in SEKI and NAGAI cells. Similar results were obtained in the xenografts. A band of IL-6 mRNA with the molecular size of 1.3 kb was detected in the MKN-1 cells as well as the xenograft. The expression of other cytokine mRNAs including IL-1α, IL-1β, TNF-α, and IFN-γ was not detected in the other cell lines or in nude mouse tumors (data not shown). The 2.0 kb band of β-actin mRNA was detected in each sample (Fig. 2).

Concentrations of cytokines, glucose and IAP The concentrations of LIF in the conditioned media of SEKI and NAGAI were 710 pg/ml and 2,600 pg/ml, respectively (Table II). LIF was detectable in the medium of LX-1, but its concentration was as low as 41 pg/ml. The concentration of IL-6 in the medium conditioned by MKN-1 was 140 pg/ml, but the other examined cytokines, TNF-α, OSM and IL-11, were not detectable in any media. Table III shows the concentrations of LIF and IL-6 in the plasma obtained from the tumor-bearing nude

Table II. Cytokine Concentrations in the Culture Media Conditioned by Cancer Cell Lines

Cell line	Cytokine levels (pg/ml)				
	LIF	IL-6	TNF-α	OSM	IL-11
SEKI	710	<35	<2.5	<16	<16
NAGAI	2,600	<35	<2.5	<16	<16
LX-1	41	<35	<2.5	<16	<16
MKN-1	<31	140	<2.5	<16	<16
LS180	<31	<35	<2.5	<16	<16

Table III. Concentrations of LIF, IL-6, Glucose and IAP in Plasma Obtained from Tumor-bearing Mice

Cell line	LIF (pg/ml)	IL-6 (pg/ml)	Glucose (mg/dl)	IAP (μg/ml)
SEKI	1,700±630[a)]	<3.1	66±6.5	700±190
NAGAI	1,400±140	<3.1	33±14.0	1,100±180
LX-1	<5.0	<3.1	47±2.3	<50
MKN-1	<5.0	<3.1	30±11.0	<50
LS180	<5.0	<3.1	40±15.0	390±45
Control[b)]	<5.0	<3.1	92±17.0	130±49

a) Mean±standard deviation.
b) Control mice not bearing tumors.

mice. The mean plasma LIF level in the SEKI- and NAGAI-bearing mice was very high, 1,700 and 1,400 pg/ml, respectively. In the MKN-1 cells, the plasma IL-6 level was less than 3.1 pg/ml, indicating that the small amount of IL-6 produced by cancer cells did not affect the plasma IL-6 level in the MKN-1-bearing mice. Table III also shows the plasma glucose and IAP levels. All of the tumor-bearing nude mice demonstrated hypoglycemia compared with the control mice. Moreover, in the SEKI- and NAGAI-bearing mice, the plasma level of IAP, an indicator of cachexia,[24] was very high.

DISCUSSION

The nude mice bearing the SEKI cell line were considered to be a good animal model for the study of cancer cachexia syndrome, since in our previous study the cachexia produced in these mice was found to be more severe than that produced in the nude mice bearing any of 264 other tumor cell lines transplantable to these mice.[5, 17] Thereafter, we found 4 new cancer cell lines with activity to induce cachexia, when transplanted to nude mice. In the present study, the body weight of the SEKI-bearing nude mice decreased to 59% of that of the control mice 3 weeks after the tumor transplantation. The body weight of the newly discovered models also decreased to 57–63% of the control at 3–6 weeks after the transplantation, indicating that the degree of cachexia produced in these mice was similar to that produced in the SEKI-bearing nude mice. Therefore, these mice are useful animal models for the study of the cancer cachexia syndrome.

In our previous studies, we isolated, from the culture medium conditioned by SEKI cells, a factor that possessed strong activity to inhibit LPL of differentiated adipocytes.[5] Amino acid sequence analysis revealed that the amino-terminal sequence of the factor was identical to that of LIF. Further studies revealed that SEKI cells expressed a large amount of LIF mRNA.[6] These findings strongly suggest that LIF is a candidate factor responsible for the cancer cachexia syndrome developing in the nude mice bearing SEKI cells. These findings also suggest that cancer cell products with activity to inhibit LPL play an important role in the development of cancer cachexia syndrome. Using the same biological assay for LPL, we examined whether the culture media conditioned by the other 4 cell lines possessed LPL-inhibitory activity, and found that all 4 had similar activities. The activity differed with the cell line; the cultured medium conditioned by NAGAI possessed stronger activity than that conditioned by SEKI, but those conditioned by the other 3 cell lines possessed weaker activity. Our study demonstrated that, in addition to LIF, other cytokines including IL-6, TNF-α,[25] IL-1,[26] IFN-γ,[27] OSM and IL-11 had activity to inhibit LPL in the assay system using differentiated adipocytes (Ohue *et al.*, manuscript in preparation). Therefore, we examined whether these cancer cell lines produce cytokines with activity to inhibit LPL. Northern blot analyses revealed that the SEKI and NAGAI cells expressed LIF mRNA and that MKN-1 cells expressed IL-6 mRNA. The immunoassay of the culture media demonstrated that SEKI and NAGAI cells produced large amounts of LIF immunoreactivity. However, the MKN-1 cells that expressed IL-6 mRNA did not exhibit IL-6 immunoreactivity. TNF-α, OSM and IL-11 were not produced by these cell lines, as determined by the respective immunoassays. These findings indicated that the cachexia syndrome developing in the SEKI- and NAGAI-bearing nude mice was caused by the LIF produced by the cancer cells, whereas in the remaining 3 cell lines, other unidentified mechanisms caused the cancer cachexia. It is worth noting that the induction of the cancer cachexia syndrome by a TNF-α-producing tumor has not yet been demonstrated.

Northern blot studies for xenografts in the nude mice revealed similar findings to those for the cultured cells. Therefore, we examined whether the serum LIF level in SEKI- and NAGAI-bearing nude mice was sufficiently elevated to induce cancer cachexia in these mice. Our previous studies revealed that intraperitoneal injection of recombinant human LIF, at the dose of 5 μg twice a day,

induced remarkable weight loss in mice; the plasma LIF concentration was approximately 7,000 pg/ml (Akiyama *et al.*, manuscript in preparation). In the present study, plasma LIF levels in SEKI- and NAGAI-bearing nude mice were 1,700 and 1,400 pg/ml, respectively, indicating that the plasma LIF level was of the same order as that in the mice presenting with LIF-induced cachexia. These findings suggest that the LIF produced by cancer cells is a causative factor for the cancer cachexia induced by these 2 cell lines.

As described above, SEKI and NAGAI cell lines had strong LPL-inhibitory activity, but the other cell lines had weaker activity. Plasma IAP levels were high in the former two cell lines. LIF and IL-6 have been demonstrated to induce IAP production in hepatocytes.[28, 29] Therefore, the plasma IAP elevation in cachexia models could be explained by the association of LIF, IL-6 or other cytokines with similar mechanisms of action.

In conclusion, in SEKI- and NAGAI-bearing nude mice, the present study revealed that the LIF produced by cancer cells increased plasma LIF levels, resulting in cachexia syndrome. LIF may fulfill the requirements for the toxohormone proposed by Nakahara and Fukuoka.[1] In contrast, the mechanisms responsible for cachexia that developed in MKN-1-, LS180- and LX-1-bearing mice remain unknown. These findings suggest that the so-called toxohormone consists of multiple factors, including various bioactive substances produced by cancer cells.

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