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Mechanism of Metabolic Abnormality of Thyroid Hormones in Walker 256 Carcinosarcoma-bearing Rats

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We examined the mechanism of abnormality of thyroid hormone metabolism in Walker 256 carcinosarcoma-bearing rats. The serum levels of thyroxine (T_4), 3,5,3′-triiodothyronine (T_3) and thyroid-stimulating hormone (TSH), and the responses of serum T_4 and T_3 to exogenous TSH in tumor-bearing rats on day 14 after inoculation of tumor cells were significantly less than those in pair-fed control (PFC) rats, suggesting that the metabolic abnormality of thyroid hormones may be caused by disorder of both peripheral and central functions, and that a certain tumor-derived factor may be involved in this abnormality. An active factor responsible for the metabolic abnormality was found in soluble cytosol fraction (SF) of the tumor cells. Administration of the SF to normal rats significantly reduced their serum T_4 and T_3 concentrations, liver 5′-deiodinase (5′-DI) activity, responsiveness of the thyroid gland to TSH and food intake compared with those of PFC rats, but, unlike the tumor, did not reduce the serum TSH level. This biologically active factor in the SF was found to be a heat-labile protein and specific to the tumor. It was tentatively named serum thyroid hormone reducing factor (STRF). STRF was partially purified from the SF by ammonium sulfate fractionation and DEAE-cellulose chromatography. Partially purified STRF preparation significantly diminished the serum T_4 and T_3 concentrations and liver 5′-DI activity and food intake of normal rats compared with those of PFC rats, mimicking the changes associated with the tumor in tumor-bearing animals. These results suggested that abnormality of thyroid hormone metabolism in tumor-bearing animals may partly be caused by STRF-mediated modulation at peripheral and thyroid gland levels. Whether STRF actually induces anorexia remains to be clarified.

Key words: Thyroid hormone — Rat — Sarcoma — Cancer cachexia — Tumor-derived factor

There are many reports on metabolic abnormalities of thyroid hormones in hosts with thyroidal tumor. However, there are few reports on these abnormalities in hosts with non-thyroidal tumors or on the correlation between thyroid hormones and cancer cachexia. There have been some studies on the effects of thyroid hormones on non-thyroidal tumor growth.[1–4] Mishkin *et al.*[5] reported increased survival of hepatoma-bearing rats made hypothyroid with propylthiouracil. They and other investigators found that hypothyroidism resulted in suppression of tumor growth and that exogenous thyroxine reversed this effect. Some kinds of hepatomas induce high plasma levels of thyroid hormones.

On the other hand, a low thyroid hormone syndrome of hosts with non-thyroidal cancers has also been reported, although patients or animals with progressive malignancy show many of the clinical or experimental features of hyperthyroidism, such as elevated energy expenditure, increased glucose, lipid, and protein metabolism and wasting of peripheral tissues. Persson *et al.*[6] reported a low T_3 (3,5,3′-triiodothyronine) syndrome without significant changes in the plasma levels of thyroxine (T_4) and thyroid-stimulating hormone (TSH) in cachectic patients with non-thyroidal tumors. Surks *et al.*[7] observed low plasma levels of T_4 and T_3, and reduced hepatic nuclear T_3 receptors in rats bearing Walker 256 carcinosarcomas. Ong *et al.*[8] reported decrease in plasma T_3 and stimulation of the conversion of T_4 to T_3 in the liver of rats with R3230AC mammary tumors. Moreover, Svaninger *et al.*[9] observed low levels of circulating thyroid hormones and an increased response of TSH to exogenous TRH in mice bearing 3-methylcholanthrene-induced sarcoma MCG 101. Most authors considered the abnormalities of thyroid hormones observed in experimental or clinical cancer cachexia to be secondary to malnutrition due to redued food intake (anorexia).

We have investigated the mechanism of development of cancer cachexia assuming that one of the main causes of its development is metabolic abnormalities or widespread direct or indirect modification of gene expressions in the host by physiologically active factors derived from the tumor and by endogenous factors such as cytokines which could be produced in excessive amounts or inappropriately as a result of host-invader interaction: such

Abbreviations used: RIA, radioimmunoassay; T_4, thyroxine; T_3, 3,5,3′-triiodothyronine; TSH, thyroid-stimulating hormone; 5′-DI, 5′-deiodinase; PFC, pair-fed normal control; FC, freely fed normal control; TB, tumor-bearing; ip, intraperitoneally; sc, subcutaneously; SF, soluble cytosol fraction.

metabolic abnormality could result in a chaotic state or cachexia syndrome, leading to imbalanced homeostasis.

In this study, the serum levels of T_4, T_3 and TSH, the responses of serum T_4 and T_3 to exogenous TSH, and the liver 5′-deiodinase (5′-DI) activity were measured as markers to examine the mechanism of abnormality of thyroid hormone metabolism in rats with Walker 256 carcinosarcomas. The values of these parameters observed in tumor-bearing (TB) animals were compared with those in pair-fed normal animals as the control, because these values were altered by the nutritional status of the animals. A biologically active factor that induced deviation of thyroid hormone metabolism was partially purified from the soluble cytosol fraction (SF) of Walker 256 carcinosarcoma cells. This factor in the SF was tentatively named serum thyroid hormone reducing factor (STRF). The effects of partially purified STRF on the above parameters in normal rats were examined.

The results obtained strongly suggest that STRF is a heat-labile protein involved in the induction of abnormality of thyroid hormone metabolism in rats with Walker 256 carcinosarcomas through direct or indirect harmful influences on peripheral tissues, especially the liver, and thyroid gland. The mechanism of genesis of cancer cachexia is discussed on the basis of these results.

MATERIALS AND METHODS

Animals Normal male Wistar rats, 5 weeks old, weighing about 90–100 g, were obtained from Japan SLC Co. (Shizuoka). Male rats were used because of the greater hormonal stability of their thyroid activity. They were fed rat chow (MF; Oriental Yeast Co. Ltd., Osaka) and allowed tap water *ad libitum*. Five rats were used per group. They were housed in temperature-controlled rooms (23 ± 1°C) with a 12 h-light/12 h-dark cycle. The food consumption per group and body weight of each rat were determined daily. Freely fed normal control (FC) and pair-fed normal control (PFC) rats were used as controls. PFC rats received the same amount of food as the TB rats or factor-treated rats during the experiment because rats bearing tumors or treated with tumor-derived factor showed decreased food consumption.

Protein determination Protein was determined by the method of Lowry *et al.*[10] using fraction V bovine albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

Inoculation of Walker 256 carcinosarcoma and treatment A Walker 256 carcinosarcoma was kindly provided by the laboratory of Shionogi Pharmaceutical Co. (Osaka). The tumor was maintained by routine passage every 10–14 days in Wistar rats. Viable tumor fragments were transplanted sc into the interscapula region under aseptic conditions and light anesthesia. To examine the effect of tumors on the animals, non-necrotic tumor parts were selected, washed with Dulbecco's PBS (−) medium and minced with scissors, and the minced tissue was dispersed by about 15 strokes of a loosely fitting Dounce homogenizer. The preparation was then filtered through nylon mesh to remove connective tissue and centrifuged at 100*g* for 3 min at 4°C. The precipitated cells were suspended in Dulbecco's PBS (−) medium. This procedure was repeated 3 times to wash the tumor cells. The resultant tumor cells were then washed twice with Williams E Medium by centrifugation by the method described above, and finally resuspended in the same medium. Then, inocula of 0.5×10^7 cells were injected sc into the interscapula region of the rats. PFC rats were treated sc with 0.1 ml of PBS. Rats were killed by decapitation on day 4, 7, 10 or 14 after inoculation of tumor cells. The stress involved in decapitation was reduced by handling the animals for several days before killing them. Blood was collected and the serum was separated and stored at −20°C until used for estimation of T_4, T_3 and TSH. The livers and kidneys were also removed immediately from the same animals, washed ice-cold saline, quickly frozen in dry ice, and stored at −80°C until used for assay of 5′-DI activity. Non-necrotic parts of tumors were also frozen on dry ice and stored at −80°C until used for preparation of tumor-derived biologically active factor as described below.

Radioimmunoassay (RIA) of thyroid hormones T_4 and T_3 concentrations were measured by the RIA method described by Mashita *et al.*,[11] which is a modification of the method of Lieblich and Utiger.[12] Antisera against T_4 and T_3 were obtained from Miles Inc. (Kankakee, IL). T_4 and T_3 for use as standards were purchased from Sigma Chemical Co. Radioactivities were counted in a γ-counter (Model MINAXI autogamma 5000 series, Packard Japan Co., Tokyo). Serum TSH was determined by RIA using the materials and protocol kindly supplied by the NIADDK. rTSH-RP-2 (AFP-5153B) was used as a standard. [^{125}I]TSH for use as a tracer was prepared by the chloramine-T method using rTSH-I-8 (AFP-8334B). Anti-rTSH-S-5 (CS1381) was used as the antiserum against TSH. The intraassay and interassay variations were 12% and 6%, respectively.

Assay of 5′-DI activity 5′-DI, which catalyzes the conversion of T_4 to T_3, was assayed by the method of Nauman *et al.*[13] with a minor modification. The T_3 generated and remaining T_4 were assayed by RIA. In this study the 5′-DI activity of the microsomal fraction was determined. Rat liver and kidney, which had been stored at −80°C as described above, were homogenized (3:1 v/w) in 20 m*M* Tris-HCl buffer, pH 7.2 containing 0.25 *M* sucrose, 3 m*M* EDTA and 5 m*M* dithioerythritol (DTE) in a Dounce homogenizer. The homogenate was filtered through 4 layers of gauze to remove cell debris and centrifuged at 10,000*g* for 10 min at 4°C. The

supernatant was recentrifuged at 105,000g for 90 min in an ultracentrifuge (Hitachi 55P-72N Hitachi Koki Co., Ibaragi) and the supernatant was removed by aspiration leaving the pellet of the crude microsomal fraction. The pellet was suspended in 20 mM Tris-HCl buffer, pH 7.2, containing 3 mM EDTA and 1 mM β-mercaptoethanol by gentle agitation and the suspension was recentrifuged at 105,000g for 60 min at 4°C. The resulting pellet was dispersed in the same buffer. The reaction mixture contained 50 mM Tris-HCl buffer (pH 6.0), 3 mM EDTA, 5 mM DTE and 50 μg of microsomal protein in a final volume of 0.4 ml. The reaction was started by adding 1.2 μM L-T_4 solution to the medium and was carried out in a shaking incubator for 20 min at 37°C. The reaction was stopped by adding 0.8 ml of ice-cold 99.5% ethanol and placing the mixture in an ice bath. The 5′-DI activity was expressed as newly generated T_3 in pmol/mg protein/min. Reaction mixture without T_4 was used to measure the generation of T_3 from endogenous substrate (blank control). In addition, the microsomal fraction was added to the reaction mixture containing 99.5% ethanol (0 time control) for measurement of endogenous T_3 or T_3 present in the substrate.

Isolation of biologically active factor (STRF) from tumor cells Non-necrotic parts of tumors that had been stored at −80°C as described above were used for preparation of the tumor-derived, biologically active factor. Three fractions of tumor cells were obtained as follows. The tumor tissue was weighed, minced with scissors, suspended in 2 volumes of 0.15 M NaCl in 20 mM Tris-HCl buffer, pH 7.5 and homogenized in a Waring blender for 3 min at 0°C. The homogenate was centrifuged at 105,000g for 60 min at 4°C. Lipid, if present, was removed from the supernatant with a Pasteur pipette. The supernatant was used as the SF. The precipitate was resuspended in the same buffer and sonicated at 4°C until no intact nuclei could be detected by microscopy. The suspension was centrifuged at 9,000g for 10 min at 4°C and the supernatant was used as the insoluble fraction (IF) of the tumor cells. The precipitate was homogenized further in a tightly fitting Dounce homogenizer and used as the cellular residue fraction (RF).

To examine whether normal tissue components possess STRF activity like tumor cells or whether STRF is specific to tumor cells, normal rat muscle and liver were fractionated by the same method as tumor cells.

Responses of serum T_4 and T_3 to exogenous TSH Bovine TSH was obtained from Sigma Chemical Co. For examination of the response of the thyroid gland to TSH in TB animals on day 14 after tumor inoculation, or STRF-treated rats, these animals were treated ip with 400 mU of TSH, and their serum levels of T_4 and T_3 were measured 0, 3, 5 and 8 h later. STRF was administered as 10 mg of SF/rat once a day for 9 days. FC and PFC rats were also treated with the same doses of TSH.

Data are expressed as means±SDs. The significance of differences between means was determined by using Student's t test. The difference was considered significant at $P<0.05$.

Chemicals Pronase was obtained from Calbiochem-Behring Corp. (CA, USA). All other chemicals were of the highest commercial grade available.

RESULTS

Effect of nutritional status on thyroid hormone metabolism in normal and TB rats Tumors were palpable but small about 10 days after inoculation, and then grew

Table I. Effects of Walker 256 Carcinosarcoma on Serum T_4, T_3 and TSH Concentrations and Liver 5′-DI Activity in Tumor-bearing Rats

		Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Serum TSH (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
	FC	7.20±1.40	0.72±0.10	1.98±0.3	13.91±0.97
Day 4	TB	8.58±0.55	0.86±0.13	1.94±0.14	12.71±1.10
	PFC	8.50±1.07	0.85±0.09	2.27±0.24	13.03±1.19
Day 7	TB	8.28±1.10	0.94±0.04	1.94±0.21	11.87±2.05
	PFC	9.66±1.20	0.80±0.13	2.28±0.21	12.46±1.92
Day 10	TB	5.55±1.86[a]	0.72±0.06	1.44±0.20[b]	9.60±1.86
	PFC	8.48±0.46	0.79±0.07	2.43±0.30	11.14±2.00
Day 14	TB	4.68±0.78[b]	0.52±0.05[b]	1.61±0.21[a]	6.75±0.83[b]
	PFC	6.32±0.35	0.67±0.06	2.02±0.17	10.83±1.74

Values are means±SDs for five rats.
Significance of difference vs. PFC: *a*), $P<0.05$; *b*), $P<0.01$.

rapidly. TB hosts are known to suffer from malnutrition because tumor growth results in decreased food intake. As shown in Table I, the serum T_4 and T_3 levels in TB rats were significantly decreased on days 10 and 14, respectively, after tumor cell inoculation. So, the food consumption and body weights of the TB rats were compared with those of FC rats (Fig. 1). The food intake of TB rats was significantly decreased on day 10 and the rate of increase in body weight gradually became slower than that of FC rats. Therefore, the serum T_4 and T_3 levels in TB rats were compared with those of PFC rats to exclude the effects of reduced food intake or anorexia during the experimental period (two weeks). As shown in the same columns in Table I, the serum T_4 concentration in TB rats became significantly decreased on day 10 and was further decreased on day 14 compared with those in PFC (PFC: 6.32 ± 0.35 μg/dl, TB: 4.68 ± 0.78 μg/dl). Serum T_3 responded to tumor growth rather more slowly than serum T_4: it was slightly decreased on day 10 and significantly decreased on day 14 (PFC: 0.67 ± 0.06 ng/ml, TB: 0.52 ± 0.05 ng/ml). These results indicate that the decreases in serum T_4 and T_3 in TB rats are not caused solely by malnutrition resulting from decreased food intake, and strongly suggest that some tumor-derived factor influences the host's metabolism.

To examine the mechanism of the metabolic deviation of thyroid hormones in TB rats, we also measured the serum TSH level and liver 5′-DI activity and compared them with those of PFC rats. As also shown in Table I, the serum TSH level was significantly decreased on day 10 (PFC: 2.43 ± 0.30 ng/ml, TB: 1.44 ± 0.20 ng/ml), indicating impairment of the central function. On the other hand, the liver 5′-DI activity, which is a marker of peripheral metabolism, was also significantly diminished on day 14 (PFC: 10.83 ± 1.74 pmol/mg protein/min, TB: 6.75 ± 0.83 pmol/mg protein/min), indicating disorder of peripheral function. These results suggest that central function was more sensitive than peripheral function to growth of the tumor. The levels of serum T_4 and T_3, and liver 5′-DI activity in PFC rats in Table I show a

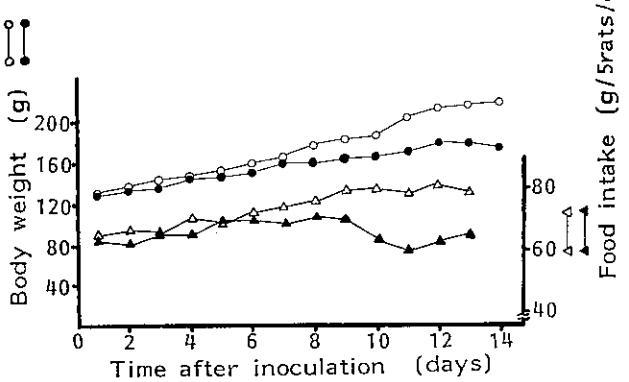

Fig. 1. Food consumption and body weight changes of Walker 256 carcinosarcoma-bearing rats. Walker 256 carcinosarcoma (0.5×10^7 cells) was injected sc into rats. (●, ○) and (▲, △) show body weights and food consumptions of rats inoculated with tumor cells and FC rats injected with 0.1 ml of PBS, respectively. Values are means for five rats.

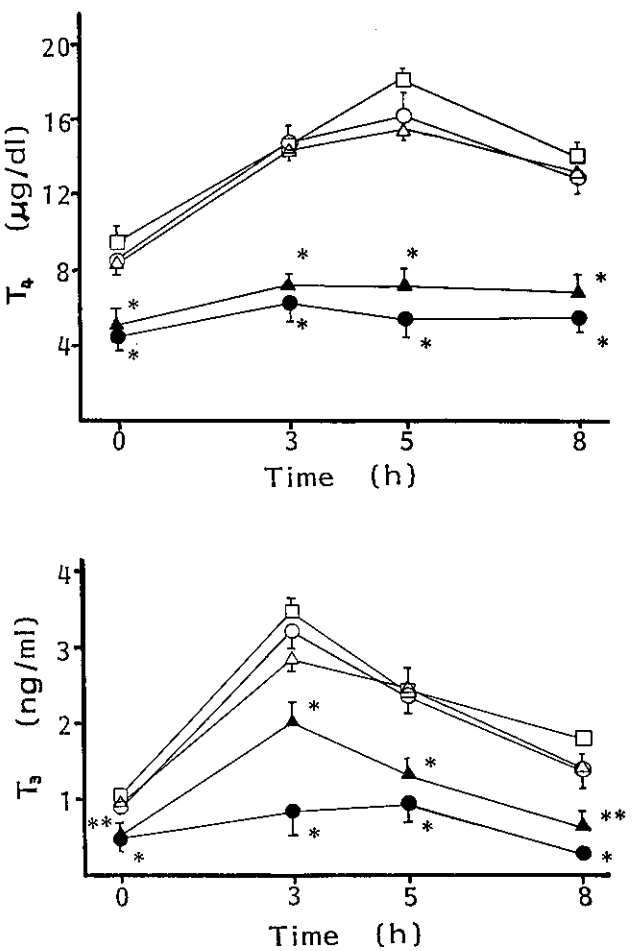

Fig. 2. Responses of serum T_4 and T_3 concentrations to exogenous TSH in rats bearing Walker 256 carcinosarcoma and rats treated with the tumor-derived factor. Bovine TSH (400 mU) was administered ip to TB rats on day 14 after tumor inoculation and to the rats treated with 10 mg of the tumor-derived factor once a day for 9 days. Serum concentrations of T_4 and T_3 were determined at the indicated times after TSH administration. (●) and (▲) show values for TB and factor-treated rats, repectively. (○) and (△) show values for the respective PFC rats treated with 0.1 ml of PBS. (□) show values for FC rats given 0.1 ml of PBS. Values are means±SDs for five rats. Significance of difference vs. PFC: * $P<0.001$, ** $P<0.01$.

tendency to decrease suggesting that these parameters are influenced by change in nutritional status, such as reduced food intake, but differences among those values were statistically not significant during the experimental period. The serum TSH concentration was also not statistically significantly changed in PFC rats, suggesting that it is not influenced so much by reduced food intake during the experimental period. Interestingly, the kidney 5′-DI activity was significantly increased on day 10 after tumor inoculation compared with that in PFC, in contrast with the 5′-DI activity in the liver (data not shown).

Effect of the tumor on the response of the thyroid gland to TSH The responses of serum T_4 and T_3 to exogenous TSH were examined to obtain additional information on the metabolic deviation in TB animals. As shown in Fig. 2, when ip injection of 400 mU of TSH/rat, which stimulated the productions of T_4 and T_3 maximally, was given to rats, the serum T_4 concentration almost doubled after 5 h in FC (from 9.11 ± 0.91 to 17.89 ± 0.73 μg/dl) and PFC (from 8.54 ± 0.29 to 16.12 ± 1.82 μg/dl) and then rapidly decreased, whereas the serum T_4 increased only slightly in TB rats on day 14 after inoculation of the tumor (from 4.68 ± 0.78 to 5.42 ± 0.33 μg/dl). Similar results were obtained for the effect of TSH on the serum T_3 concentration: TSH increased the serum T_3 concentration after 3 h substantially in FC (from 0.96 ± 0.04 to 3.19 ± 0.38 ng/ml) and PFC rats (from 0.81 ± 0.07 to 3.37 ± 0.35 ng/ml), respectively, but only slightly in TB rats (0.51 ± 0.06 to 0.96 ± 0.20 ng/ml). These results strongly suggest that the responsiveness of the thyroid gland to TSH was greatly blunted in TB hosts.

Isolation of a biologically active factor from Walker 256 carcinosarcoma Next, we tried to isolate a factor responsible for the reduced serum concentrations of thyroid hormones. We separated three fractions of tumor cell components, SF, IF (extractable from insoluble cell components by sonication), and RF (components that were not solubilized by sonication) as described in "Materials and Methods," and tested their effects on the serum levels of T_4, T_3 and TSH, and the liver 5′-DI activity. We found that only SF had the biological activity responsible for metabolic deviation of thyroid hormones. As shown in Table II, when 10 mg of SF was administered ip to

Table II. Effects of Soluble Cytosol Fraction of Tumor Cells on Serum T_4, T_3 and TSH Concentrations and Liver 5′-DI Activity in Rats

		Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Serum TSH (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
	FC	7.20 ± 1.40	0.72 ± 0.10	1.96 ± 0.30	13.91 ± 0.97
Day 4	SF	6.93 ± 1.14	0.74 ± 0.04	1.86 ± 0.22	16.84 ± 1.67
	PFC	7.46 ± 0.73	0.87 ± 0.16	1.98 ± 0.30	15.15 ± 1.47
Day 7	SF	$5.59\pm0.90^{a)}$	0.66 ± 0.12	1.70 ± 0.15	14.12 ± 1.11
	PFC	6.94 ± 0.41	0.79 ± 0.11	1.98 ± 0.55	13.46 ± 1.43
Day 10	SF	$3.21\pm0.47^{b)}$	$0.41\pm0.05^{a)}$	1.88 ± 0.17	$8.30\pm1.39^{a)}$
	PFC	6.52 ± 0.81	0.58 ± 0.07	1.81 ± 0.21	11.40 ± 1.39

Values are means±SDs for five rats.
Significance of difference vs. PFC: *a*), $P<0.05$; *b*), $P<0.001$.

Table III. Effects of Extracts from Normal Liver and Muscle, and Sera from Normal and Tumor-bearing Rats

	Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
FC	7.20 ± 1.40	0.72 ± 0.10	10.60 ± 1.65
Normal rat liver	8.46 ± 1.20	0.80 ± 0.03	11.49 ± 1.64
Normal rat muscle	6.92 ± 1.10	0.81 ± 0.10	13.08 ± 1.15
Serum of normal rat	8.65 ± 1.30	0.60 ± 0.08	9.00 ± 1.10
Serum of tumor-bearer	7.64 ± 0.53	0.88 ± 0.07	10.19 ± 1.84

Values are means±SDs for five rats.
Values were not significantly different from those in FC.

normal rats once a day, the serum T_4 level was significantly decreased on day 7 and the decrease was greater on day 10 (PFC: 6.52±0.07 μg/dl, SF: 3.21± 0.47 μg/dl). The serum T_3 concentration was also significantly decreased on day 10 of treatment (PFC: 0.58±0.07ng/ml, SF: 0.41±0.05 ng/ml). However, the serum TSH level was not significantly changed by treatment with SF for 10 days (PFC: 1.81±0.21, SF: 1.88± 0.17 ng/ml) though that of TB rats was reduced at this time (Table I). The liver 5′-DI activity was also significantly decreased by treatment with SF for 10 days (PFC: 11.40±1.39 pmol/mg protein/min, SF: 8.30± 1.39 pmol/mg protein/min). The effects of SF on the serum levels of T_4 and T_3, and the liver 5′-DI activity in normal rats showed dose dependency (data not shown). On the other hand, administration of 10 mg of the IF or RF ip to normal rats once a day for 9 days did not affect these parameters significantly in comparison with those of PFC (data not shown). As shown in Table III, extracts of liver and muscle of normal rats and sera from normal and TB rats at daily doses of 10 mg/rat for 9 days also did not significantly reduce the serum concentrations of T_4, T_3 and TSH, or the liver 5′-DI activity compared with those of PFC rats treated with PBS, suggesting that the effect of the SF on thyroid hormone metabolism was tumor-specific. The reason why the serum from TB rats did not induce metabolic deviation of thyroid hormones will be discussed later. This tumor-derived factor was tentatively named serum thyroid hormone reducing factor (STRF).

Effect of crude STRF (SF) on the response of the thyroid gland to TSH The responses of serum T_4 and T_3 to exogenous TSH in the factor-treated rats were examined to obtain information on the mechanism of reduced function of the thyroid gland in TB rats. As shown in Fig. 2, when 400 mU of TSH/rat was administered ip to rats treated with 10 mg of crude STRF/rat once a day for 9 days, the increase in serum T_4 concentration after 5 h (from 5.24±0.58 to 7.31±0.38 μg/dl) was greatly diminished like that in TB rats (from 4.68±0.78 to 6.24 ±0.44 μg/dl) compared with that in FC (from 9.11± 0.91 to 17.89±0.73 μg/dl) or PFC rats (from 8.35±0.34 to 15.60±0.91 μg/dl). However, the increase in serum T_3 concentration after 3 h (from 0.59±0.04 to 2.21±0.07 ng/ml) was significantly diminished compared with that in FC (from 0.96±0.04 to 3.19±0.38 ng/ml) or PFC (from 0.83±0.06 to 2.98±0.10 ng/ml), but was rather higher than that in TB rats (from 0.51±0.06 to 0.96± 0.20 ng/ml). These results suggest that administration of STRF to normal rats blunted or impaired the responsiveness of the thyroid gland to TSH, mimicking the effect of tumors.

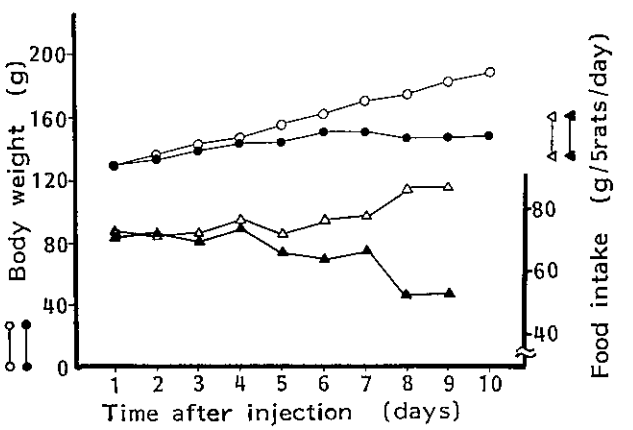

Fig. 3. Effect of the tumor-derived factor on food consumption and body weight. SF (10 mg) of tumor cells was administered ip to normal rats once a day throughout the experiment. (●, ○) and (▲, △) indicate values for body weight and food consumption of rats treated with tumor-derived factor and PFC rats treated with 0.1 ml of PBS, respectively. Values are means for five rats.

Table IV. Stability of the Factor

Treatment	Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
FC	8.20±1.40	0.82±0.10	10.60±1.65
No treatment	5.05±0.79[a)]	0.59±0.07[a)]	7.12±0.36[a)]
PFC	7.77±0.65	0.84±0.05	12.10±0.37
Incubation at 55°C for 5 min	9.08±1.01[c)]	1.00±0.14[c)]	11.29±0.83[c)]
37°C for 1 h	5.96±0.76[b)]	0.53±0.07[a)]	6.79±0.42[a)]
PFC	8.09±0.49	0.85±0.05	12.16±0.41
Pronase	8.59±0.63[c)]	0.94±0.09[c)]	11.05±0.72[c)]

Values are means±SDs for five rats.
Significance of differences vs. PFC: *a*), $P<0.001$; *b*), $P<0.01$. *c*), Values were not significantly different from those in FC.

Table V. Ammonium Sulfate Fraction of the Tumor Factor

Fraction	Recovery of protein (mg)	(%)	Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
Original[d]	4186	100.0	4.27±0.05[a]	0.59±0.04[a]	8.40±1.13[b]
PFC			7.52±1.26	0.72±0.02	10.24±0.66
0–35%	1432	34.2	8.00±1.13	0.90±0.11	10.82±0.59
PFC			9.28±0.33	0.94±0.30	10.71±0.61
36–50%	869	20.8	4.36±0.33[c]	0.63±0.05[c]	7.83±0.54[a]
PFC			8.45±1.25	0.89±0.04	11.10±1.25
51–65%	603	14.4	6.72±1.16[b]	0.58±0.05[a]	8.64±0.93[a]
PFC			8.79±0.85	0.80±0.07	11.76±1.29
66–75%	508	12.1	9.35±0.63	0.89±0.09	12.23±0.82
PFC			9.22±0.63	0.87±0.10	12.41±0.31
Total	3412	81.5			

Values are means±SDs for five rats.
Significance of difference vs. PFC: *a*), $P<0.01$; *b*), $P<0.05$; *c*), $P<0.001$.
d) Original means the soluble cytosol fraction of tumor cells (crude STRF).

Effects of crude STRF on food consumption and body weight Injection of 10 mg of crude STRF/rat into normal rats once a day for 9 days decreased their food consumption and blunted the increase in their body weight, as tumors did (Fig. 3).

Properties of the STRF The effects of heat treatment and pronase treatment of STRF on its activity were examined. Table IV shows the influences of these treatments on the effects of crude STRF on the serum levels of T_4 and T_3, and the liver 5′-DI activity, when injected at 10 mg a day for 9 days. Heat treatment at 37°C for one hour did not significantly inactivate the activity of STRF. However, heating at 55°C for 5 min or treatment with pronase at 2.5 U/mg protein at 37°C for one hour and then overnight at 4°C resulted in loss of activity. These results indicate that the factor is a heat-labile protein.

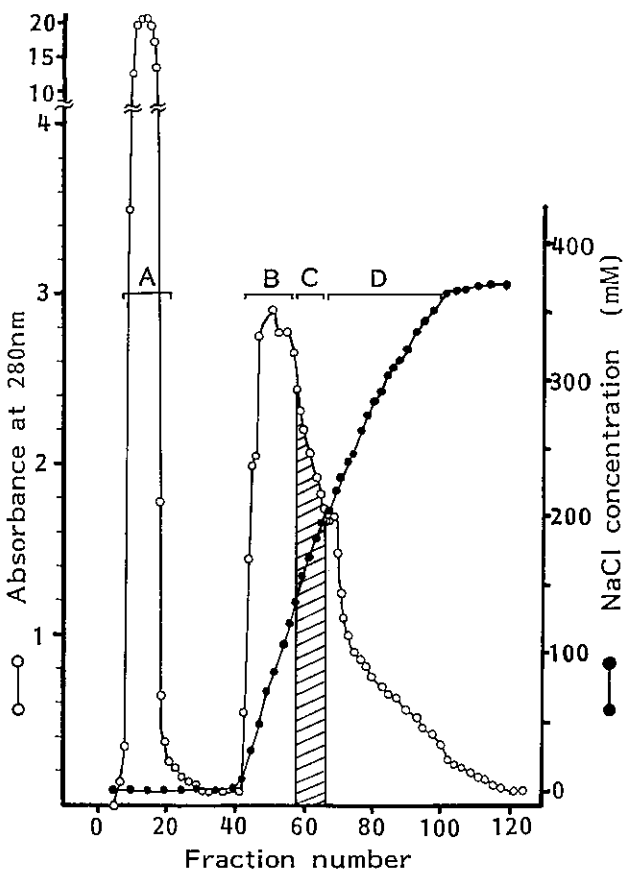

Fig. 4. Elution profile of STRF from a DEAE-cellulose column. Ammonium sulfate fractions with activity were dialyzed against 10 m*M* Tris-HCl buffer, pH 7.5, combined and applied to a DEAE-cellulose column (2×25 cm) equilibrated with the same buffer. The flow rate was 1 ml/min throughout, and the eluate was collected in 10 ml fractions. After collection of an unadsorbed fraction, adsorbed material was eluted with a linear gradient of 0–400 m*M* NaCl in 10 m*M* Tris-HCl buffer, pH 7.5. (○) and (●) show the absorbance at 280 nm and electric conductivity or NaCl concentration of the eluate, respectively. A represents the unadsorbed fraction. Adsorbed fractions were pooled in three fractions, B, C and D. Fraction C, shown by the hatched area, had STRF activity as described in the text.

Table VI. Partial Purification of the Tumor Factor by DEAE-Cellulose Chromatography

Fraction	Recovery of protein (mg)	(%)	Serum T_4 (μg/dl)	Serum T_3 (ng/ml)	Liver 5′-DI activity (pmol/mg protein/min)
Original[d]	3468	100.0	6.18±0.67[a]	0.68±0.06[b]	8.87±0.55[b]
PFC			8.07±0.68	0.84±0.04	11.28±0.61
Fraction A	1770	51.0	11.36±2.41	1.06±0.15	10.05±1.17
PFC			10.71±1.08	0.94±0.09	11.56±0.26
Fraction B	420	12.1	10.05±1.34	0.89±0.02	10.96±0.58
PFC			10.71±1.08	0.90±0.08	11.56±0.26
Fraction C	289	8.3	6.36±1.08[a]	0.67±0.05[a]	7.34±0.37[c]
PFC			8.16±0.38	0.80±0.07	11.27±0.44
Fraction D	358	10.3	9.80±0.97	0.92±0.09	11.43±0.83
PFC			10.71±1.08	0.94±0.09	11.56±0.26
Total	2837	81.7			

Values are means±SDs for five rats.
Significance of difference vs. PFC: *a*), $P<0.05$; *b*), $P<0.01$; *c*), $P<0.001$.
d) Original means the combination of the two fractions of STRF precipitated with 36–50 and 51–65% saturation of ammonium sulfate.

Partial purification of the factor from crude STRF by ammonium sulfate fractionation and DEAE-cellulose chromatography (i) Ammonium sulfate fractionation: The SF extracted from tumor cells as described in "Materials and Methods" was fractionated by treatment with 0–35%, 36–50%, 51–65% and 66–75% saturation of ammonium sulfate. Each fraction was dialyzed against 0.15 *M* NaCl in 10 m*M* Tris-HCl buffer, pH 7.5, to remove ammonium sulfate. Table V shows the effects of these fractions injected at 10 mg protein/rat for 9 days on the serum levels of T_4 and T_3 and the liver 5′-DI activity in comparison with values in PFC rats treated with PBS. The 36–50% and 51–65% fractions, containing about 35% of the total protein, were active, causing significant decreases in the levels of these parameters on day 10. Both fractions also reduced food intake (data not shown).

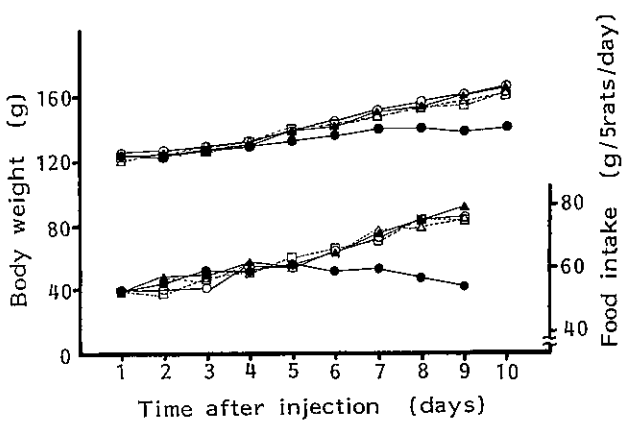

Fig. 5. Effect of partially purified STRF on food consumption and body weight. Doses of 5 mg/rat of the four fractions separated by DEAE-cellulose column chromatography were administered ip to normal rats once a day for 9 days. (▲), (△), (●), (○), and (□) indicate values for FC rats treated with fractions A, B, C, D, and 0.1 ml of PBS, respectively. The upper and lower graphs show food consumption and body weight changes, respectively.

(ii) Purification by DEAE-cellulose column chromatography: The active fraction separated by ammonium sulfate precipitation was purified further by DEAE-cellulose column chromatography. For this, the two active fractions were combined and dialyzed against 10 m*M* Tris-HCl buffer, pH 7.5. The dialyzed material was applied to a DEAE-cellulose column (2.2×25 cm) equilibrated with the same buffer, and the column was washed with the same buffer at a flow rate of 1 ml/min until the absorbance of the eluate at 280 nm returned to the base line. The adsorbed materials were eluted with a linear gradient of 0–400 m*M* NaCl in the same buffer. The eluate was collected in 10 ml fractions. Fig. 4 shows the elution profile of the adsorbed materials from the DEAE-cellulose column. Fraction A is unadsorbed material. The eluate was collected in three fractions, fractions B (tubes No. 43–57), C (tubes No. 58–66) and D (tubes No. 67–100). Each fraction was concentrated by addition of crystalline ammonium sulfate and centrifugation and dialyzed against 0.15 *M* NaCl containing 10 m*M* Tris-HCl, pH 7.5. Then the effects of each fraction injected

daily at 5 mg protein/rat for 9 days on the serum levels of T_4 and T_3, and the liver 5′-DI activity were examined in comparison with the values in PFC rats treated with PBS. Only fraction C eluted with 140–200 m*M* NaCl and corresponding to 8.3% of the applied protein was active, as shown in Table VI. This preparation also reduced food intake and blunted increase in body weight, when injected at 5 mg/rat ip into normal rats once a day for 9 days as shown in Fig. 5.

DISCUSSION

There are only a few reports on metabolic abnormalities of thyroid hormones in hosts with non-thyroidal tumors and on the relationship between these abnormalities and cancer cachexia. Most investigators have considered that these abnormalities are secondary to malnutrition due to reduced food intake (anorexia).

We have investigated the mechanism of cancer cachexia assuming that as well as reduced food intake, some tumor-derived, physiologically active factor is important in the development of cancer cachexia.[14] In this study, we measured the serum T_4, T_3 and TSH levels, the responses of T_4 and T_3 to exogenous TSH, and the liver 5′-DI activity as markers of thyroid hormone metabolism in rats with Walker 256 carcinosarcomas. We compared the values for these parameters with those in PFC and FC rats, because these parameters are influenced by the nutritional state of animals.

We found that the serum levels of T_4 and TSH were more sensitive than other parameters to tumor growth and were significantly reduced on day 10 after inoculation of tumor cells, whereas the serum T_3 concentration, the liver 5′-DI activity and responsiveness of the thyroid gland to TSH were significantly diminished on day 14. These findings indicate that the hypothalamic-pituitary axis (central function) is more sensitive to tumor growth than peripheral function or the thyroid gland.

Our results also showed that the changes were not simply due to malnutrition caused by reduced food intake (anorexia), and strongly suggested that some biologically active factor derived from tumor cells was involved in the abnormalities. Therefore, we next tried to isolate the factor responsible for the metabolic deviation from tumor cells. We fractionated Walker 256 carcinosarcoma cells into SF, IF (extracted by sonication of the precipitate obtained by ultracentrifugation) and RF (cellular residue after sonication) and found that the activity of the factor was present in the SF of tumor cells. Injection of 10 mg of SF protein/rat daily for 9 days into normal rats significantly reduced their serum T_4 and T_3 levels, the responses of T_4 and T_3 to exogenous TSH and their liver 5′-DI activity in comparison with those in PFC rats. However, it did not significantly affect their serum level of TSH, whereas that in TB rats decreases significantly. This finding suggests that the biological factor in the SF did not impair the hypothalamic-pituitary axis. These results indicated that some biological factor(s) in the SF was involved in inducing abnormality of thyroid hormone metabolism through direct or indirect harmful effects in the periphery and thyroid gland and that it induced many of the changes associated with tumors in TB rats. This SF also reduced food intake when administered to normal rats. Similar fractions prepared from muscle and liver of normal rats by the method used for preparation of fractions of tumor tissues and serum of normal animals did not induce significant changes in serum T_4 and T_3 concentrations and the liver 5′-DI activity, indicating that the factor in the SF is specific to tumor cells. They also did not reduce food intake significantly. The serum from TB animals also did not significantly influence the parameters, suggesting that the circulating level of the factor is too low to induce any change of thyroid hormone metabolism on injection of unconcentrated serum, or that the factor is very labile in serum. This factor was tentatively named serum thyroid hormone reducing factor (STRF).

This factor was partially purified from the SF by ammonium sulfate fractionation and DEAE-cellulose chromatography. STRF activity was precipitated with 36–65% saturation of ammonium sulfate. This fraction was further purified by DEAE-cellulose column chromatography with a linear gradient of 0–400 m*M* NaCl. STRF activity was recovered in fraction C, which was eluted with 140–200 m*M* NaCl. Daily injection of the partially purified preparation from the DEAE-cellulose column into normal rats, at a dose of 5 mg/rat for 9 days, significantly decreased their serum T_4 and T_3 concentrations and liver 5′-DI activity in comparison with those of PFC rats, thus mimicking the changes induced by Walker 256 carcinosarcoma. This partially purified preparation also induced anorexia. Thus, partially purified STRF seems to be bifunctional, inducing both metabolic deviation and anorexia. However, whether STRF actually has binary effects as an inducer of abnormality of thyroid hormone metabolism and anorexia remains to be clarified because it is possible that the partially-purified STRF may contain two different factors with the respective activities.

The activity of the STRF preparation was destroyed by pronase or heat treatment, indicating that the factor is a heat-labile protein. These results indicate that suppression of metabolism of thyroid hormones in TB hosts is not only secondary to change in the nutritional state resulting from reduced food intake associated with tumor but also caused by direct or indirect harmful influences of STRF on thyroid hormone metabolism in peripheral tissues (mainly the liver) and on responsiveness of the

thyroid gland to TSH through synthesis of T_4 or secretion of T_4 or both.

The results suggest that TSH secretion or the function of the hypothalamic-pituitary axis may also be influenced by some other factor besides STRF, because the serum TSH level was not significantly changed in STRF-treated rats, whereas that in rats with tumors was significantly reduced. As TB host tissues may be chronically exposed to tumor-derived factors and endogenous factors (host-derived factors) such as cytokines, which may be produced in excess or inappropriately through interaction of the host with invading cells such as microorganisms or cancer cells, the two types of factors may have additive or synergistic effects on the metabolism of the host. Differences between the values for parameters in TB and STRF-treated rats may, therefore, be due to the influences of endogenous factors or other tumor-derived factors in the tumor hosts. This idea is supported by the findings that administration of cytokines such as IL-1[15, 16] and TNF/cachectin[17] to mice and rats strongly modified thyroid hormone metabolism and also induced deviation in the isozyme pattern of pyruvate kinase, a glycolytic key enzyme (our unpublished results). Therefore, these factors may induce impairment of the hypothalamic-pituitary axis in TB animals. In fact, Dubuis *et al.*[16] recently reported that administration of human recombinant IL-1β to normal rats reduced the serum concentration of TSH. In addition, we recently found that an ornithine decarboxylase inducing factor (ODC-factor),[18, 19] which was highly purified from cell-free ascites fluid of Ehrlich ascites tumor also induced abnormality of thyroid hormone metabolism reducing serum levels of T_4 and T_3 upon administration to normal mice (unpublished results). The mechanism of the deviation of thyroid hormone metabolism induced by the ODC-factor is under investigation.

The present results indicate that abnormality of thyroid hormone metabolism in rats with Walker 256 carcinosarcomas is at least partly caused by impaired modulation at the peripheral level (mainly the liver) and at the level of the thyroid gland, directly and indirectly mediated by tumor-derived STRF. These findings support the idea that chronic release of a physiologically active factor from progressive cancer may be a signal or potent mediator of cancer cachexia through imbalance of homeostasis of energy metabolism. There have been several reports on the relationship between cytokines and cachexia.[20–22] To understand cancer cachexia in more detail at the level of host metabolism, therefore, studies are required on whether the combined effects of tumor-derived factors and host-derived factors such as cytokines (IL-1 and TNF/cachectin) on thyroid hormone metabolism are additive or synergistic. The problem of whether STRF actually has binary effects as an inducer of abnormality of thyroid hormone metabolism and anorexia must await purification of STRF.

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