Apoptosis of Muscle Cells Causes Weight Loss Prior to Impairment of DNA Synthesis in Tumor-bearing Rabbits

Osamu Ishiko,[1, 3] Toshiyuki Sumi,[1] Kouzo Hirai,[1] Ken-ichi Honda,[1] Shinichi Nakata,[2] Hiroyuki Yoshida[1] and Sachio Ogita[1]

[1]*Department of Obstetrics and Gynecology, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585 and* [2]*Department of Obstetrics and Gynecology, Osaka City General Hospital, Miyakojima, Osaka 534-0021*

The mechanism of weight loss induced by the growth of malignant tumors is still unknown. We investigated it by focusing on apoptosis of skeletal muscle. VX2-tumor was implanted into rabbits and the apoptotic index (AI) of skeletal muscle was measured by *in situ* end-labeling assay. Plasma of the tumor-bearing rabbits was perfused repeatedly through non-coated charcoal resin. The AI reached 54.6% early after tumor implantation, when weight loss amounted to an 18% decrease in lean body mass (LBM) without change in muscle DNA synthesis or urinary 3-methylhistidine/ creatinine ratio (3-MH/Cr). When the decrease of LBM reached 30%, DNA synthesis was decreased by 48% and 3-MH/Cr was increased by 104%, whereas AI was only 4.7%. The plasma perfusion did not prevent apoptosis in muscle, but improved LBM, DNA synthesis, and 3-MH/Cr. There may be two mechanisms of muscle depletion during the tumor growth: apoptosis in the early stage and metabolic abnormalities in muscle in the late stage.

The weight loss induced by the progression of malignant tumors is the symptom that has attracted the greatest attention among investigators attempting to identify the etiology of cancer cachexia, a condition that gives rise to a variety of symptoms. Cancer cachexia is generally considered to be characterized metabolically by proteolysis and lipolysis rates that exceed protein synthesis and lipogenesis, but no conclusion has been reached concerning how these metabolic abnormalities occur. Todorov *et al.* succeeded in purifying a cancer cachectic factor that induces skeletal muscle catabolism from mice transplanted with MAC16 tumors.[1] Other humoral factors such as cachectin, tumor-necrosis factor, interleukin (IL)-1, IL-6, interferon (IFN)-γ, leukemia-inhibitory factor (LIF), and lipid-mobilizing factor have also been reported to act as mediators inducing cancer cachexia.[2–4] We purified an anemia-inducing substance (AIS) that depresses erythrocyte and immunocompetent cell functions, as reported in our previous papers,[5, 6] and AIS has been recently shown to have another biological action, i.e., lipolytic activity. As stated above, many substances have been reported to be causative agents of cancer cachexia, and at present it is difficult to discuss the etiology of this condition in a coherent fashion.

In the present study using tumor-bearing rabbits, we examined the relationship between weight loss induced during the progression of malignant tumors and the morphological and energy metabolism changes in muscle tissue.

MATERIALS AND METHODS

Experimental animals   Male Japanese White rabbits implanted with VX2-carcinoma[7] in the muscles of their right thigh were supplied by Tsukuba Animal Research Laboratories Co. (Tsukuba) and fed essential CR-3 (Clea, Tokyo) and water. When the VX2-carcinoma in the right thigh of the rabbits grew to more than 5 cm in diameter, it was resected, and cell suspensions (1×10^5 cells in 1 ml of 0.15 M NaCl) were injected intramuscularly into the right thigh of normal rabbits weighing about 3 kg. Body weight and lean body mass (LBM) were measured and urine was sampled to determine 3-methylhistidine (3-MH) and creatinine (Cr) concentrations by HPLC [8, 9] every 10 days after tumor implantation.

As the control group, normal rabbits about 3 kg were injected with 1 ml of 0.15 M NaCl into the right thigh and were fed the same amount that the VX2-implanted animals ate (group C).

Monitoring of LBM   Rabbits intravenously anesthetized with pentobarbital (25 mg/kg) were subjected to measurement of the total body electrical conductivity (TOBEC) with a TOBEC Small Animal Body Composition Analysis System (Model 3152, EM-SCAN Inc., Springfield, IL; 203 mm in inner diameter and 617 mm long). The measurements were made by observing changes in impedance upon exposure to a 10-MHz magnetic field in a measurement chamber. The LBM (g) of the rabbits was calculated by using the formula: $1.536 \times$ TOBEC value $+475.1$.[10]

Method of cyclic plasma perfusion   A Teflon catheter (24-gauge Insite, Terumo Co., Tokyo) was inserted into

[3] To whom correspondence should be addressed.
  E-mail: ishikoo@msic.med.osaka-cu.ac.jp

the rabbit's auricular artery and vein, and blood flowing out of the artery at about 5 ml/min was separated with a membrane-type plasma separator (pore size: 0.2 μm, membrane area: 0.1 m^2). The separated plasma was then passed through an adsorptive resin column containing non-coated charcoal as the base material at a flow rate of about 3 ml/min, after which the cellular component and plasma component were re-mixed and returned into the rabbit's auricular vein. The quantity of plasma perfused each time was equivalent to the rabbit's total plasma volume (the total circulating plasma volume of each rabbit was calculated as 38.8 ml/kg of the animal's body weight). Plasma perfusion was performed twice a week beginning 10 days after implantation of VX2-carcinoma (group A).[11, 12]

As the control group, the perfusion was performed for the same duration and with the same frequency on VX2-implanted rabbits without the non-coated charcoal column (group B).

Determination of the apoptotic index (AI) A femoral muscle tissue specimen was collected from each rabbit before, and on days 20 and 40 after VX2-tumor implantation, fixed with 10% neutral formalin, embedded in paraffin, and sectioned. Apoptotic cells were detected *in situ* by the TUNEL (TdT-mediated dUTP-biotin nick-end labeling) method using the Apop Tag kit (Oncor, Inc., Gaithersburg, MD).[13] The AI was defined as the percentage of TUNEL-method-positive cells in a 200-times magnified field, and the AI was compared between the experimental groups. To ensure lack of investigator bias, two different persons, who were blinded as to the treatment group, read the AI separately.

DNA laddering Skeletal muscle tissue collected from each rabbit every 10 days after tumor implantation was homogenized, and the DNA was extracted by the methods described previously.[14] The extracts were electrophoresed in 1.5% agarose gel, stained with SYBR Green I (Molecular Probes, Eugene, OR), and visualized under UV illumination.

Primary myoblast culture and DNA synthesis Approximately 3 ml of hypertonic saline (20% w/v) was injected intramuscularly into the left thigh of normal rabbits and rabbits implanted with VX2 carcinoma 20 days or 40 days before, and either treated or not treated by cyclic plasma perfusion, and a specimen of regenerating muscle (about 1 g) was resected from the injection area 72 h later. The muscle specimen was washed with Hank's balanced salt solution (HBSS; Wako Pure Chemical Ind., Osaka), minced with scissors, and incubated in 20 ml of HBSS supplemented with 5 mM CaCl$_2$, 10 mM N-2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES; Wako), 0.8 mg/ml of collagenase (Wako), and 1000 protein units/ml of Dispase (protease; Godo Shusei Co., Tokyo) for 1 h at 37°C. The dispersed cells were centrifuged at 450g for 5 min, and washed twice with HBSS. Then aliquots of

6×10^5 cells were cultured in 3 ml of Williams' medium E (Gibco BRL, Life Technologies Inc., Grand Island, NY), supplemented with 10^{-8} M insulin, 10^{-8} M dexamethasone, 30 μg/ml kanamycin, and 17% fetal bovine serum (Gibco BRL) on 6-cm diameter collagen-coated plastic dishes (Iwaki Co., Funabashi) under an atmosphere containing 5% CO$_2$ at 37°C. Fibroblasts were removed by means of the selective plating procedure described by Yaffe.[15]

Three days after the start of primary culture of the myoblasts, 10 μCi of [^{3}H]thymidine (New England Nuclear Co., Boston, MA; 11 Ci/mmol) was added to each dish, and the cultures were incubated under an atmosphere containing 5% CO$_2$ at 37°C for 6 h. The cells in each dish were then washed twice with PBS and solubilized in 1.5 ml of 0.2% sodium dodecyl sulfate. The DNA in the solubilized cells was precipitated by addition of 1.5 ml of 10% trichloroacetic acid on ice for 10 min and centrifugation at 10 000g for 10 min. Incorporation of [^{3}H]thymidine into DNA was measured by counting the scintillation rates of the precipitates in 10 ml of scintillation liquid (Emulsifier-Safe; Packard Instruments B.V., Groningen, the Netherlands), and incorporation of [^{3}H]thymidine per 1×10^5 myoblasts was recorded as the DNA synthesis rate.[8]

Data analysis Student's t test was used to analyze the data for significant differences, and differences were considered statistically significant when the P value was <0.05. All data are expressed as means±SD.

RESULTS

Rabbits were divided into three groups as follows: group A, VX2-implanted rabbits with cyclic plasma perfusion through non-coated charcoal; group B, VX2-implanted rabbits with sham-perfusion; group C, rabbits injected with 1 ml of 0.15 M NaCl solution instead of VX2 tumor cells.

There were no significant differences in body weight and LBM at day 0 of the experiment between the three groups. The tumor implanted in the right thigh showed almost the same growth in both group A and group B before the start of plasma perfusion.

In group A, total body weight and LBM at 20 days after implantation significantly decreased to 0.86±0.14 and 0.85±0.19 (values are mean±SD expressed as the ratio to the values on day 0), respectively, showing no significant difference from those in group B (Fig. 1). At that time, myoblast DNA synthesis (DNAS) was 0.99±0.08 and 0.99±0.13 in group A and group B, respectively (Fig. 2A), showing no significant decrease in both groups. At 40 days after implantation, total body weight, LBM, and DNAS in group B decreased to 0.64±0.14, 0.69±0.22, and 0.54±0.18, respectively, while those in group A improved significantly to 0.90±0.19, 0.94±0.20 and 0.86±0.24, respectively (Figs. 1, 2A). No apoptotic cells were

detected by the TUNEL method in group C. By contrast, the AI values in group A on 10, 20, 30, and 40 days after implantation were 3.5 ± 1.6, 47.9 ± 12.5, 37 ± 12.8, and 5.0 ± 4.1, respectively, and there was no significant difference in AI between group A and group B (Figs. 2B, 3). We also assessed apoptosis in terms of DNA laddering. A DNA laddering pattern characteristic of DNA fragmentation was visible in the samples from animals with higher percentages of TUNEL-positive nuclei (Fig. 4).

The urinary 3-MH/Cr, a probable index of muscle catabolism, had not changed as of 20 days after implantation in both group A and group B. However, 3-MH/Cr in group B had increased by 2.1 fold at 40 days after implantation compared with pre-implantation values, while it was 152 ± 41.7 nmol/mg (almost the same as before implantation) in group A (Fig. 5).
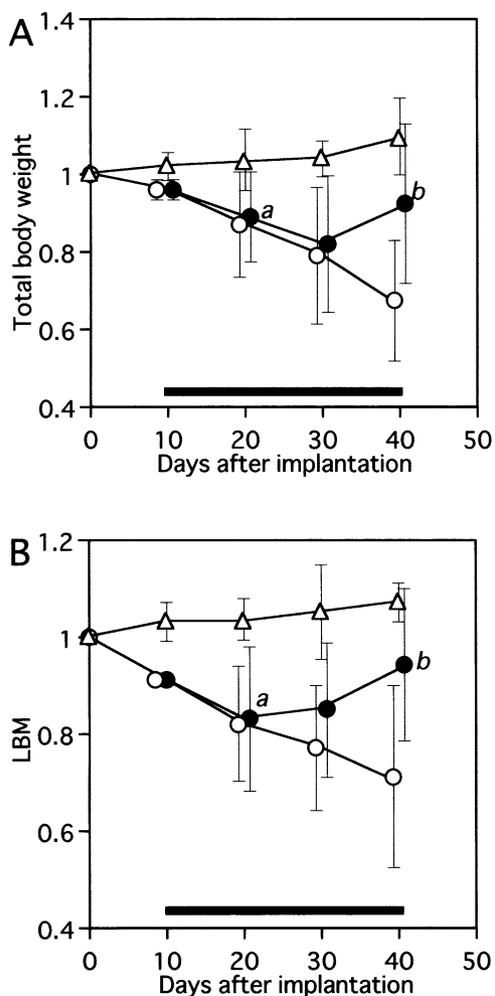

Fig. 1. Total body weight (A) and lean body mass (B) of VX2 carcinoma-bearing rabbits in response to cyclic plasma perfusion. Closed circles, VX2-implanted rabbits with cyclic plasma perfusion through non-coated charcoal ($n=8$, group A); open circles, VX2-implanted rabbits with sham-perfusion ($n=8$, group B); open triangles, rabbits injected with 1 ml of 0.15 M NaCl solution ($n=8$, group C); horizontal bars, during cyclic plasma perfusion. Values are means$\pm$SD expressed as the ratio to values on day 0. Statistical significance is as follows: a, $P<0.05$ group A, B vs. group C at 20 days after implantation; b, $P<0.01$ group A vs. group B at 40 days after implantation.
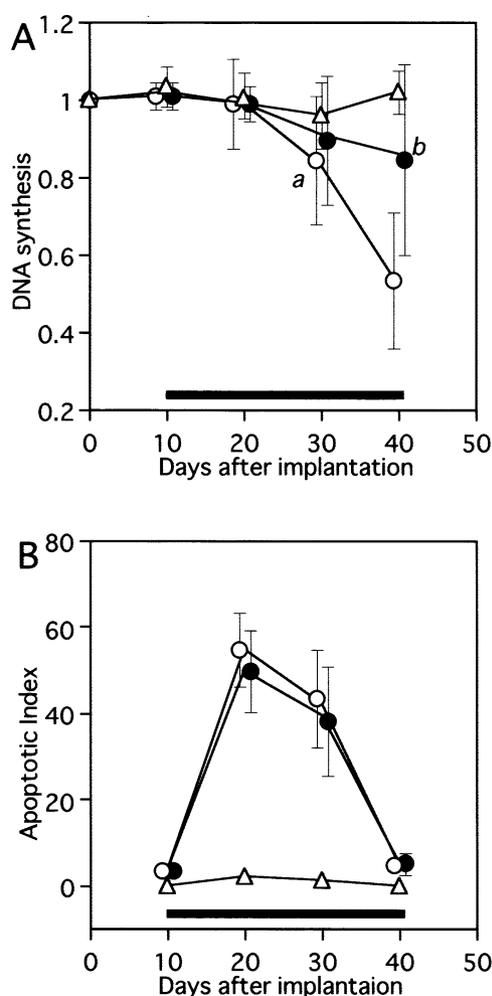
Fig. 2. DNA synthesis (A) and apoptotic index (B) of VX2 carcinoma-bearing rabbits in response to cyclic plasma perfusion. Closed circles, VX2-implanted rabbits with cyclic plasma perfusion through non-coated charcoal ($n=8$, group A); open circles, VX2-implanted rabbits with sham-perfusion ($n=8$, group B); open triangles, rabbits injected with 1 ml of 0.15 M NaCl solution ($n=8$, group C); horizontal bars, during cyclic plasma perfusion. Values are means$\pm$SD expressed as the ratio to values on day 0 (A) and as occurrence of TUNEL-positive nuclei within five fields at $\times$200 (B). Statistical significance is as follows: a, $P<0.05$ group B vs. group C at 30 days after implantation; b, $P<0.001$ group A vs. group B at 40 days after implantation.

DISCUSSION

The pathogenesis of the cancer cachexia associated with the progression of malignant tumors has yet to be elucidated. Although most studies have postulated cytokines, such as tumor necrosis factor-α (TNF-α), IL-1, IL-6, IFN-γ, and LIF, as causative substances, some researchers are pursuing potential causative substances produced by the tumor, such as lipid mobilizing factor (LMF).[16–18] In 1987, we discovered an AIS that reduces the functions of erythrocytes and immunocompetent cells in the plasma of advanced ovarian cancer patients.[5, 6] Recently it has also been shown to possess a lipolytic effect.[19] That series of studies dealt with weight loss in terms of decreases in fats. Our previous study on the depletion of total body fat in tumor-bearing subjects showed that the main reason for the decrease in adipose tissue was adipocyte apoptosis in the early stage of tumor progression, while it was metabolic imbalance in the late stage of tumor progression.[20] Does a similar phenomenon occur in muscle tissue, which accounts for a large proportion of the body components?
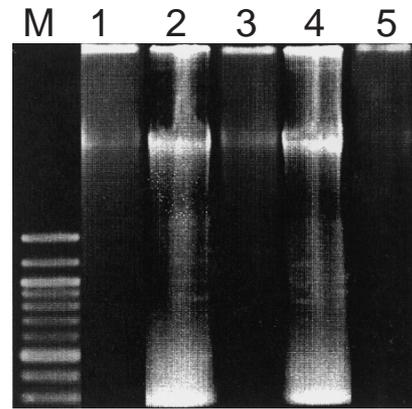

Fig. 4. DNA fragmentation assay of the skeletal muscle of a VX2-bearing rabbit. Lane M: DNA ladder marker. Lane 1: a non-tumor-bearing rabbit (group C). Lane 2: a rabbit with sham-plasma perfusion at 20 days after implantation (group B). Lane 3: a rabbit with sham-plasma perfusion at 40 days after implantation (group B). Lane 4: a rabbit with plasma perfusion at 20 days after implantation (group A). Lane 5: a rabbit with plasma perfusion at 40 days after implantation (group A).
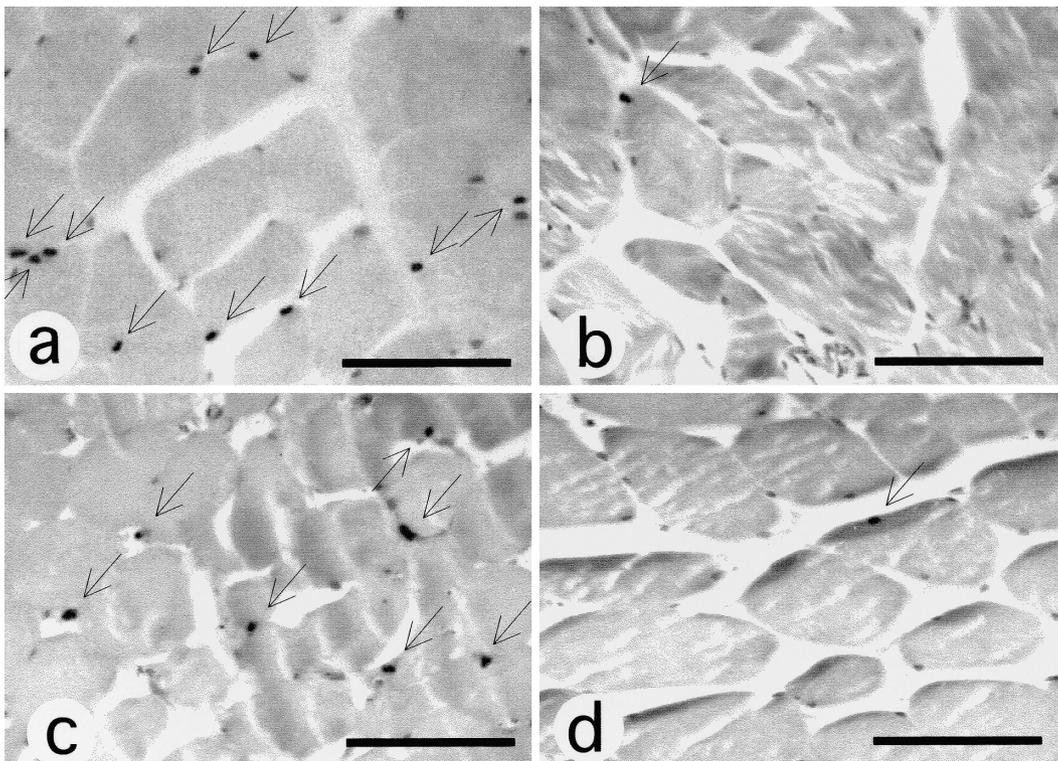

Fig. 3. TUNEL signals in the skeletal muscle of a VX2 carcinoma-bearing rabbit. (a) Intense TUNEL signals at 20 days after implantation in the nuclei of some muscle cells of a rabbit that underwent sham-plasma perfusion (group B), showing an AI of 40.3% (arrows), (b) a rabbit at 40 days after implantation that underwent sham-plasma perfusion (group B), showing an AI of 4.1% (arrow), (c) a rabbit at 20 days after implantation that underwent plasma perfusion (group A) and had an AI of 39.4% (arrows), and (d) a rabbit at 40 days implantation (group A) that underwent plasma perfusion and had an AI of 4.0% (arrow). Scale bars, 30 μm.
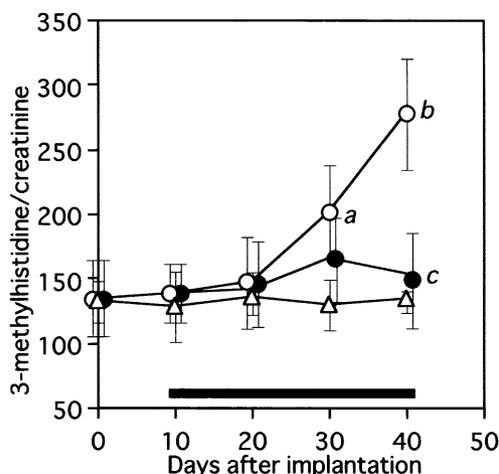
Fig. 5. Urinary 3-methylhistidine/creatinine ratio of VX2 carcinoma-bearing rabbits in response to cyclic plasma perfusion. Closed circles, VX2-implanted rabbits with cyclic plasma perfusion through non-coated charcoal ($n=8$, group A); open circles, VX2-implanted rabbits with sham-perfusion ($n=8$, group B); open triangles, rabbits injected with 1 ml of 0.15 M NaCl solution ($n=8$, group C); horizontal bar, during cyclic plasma perfusion. Statistical significance is as follows: a, $P<0.05$ group B vs. group C; b, $P<0.01$ group A vs. group B and group B vs. group C; c, not significant group A vs. group C.

In this preliminary study we examined the relationship between weight loss induced during the progression of malignant tumors and the morphological and energy metabolism changes in muscle tissue in order to develop a new hypothesis for the cause of weight loss in cancer patients.

It is known from the results of animal model studies that body weight, especially total body fat, decreases early after tumor implantation.[19–21] Are the mechanisms of the weight loss observed in the early stage of tumor bearing the same as those of the late phase? Body weight and LBM had significantly decreased 20 days after tumor implantation compared with the pre-implantation values, but there was no change in DNAS or urinary 3-MH/Cr, a probable index of muscle catabolism, and 54.6% of muscle cells were positive for apoptosis. These findings strongly suggested that weight loss in the early stage of cancer bearing is mainly attributable to apoptosis in skeletal muscle, not metabolic catabolism. At 40 days after implantation, however, DNAS had decreased by 54% and 3-MH/Cr had increased by 107%, compared with their pre-implantation values, whereas AI was just 5.0%. Both DNAS and 3-MH/Cr returned to their pre-implantation values after plasma perfusion. This means that plasma perfusion removed humoral factors, which induce catabolic changes in skeletal muscle, from the plasma. In other words, this finding indicates that substances that increase muscle catabolism and can be removed by plasma perfusion are involved in the late stage of cancer bearing. However, plasma perfusion did not affect the apoptosis of muscle cells. This shows that weight loss in the late stage of cancer bearing is related to decreased protein synthesis and increased proteolysis.

Why is apoptosis of normal skeletal muscle cells induced in the early stage of cancer bearing and decreased in the late stage? One possibility is that the apoptosis of tumor cells is induced as a mechanism to suppress tumor proliferation in the early stage of cancer and the apoptosis of normal muscle tissue is induced as an overreaction, and that the mechanism that inhibits tumor proliferation is already reduced in the late stage of cancer bearing, which results in the inhibition of apoptosis in muscle tissue. We are attempting to identify the genes involved in the induction and inhibition of the apoptosis of normal muscle cells in order to elucidate the mechanisms of the apoptosis.

Based on the results of this study, we hypothesize that there are at least two major causes of the weight loss induced by the growth of malignant tumors, which may play central roles in muscle depletion: apoptosis in the early stage and metabolic abnormality in muscle in the late stage. Similar studies need to be conducted in human subjects.

REFERENCES

1) Todorov, P., Cariuk, P., McDevitt, T., Coles, B., Fearon, K. and Tisdale, M. Characterization of a cancer cachectic factor. *Nature*, **379**, 739–742 (1996).
2) Nakahara, W. and Fukuoka, F. Toxohormone: a characteristic toxic substance produced by cancer tissue. *Jpn. J. Cancer Res.*, **40**, 45–69 (1949).
3) Langstein, H. N., Doherty, G. M., Franker, D. L., Buresh, C. M. and Norton, J. A. The roles of gamma-interferon and tumor necrosis factor alpha in an experimental rat model of cancer cachexia. *Cancer Res.*, **51**, 2302–2306 (1991).
4) Mori, M., Yamauchi, K. and Abe, K. Purification of a lipoprotein lipase-inhibiting protein produced by a melanoma cell line associated with cancer cachexia. *Biochem. Biophys. Res. Commun.*, **160**, 1085–1092 (1989).

5) Ishiko, O., Sugawa, T., Tatsuta, I., Shimura, K., Naka, K., Deguchi, M. and Umesaki, N. Anemia-inducing substance (AIS) in advanced cancer: inhibitory effect of AIS on the function of erythrocytes and immunocompetent cells. *Jpn. J. Cancer Res.* (Gann), **78**, 596–606 (1987).

6) Honda, K., Ishiko, O., Tatsuta, I., Deguchi, M., Hirai, K., Nakata, S., Sumi, T., Yasui, T. and Ogita, S. Anemia-inducing substance from plasma of patients with advanced malignant neoplasms. *Cancer Res.*, **55**, 3623–3628 (1995).

7) Kidd, J. G. and Rous, P. A transplantable rabbit carcinoma originating in a virus-induced papilloma and containing the virus in masked or altered form. *J. Exp. Med.*, **71**, 813–838 (1940).

8) Nakata, S., Ishiko, O., Honda, K., Hirai, K., Deguchi, M. and Ogita, S. Increased proliferation of myoblasts after cyclic plasma perfusion of tumor-bearing rabbits. *Jpn. J. Cancer Res.*, **89**, 578–582 (1998).

9) Young, V. R. and Mumro, H. N. N-Methylhistidine (3-methylhistidine) and muscle protein turnover: an overview. *Fed. Proc.*, **37**, 2291–2300 (1978).

10) Yasui, T., Ishiko, O., Sumi, T., Honda, K., Hirai, K., Nishimura, S., Matsumoto, Y. and Ogita, S. Body composition analysis of cachectic rabbits by total body electrical conductivity. *Nutr. Cancer*, **32**, 190–193 (1998).

11) Ishiko, O., Deguchi, M., Tatsuta, I., Naka, K., Hirai, K., Nakata, S., Honda, K. and Sugawa, T. Removal of immunosuppressive substance in cancer patients' serum. *Jpn. J. Cancer Res.*, **81**, 564–566 (1990).

12) Ishiko, O., Hirai, K., Nishimura, S., Sumi, T., Honda, K., Deguchi, M., Tatsuta, I. and Ogita, S. Elimination of anemia-inducing substance by cyclic plasma perfusion of tumor-bearing rabbits. *Clin. Cancer Res.*, **5**, 2660–2665 (1999).

13) Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. Identifica-tion of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.*, **119**, 493–501 (1992).

14) Lu, W., Hirayasu, K., Ishizawa, M. and Kobayashi, Y. Purification of genomic DNA from human whole blood by isopropanol-fractionation with concentrated NaI and SDS. *Nucleic Acids Res.*, **22**, 1774–1775 (1994).

15) Yaffe, D. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA*, **61**, 477–483 (1968).

16) Kawakami, M. and Cerami, A. Studies of endotoxin-induced decrease in lipoprotein lipase activity. *J. Exp. Med.*, **154**, 631–639 (1981).

17) Fong, Y., Moldawere, L. L., He, W., Marano, M., Keogh, C. V., Gershenwald, J. and Lowry, S. F. Tumor location influences local cytokine production and host metabolism. *Surg. Oncol.*, **1**, 65–71 (1992).

18) Hirai, K., Hussey, H. J., Barber, D., Price, S. A. and Tisdale, M. J. Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. *Cancer Res.*, **58**, 2359–2365 (1998).

19) Ishiko, O., Yasui, T., Hirai, K., Honda, K., Sumi, T., Nishimura, S. and Ogita, S. Lipolytic activity of anemia-inducing substance from tumor-bearing rabbits. *Nutr. Cancer*, **33**, 201–205 (1999).

20) Nishimura, S., Ishiko, O., Hirai, K., Sumi, T., Yasui, T. and Ogita, S. Effects of cyclic plasma perfusion on adipocyte apoptosis and lipolytic activity in VX2 carcinoma-bearing rabbits. *Anticancer Res.*, **20**, 1657–1662 (2000).

21) Ishiko, O., Nishimura, S., Yasui, T., Sumi, T., Hirai, K., Honda, K. and Ogita, S. Metabolic and morphologic characteristics of adipose tissue associated with the growth of malignant tumors. *Jpn. J. Cancer Res.*, **90**, 655–659 (1999).