Increased Proliferation of Myoblasts after Cyclic Plasma Perfusion of Tumor-bearing Rabbits

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Rabbits implanted with VX2 carcinoma and treated by cyclic plasma perfusion showed lower weight loss and longer survival than untreated rabbits. The urinary 3-methylhistidine/creatinine ratio was less elevated in the treated rabbits than in the untreated rabbits. Myoblasts from untreated rabbits with VX2 carcinoma showed lower proliferation than those from normal rabbits, while those from rabbits treated by cyclic plasma perfusion showed intermediate proliferation. Furthermore, the DNA synthesis rate of myoblasts from treated rabbits was much better maintained than in the case of untreated rabbits.

Key words: Anemia-inducing substance — Plasma perfusion — 3-Methylhistidine — Myoblast — Cancer cachexia

Cancer cachexia is characterized by progression of wasting in adipose tissue and muscle following tumor growth. Tumor necrosis factor (TNF), interleukin (IL)-6, interferon-γ, and lipid-mobilizing factors have been shown to convert adipose tissue to a catabolic state.1) Muscle is more important to the quality of life of cancer patients, but the cause of muscle wasting is not well understood.2) Increased release of 3-methylhistidine (3-MH) from skeletal muscles is thought to reflect increased breakdown of myofibrillar proteins,3) and an elevated urinary 3-methylhistidine/creatinine (3-MH/Cr) ratio has been reported in cancer patients.4) However, there is another report showing lower 3-MH levels in the leg vein blood of cancer patients than that of healthy subjects.5)

Ishiko et al. showed that plasma perfusion through a non-coated charcoal column removes deleterious factors for red blood cells (RBCs) from rabbit plasma.6, 7) In this study, we repeatedly perfused the plasma of rabbits implanted with VX2 carcinoma, and found beneficial effects on body weight and the urinary 3-MH/Cr ratio.

We surmised that some plasma factor has an inhibitory effect on muscles, and that the ability of muscle cells to regenerate decreases following tumor growth. Muscles regenerate by activation of myogenic precursor cells, known as satellite cells, to generate myoblasts.8) If the putative plasma factor could be removed by plasma perfusion, myoblasts should proliferate in the muscles. We therefore studied the effect of plasma perfusion on the proliferation of myoblasts from tumor-bearing rabbits.

MATERIALS AND METHODS

Rabbits implanted with VX2 carcinoma Male Japanese white rabbits implanted with VX2 carcinoma in the muscles of their right thigh were supplied by Tsukuba Animal Research Laboratories, Chiyoda Kaibatsu Co. (Tsukuba), and fed 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 implanted intramuscularly into the right thigh of normal rabbits weighing about 3 kg. Body weight and the diameter of the implanted tumor were measured weekly, and urine was sampled to determine 3-MH and creatinine (Cr) concentrations.

Plasma perfusion Teflon catheters (24-gauge Insite, Terumo Co., Tokyo) were inserted into the rabbit’s auricular artery and vein, and blood flowing out of the artery at about 8 ml/min was separated with a membrane-type plasma separator (AP-02H; pore size 0.2 μm, membrane area 0.1 m², Asahi Medical Inc, Tokyo). 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 5 ml/min, after which the cellular component and plasma component were mixed back together and returned into the rabbit’s auricular vein (Fig. 1). The quantity of plasma perfused each time was made equivalent to the rabbit’s total plasma volume, and the total circulating plasma volume of each rabbit was calculated as 38.8 ml/kg of the animal’s body weight.8) Plasma perfusion was performed twice a week from 40 days after implantation of VX2 carcinoma. The rabbits were weighed before each plasma perfusion.

Primary culture of rabbit myoblasts About 3 ml of hypertonic saline (20% w/v) was injected intramuscularly
Myoblasts from Tumor-bearing Rabbits

into the left thigh of normal rabbits and rabbits implanted with VX2 carcinoma 55 days before and either treated or not treated by cyclic plasma perfusion, and 72 h later, a specimen of regenerating muscle (about 1 g) was resected from the injection area.11) The muscle specimen was washed with Hanks’ balanced salt solution (HBSS; Wako Pure Chemical Industries, Osaka), minced with scissors, and incubated in 20 ml of HBSS supplemented with 5 mM CaCl₂, 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 6×10⁵ cells each 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% CO₂ at 37°C. Fibroblasts contaminating the cell population alone were removed by means of the selective plating procedure described by Yaffe.12)

Determinations of cell counts and DNA synthesis rate
The cells on the bottom of the culture dish were isolated by incubation in 0.25% trypsin, and the number of living cells stained by Trypan Blue in phosphate-buffered saline was counted after 1, 3, 5, and 7 days of primary culture of the myoblasts.

In addition, 3 days after the start of primary culture of the myoblasts, 10 µCi of [³H]thymidine (New England Nuclear Corp., Boston; 11 Ci/mmol) was added to each dish, and the cultures were incubated under an atmosphere containing 5% CO₂ 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 [³H]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 [³H]thymidine per 1×10⁵ cells of myoblasts was recorded as the DNA synthesis rate.

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

Body weight of the VX2-carcinoma-bearing rabbits
(Fig. 2) The VX2 carcinoma became palpable at about 20 days after implantation, and rapidly grew larger, reaching as much as about 7.5 cm in diameter by 40 days. The VX2 carcinoma did not grow thereafter and often rap-

Fig. 1. Schema of extracorporeal plasma perfusion. Blood from an auricular artery (A) of a rabbit was pumped at a flow rate of approximately 8 ml/min and passed through a plasma separator (a) (pore size, 0.2 µm; membrane area, 0.1 m²). The separated plasma, after having been passed through an adsorptive resin column containing non-coated charcoal (b), was returned into an auricular vein (V).

Fig. 2. Growth of VX2 carcinoma in rabbits and effects of cyclic plasma perfusion on body weight. Data are shown for rabbits treated by plasma perfusion (●) and not treated by plasma perfusion (○), and the days of plasma perfusion (▲) are indicated. Percentages of body weight before implantation of VX2 carcinoma (lines) and tumor diameter (columns) are indicated on the vertical axis. * Significantly different at P<0.05. n=7.
tured with the release of necrotic contents, especially after 50 days. There was no significant difference in the diameters of VX2 carcinoma or the rates of rupture between untreated rabbits and rabbits treated with cyclic plasma perfusion.

The body weight of the VX2-carcinoma-bearing rabbits began to decrease about 2 weeks after implantation. At 40 days, weight loss was about 10% of body weight before implantation, and the decrease in body weight accelerated thereafter. The rabbits treated by cyclic plasma perfusion, however, showed less weight loss than the untreated rabbits.

**Urinary 3-MH/Cr ratio** (Fig. 3) At 45 to 50 days after implantation of VX2 carcinoma, the urinary 3-MH/Cr ratio was significantly higher in the untreated rabbits than in the normal control rabbits, but it was not significantly higher in the rabbits treated by cyclic plasma perfusion than in the normal control rabbits.

**Proliferation and DNA synthesis rates of myoblasts from VX2-carcinoma-bearing rabbits** Myoblasts from rabbits with VX2 carcinoma showed lower proliferation rates than myoblasts from normal rabbits, the difference being statistically significant at 5 days of culture. However, the proliferation rates of the myoblasts from rabbits treated by cyclic plasma perfusion were not reduced as much (Fig. 4). The DNA synthesis rates of myoblasts from the untreated rabbits were significantly lower after 3 days of culture, but remained almost unchanged after treatment by cyclic plasma perfusion, as compared with those for normal rabbits (Fig. 5).

![Fig. 3. Urinary 3-MH/Cr ratio of rabbits with VX2 carcinoma. Data for rabbits treated by plasma perfusion (●) and not treated by plasma perfusion (○) are indicated. * Significantly different at $P<0.05$.](image)

![Fig. 4. Effect of plasma perfusion on proliferation of myoblasts from rabbits with VX2 carcinoma. Proliferation of myoblasts from normal rabbits (○), and from rabbits implanted with VX2 carcinoma 55 days before and not treated (●) or treated by cyclic plasma perfusion for 2 weeks (■) are indicated. The vertical axis shows numbers of myoblasts as multiples of the cell counts at the start of culture. * Significantly different at $P<0.05$. For each group, $n=9$.](image)

![Fig. 5. Effect of cyclic plasma perfusion for 2 weeks on DNA synthesis rates of myoblasts from rabbits implanted with VX2 carcinoma 55 days before. [$^{3}$H]Thymidine incorporation per $1\times10^5$ myoblasts after 3 days of culture was recorded as the DNA synthesis rate. The vertical axis shows percentage [$^{3}$H]thymidine incorporation per $1\times10^5$ myoblasts from normal rabbits (control). * Significantly different at $P<0.01$, respectively. For each group, $n=9$. PP, plasma perfusion.](image)
DISCUSSION

Progression of malignant tumors has been found to cause loss of lean body mass in both humans and experimental animals. Toxohormone extracted from cancer tissue was found to suppress the function of catalase in the liver and to interfere with iron metabolism in rats, but it was not reported to have any effect on muscle metabolism. Administration of TNF and IL-1 has been reported to decrease levels of the mRNAs of myofibrillar proteins and the content of these proteins, but production of TNF to decrease levels of the mRNAs of myofibrillar proteins was not reported to have any effect on muscle metabolism. Administration of TNF and IL-1 has been reported to cause loss of lean body mass in both humans and experimental animals. Toxohormone extracted from cancer tissue was found to suppress the function of catalase in the liver and to interfere with iron metabolism in rats, but it was not reported to have any effect on muscle metabolism. Administration of TNF and IL-1 has been reported to decrease levels of the mRNAs of myofibrillar proteins and the content of these proteins, but production of TNF to decrease levels of the mRNAs of myofibrillar proteins was not reported to have any effect on muscle metabolism. Administration of TNF and IL-1 has been reported to cause loss of lean body mass in both humans and experimental animals.

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