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TUMOR NECROSIS FACTOR-α ALTERS PROTEIN METABOLISM AND CELL-CYCLE KINETICS IN MALIGNANT TUMOR

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The effects of tumor necrosis factor-α (TNF) on protein metabolism and cell-cycle kinetics were investigated in malignant tumor. Sprague-Dawley rats, subcutaneously inoculated with Walker 256 carcinosarcoma, were injected intraperitoneally with recombinant human TNF at a dose of 4.75×10^6 U/kg for 3 consecutive days. Tumor protein metabolism and cell-cycle kinetics were analyzed. The results showed a significant decrease in tumor volume and weight in comparison with control. TNF resulted in significant decrease in tumor protein fractional synthesis rate, protein synthesis and fractional growth rate, but no change of tumor protein fractional degradation rate. TNF also resulted in remarkable decline in labelling index and G1 phase increase of tumor cells, 6 hours after bromodeoxyuridine injection, by cytometry. The results indicated that TNF inhibits tumor growth as a result of decreases in tumor cell DNA and protein syntheses.

Key words: Tumor necrosis factor (TNF), Protein metabolism, Cell-cycle, Tumor experimental.

With a better understanding of molecular biology and the rapid development of biotechnology, biologic therapy has emerged as a fourth modality for the treatment of cancer, and significant progresses have been made in recent decade.[1] Tumor necrosis factor-α (TNF), a cytokine with anticancer activity, plays an important role in cancer biological therapy.[2] The metabolic effects of TNF, however, is poorly understood. The effects of TNF on protein metabolism and cell-cycle kinetics in malignant tumor were dealt with in this study.

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MATERIALS AND METHODS

Animals and Tumors

Male Sprague-Dawley rats were housed in stainless-steel suspansion cages and maintained with a 12 hour light/dark cycle as well as ambient temperature and humidity. The rats were subcutaneously (s.c.) inoculated with 10^7 cells of Walker 256 carcinosarcoma into the right flank and were randomly divided into two groups. The rats in both TNF treatment group and control group were injected intraperitoneally (i.p.) with 4.75×10^6 U/kg of recombinant human TNF, generously provided by Genentech Inc. (South San Francisco, CA), and 0.2 ml of normal saline, respectively, for consecutive three days. Daily measurement of tumor volume was carried out after tumor implantation. Tumor volumes were estimated with the formula for a prolate spheroid: $V=\pi/6\times L\times W\times D$, where V, L, W and D are the volume, length, width and depth, respectively, of the solid tumors measured with calipers.[3]

Protein Metabolism

On day 14 after tumor implantation, and injection containing flooding dose of 200 μCi/kg (7400 kBq/kg) of L-[1-^{14}C]-leucine (ICN Radiochemicals, Irvine, CA) and 1 mmol/kg of leucine was i.p. administered.[4] Fifteen minutes after bolus injection of precursor leucine, these animals were sacrificed by decapitation, and the tumor was quickly removed. The entire tumors were frozen in liquid nitrogen, immediately after weighing, to halt all the metabolic processes. Two pieces of tumor were separately weighed and placed in 5 ml of 10% sulfosalicyclic acid (SSA) and 5 ml of saline respectively. The analytical procedure for estimating protein metabolism of tumor was generally same as previously described.[5] Briefly, the tumor samples in 10% SSA were homogenized and centrifuged to separated the protein (precipitate) and the free intracellular (acid-soluble) amino acids for determination of leucine-specific activities in these two fractions. The supernatant was further spun down to remove contamination from the protein-bound fraction. A portion of which was analyzed by HPLC for leucine concentration. Another portion, treated with 30% H_2O_2, was incubated, centrifuged and added with scintillant Monofluor (National Diagnostics, Manville, NJ) for measurement of ^{14}C-leucine radioactivity. Intracellular-free, leucine-specific activity (SAi) was calculated from the radioactivity counts and leucine concentration in the SSA-soluble fraction. The precipitate was washed with 2% SSA and then dried. The dried samples were weighed and solubilized in BTS-450 (Beckman, Fullerton, CA) followed by adding scintillant Ultrafluor (National Diagnostics, Manville, NJ) for determining ^{14}C-leucine radioactivity. A second dried sample was analyzed for tissue nitrogen by micro-Kjeldahl digestion. The protein-bound specific activity (SAb) of leucine was calculated from the radioactivity counts, the measured nitrogen contents in the precipitated fraction, and the average percentages of leucine contents in tissue protein. The tumor sample in saline was homogenized for measurement of total nitrogen by micro-Kjeldahl digestion and spectrophotometry.[3]

Flow Cytometry

On day 7 after tumor implantation, all the rats received i.p. injection of bromodeoxyuridine (BrdU) at the dose of 750 μg/kg, following a 15 minute pretreatment of i.p. injection with 150 mg/kg of fluorodeoxyuridine (FdU). Half of the rates in each group were killed by decapitation at 1 and 6 hours after the pulse administration of BrdU. The tumors were quickly excised and placed in phosphate-buffered saline (PBS) at pH 7.2–7.4, and were dissected mechanically and filtered through cotton gauze. After washing with PBS (pH 7.2) and refiltering through a 35 μm nylon mesh, the single-cell suspension of tumor was centrifuged and fixed in ice-cold 70% ethanol. For analysis by flow cytometry, 2 ml of cell suspension were added to 2 ml of 4 N HCl with 0.5% Triton X-100 in order to denature the DNA to produce single-stranded molecules. After incubation for 30 minutes at room temperature in the dark, the cell suspension was centrifuged and the pellet was washed and resuspended in 0.1 M sodium tetraborate (pH 8.5) to neutralize the acid. Then $1–1.5\times10^6$ cells were washed twice with PBS containing 0.5% Tween-20 to achieve a pH of approximately 7.0, and resuspended in 1% FCS in PBS containing 0.5% Tween-20 (pH 7.2–7.4). Twenty μl of FITC-conjugated anti-BrdU antibody were added, and the nuclei were incubated at room temperature in the dark for 30 minutes. After washing once with 2 ml of 1% FCS/PBS containing 0.5% Tween-20 and resuspended in 1 ml of PBS containing 0.01 mg of propidium iodide (PI) for 15 minutes of staining, the cells were analyzed by flow cytometry performed on a FACSCAN (Becton Dickinson, Mountainview, CA). The cells were excited with a 15 mV laser at 488 nm. The red fluorescence was collected through a 585 nm bandpass filter and recorded as the total amount of DNA. The green fluorescence of the BrdU-labelled cells was collected through a 530 nm bandpass filter and recorded as the amount of incorporated BrdU. The data were collected in a 64×64 channel distribution showing the total DNA (red) against the logarithmic amount of incorporated BrdU (green).[6]

Calculation

The individual fractional growth rate (Kg) was calculated according to daily changes of tumor volume during the period of measurable tumor growth, using the following formula: $Kg=(LnV_2-LnV_1)/(T_2-T_1)$, where LnV_2 and LnV_1 are the natural logarithms of the tumor volume at time T_2 and T_1 respectively, and (T_2-T_1) is the time period in days.[3] The fractional synthesis rate (Ks) is determined as represented by the equation: $Ks=SAb/(SAi\times T)\times100\%$, where SAb is the

specific activity of leucine bound into protein after time T (days), and SAi is the acid-soluble intracellular leucine-specific radioactivity which falls slowly and linearly with time.[3] Protein synthesis (PS) is calculated by multiplying Ks by the tissue nitrogen mass at sacrifice and is expressed as g of protein per day. The fractional degradation rate (Kd) of tumor protein is calculated as the difference between Ks and Kg. Labelling index (LI), relative movement (RM) and G_1 phase increase (G_1I) of the tumor cells were calculated from analysis of cell-cycle kinetics. LI is the fraction of tumor cells synthesizing DNA. RM is the movement of these S-phase cells relative to the positions of G_1 and G_2 to estimate DNA synthesis time. G_1I reflects the increase of tumor cells undergoing the cell-cycle.[7] All data are represented as mean ± standard error of the mean. The student's *t* test was used for comparison between TNF and control groups.

RESULTS

Alteration of Tumor Volume

Alteration of tumor volume in Walker 256 carcinosarcoma-bearing rats after TNF treatment was shown in Figure 1. Tumor growth became slowly, and tumor volume was smaller from day 4 of postinoculation of the tumor cells in TNF group, compared to control group. The differences of tumor volumes between these two groups were statistically significant from day 6 ($P<0.01$). The tumor weight of TNF group (4.3±1.0 g) was significantly lower than that of control group (15.9±3.2 g, $P<0.05$) on day 14.

Change of Tumor Protein Metabolism

Change of tumor protein metabolism after TNF treatment was revealed that both tumor growth rate and protein synthesis declined remarkably while tumor protein degradation showed no significant change after i.p. injection of TNF (Table 1).

Flow Cytometry of Tumor Cell

Flow cytometry of tumor cells showed significant decrease of LI and G_1I, 6 h after BrdU injection, in TNF group, indicating that the number of tumor cells entering DNA synthesis period significantly declined (Table 2).

Table 1. Alterations of protein metabolism in Walker 256 carcinosarcoma with TNF treatment

Groups	TNF	Control
Ks (%/d)	25.3± 3.5*	42.9± 2.7
PS (g/d)	0.20± 0.05**	1.14± 0.20
Kg (%/d)	24.3± 2.1*	35.8± 2.3
Kd (%/d)	1.0± 2.8	7.1± 2.9

* $P<0.01$, ** $P<0.001$ versus control.

Table 2. Cell cycle kinetics of Walker 256 carcinosarcoma with TNF treatment

	Hrs. after BrdU injection	TNF group	Control group
LI (%)	1	28.7±2.0	32.4±2.2
	6	30.8±4.2*	44.7±4.2
RM (%)	1	75.3±2.8	78.2±1.6
	6	85.8±1.6	88.2±3.0
G_1I	1	5.5±0.6	5.4±0.3
	6	6.0±1.3*	12.2±2.5

* $P<0.01$ versus control.

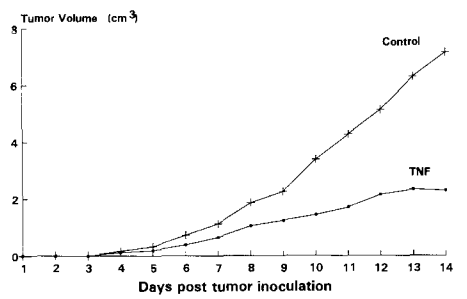

Fig. 1. Changes of tumor volume of walker 256 carcinosarcoma with TNF treatment

DISCUSSION

Diverse effects of TNF on tumor and the host were reported with different doses as well as administration ways. TNF can cause cachexia when subtotal doses are administered continuously but not

with bolus injection,[8] while repeated administration of TNF at low dose induced an attenuation of tumor growth.[9] The present study showed that i.p. injection of high dose of recombinant human TNF for continuous 3 days in tumor-bearing rats resulted in antitumor response of slow tumor growth lasting for 10 days after TNF injection.

The *in vivo* anticancer effects of TNF are associated with its direct cytotoxicity as well as diverse host responses. A short-term i.v. continuous infusion of low-dose of TNF caused a decrease in tumor protein synthesis and an increase in tumor protein breakdown in Yoshida sarcoma-bearing rats,[10] whereas administration of single dose of TNF seemed to increase tumor protein breakdown rather than to increase protein synthesis in Walker 256 carcinosarcoma.[11] The results of this study showed that the influence of high-dose of TNF on tumor protein metabolism were mainly significant decrease in protein synthesis, causing an inhibition of tumor growth, but no significant change of degradation.

In vitro study suggested that TNF is relatively cell-cycle specific and inhibits cells primarily during the G_2 period of the cell-cycle.[12] This study indicated that *in vivo* mechanism of TNF may also be inhibition of G_2 period of the cell-cycle, causing decrease in DNA synthesis of tumor cells, thus influencing tumor protein synthesis and resulting in abrogation of tumor growth.

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