Hiroshima J. Med. Sci.
Vol.38, No.3, 103~107, September, 1989
HIJM 38-17

Antitumor Effect of Recombinant Human Lymphotoxin on a Tumor Line of Human Malignant Glioma

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ABSTRACT

We examined the antitumor effect of recombinant human lymphotoxin (rHuLT) on a xenotransplantable human malignant glioma line. Tumor-bearing nude mice were treated with rHuLT for three weeks following four schedules: intratumoral injection of rHuLT 20,000 units once a week, twice a week, intravenous injection once a week and twice a week. The inhibition rate of tumor growth was 98.8%, 99.1%, 92.1% and 98.8%, respectively. Histologically, necrotic lesions were observed in the tumors of all treated mice. Thrombo-obstructive changes of tumor vessels were also seen in the tumors of mice after intravenous injection of rHuLT. None of the mice died as a result of this treatment in spite of significant body weight loss. These results indicate that rHuLT has a strong antitumor effect on a xenotransplantable human malignant glioma line.

Key words: *Lymphotoxin, Tumor necrosis factor, Glioma, Xenotransplantable tumor*

Lymphotoxin (LT), one of the lymphokines secreted by activated lymphocytes, is known to have cytotoxicity for a variety of tumor cells[5,8,12]. Following the development of molecular biology, it has become possible to obtain large amounts of recombinant human lymphotoxin (rHuLT)[1,9,11]. The antitumor effects and antitumor mechanisms of human LT have been variously reported[4,10,14,16,21,22], but its effect on human brain tumor has not yet been estimated. In this paper, the antitumor effect of rHuLT on a tumor line of human malignant glioma transplanted subcutaneously into nude mice is reported, and antitumor mechanisms *in vivo* are discussed.

MATERIALS AND METHODS

Tumor

The 12th passage of human malignant glioma maintained subcutaneously in BALB/c nu/nu nude mice in our institute was used.

Experimental animal

The animals used in this series were six-week-old BALB/c nu/nu female nude mice, 16—18g each, purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan.

Reagent

Recombinant human lymphotoxin (rHuLT), produced by transformed Chinese hamster ovary cells, was supplied by Kanegafuchi Chemical Ind. Co., Hyogo, Japan. The purified rHuLT is a glycoprotein and composed of subunits with a molecular weight of 21,000—26,000[11]. The titer of rHuLT is expressed as a house unit defined accordingly by cytotoxicity against L929 cells. Seven house units are equal to one Japanese reference unit (JRU).

Transplantation

Some blocks of tumor, 2—3mm in diameter, were transplanted subcutaneously into nude mice by means of a trocar.

Measurement of tumor size

The shortest diameter (a, mm) and the longest diameter (b, mm) were measured by means of a slide caliper twice a week. Relative tumor weight (RW, mg) was calculated by using the following formula according to the method of Battelle Columbus Laboratories. RW (mg) $=a^2 \times b/2$.

Treatment

Treatment was initiated when tumor size reached about 10—12mm in longest diameter. Tumor-bearing mice were treated with rHuLT for three weeks according to the following schedules: intratumoral injection of rHuLT 20,000 units once a week (it1) and twice a week (it2), intravenous injection of rHuLT 20,000 units once a week (iv1) and twice a week (iv2). Each treated group consisted of five mice. Six mice in the control group were treated with intratumoral injection of solvent twice a week.

Evaluation of antitumor effect

The antitumor effect was estimated from the inhibition rate of tumor growth at the end of the treatment.

Table 1. Antitumor effect of rHuLT in glioma-bearing nude mice

Group[a)]	No. of mice	% of initial tumor weight	T/C[b)]	Tumor growth inhibition rate (%)
control	6	1164.7 ± 110.8[c)]	1.000	
it1	5	13.8 ± 5.4**	0.012	98.8
it2	5	10.0 ± 2.6**	0.009	99.1
iv1	5	92.2 ± 23.5*	0.079	92.1
iv2	5	13.6 ± 6.1**	0.012	98.8

a) it1, intratumoral injection of rHuLT 20,000 units once a week; it2, twice a week; iv1, intravenous injection once a week; iv2, twice a week.
b) Comparison of the relative tumor weight between treated and control group.
c) Mean ± SE.
Statistically significant by Student's t-test as compared with that of control group (*$p<0.05$, **$p<0.01$).

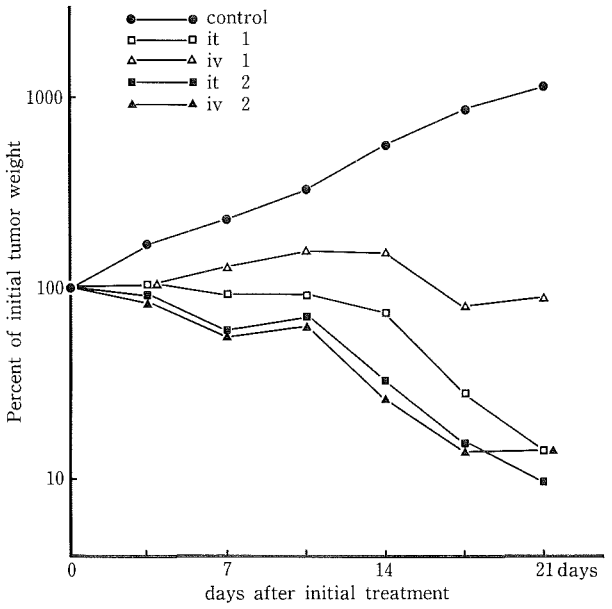

Fig. 1. Antitumor effect of rHuLT in glioma-bearing nude mice. it1, intratumoral injection of rHuLT 20,000 units once a week; it2, twice a week; iv1, intravenous injection once a week; iv2, twice a week.

Table 2. Induction of necrosis by rHuLT in glioma-bearing nude mice

Group[a)]	Macroscopic necrosis	Necrosis index (%)[b)]	Microscopic necrosis
Control	0/6[c)]	0	0/6[c)]
it1	5/5	83.1	5/5
it2	5/5	80.6	5/5
iv1	5/5	15.4	5/5
iv2	5/5	70.7	5/5

a) it1, intratumoral injection of rHuLT 20,000 units once a week; it2, twice a week; iv1, intravenous injection once a week; iv2, twice a week.
b) (Relative macroscopic necrosis weight/relative tumor weight) ×100.
c) Number of mice with necrosis/number of mice treated.

Histological examination

All tumors were studied histologically after treatment.

RESULTS

Antitumor effect

As shown in Fig. 1, strong antitumor effects were observed in treated mice. The inhibition rate of it1, it2, iv1 and iv2 was 98.8%, 99.1%, 92.1% and 98.8% respectively (Table 1). Complete regression was observed in some cases treated in it1, it2 and iv2. The T/C ratio of it1, it2, iv2 and iv2 was 0.012, 0.009, 0.079 and 0.012 respectively. In all four groups, rHuLT showed significant inhibitory effects in comparison with that in control group (it1, it2, iv2: $p<0.01$, iv1: $p<0.05$). There were no significant differences in antitumor effects among these four groups.

Histological examination

Macroscopical necroses of the tumors with crusts were observed in all treated mice. Necrosis index of it1, it2, iv1 and iv2 were 0.831, 0.806, 0.154 and 0.707, respectively (Table 2).

Microscopically, this tumor in the control group (Fig. 2A) was highly cellular, and the tumor cells showed pleomorphism with irregular hyperchromatic nuclei. Some mitotic figures were also seen. Immunostaining for glial fibrillary acidic protein was positive (not shown in figures). Necrosis or thrombosis were not seen in the control group. In the it group (Fig. 2B), massive necrosis with nuclear debris could be seen in the central area of the tumor. Moreover, thrombotic and obstructive changes of tumor vessels were observed around the necrotic area in the iv group (Fig. 2C). Infiltration of neutrophils and mononuclear cells were observed around the necrosis in the it and iv groups.

Side effect

Ten percent loss of body weight was observed for two weeks after the injection of rHuLT (Fig. 3). None of the mice died from this series of treatment.

DISCUSSION

The term 'lymphotoxin (LT)' was first introduced by Granger et al[7)] in 1968 to denote the soluble product of antigen- or mitogen-stimulated lymphocytes. Since then, studies from a number of laboratories indicated that LT specifically inhibited tumor

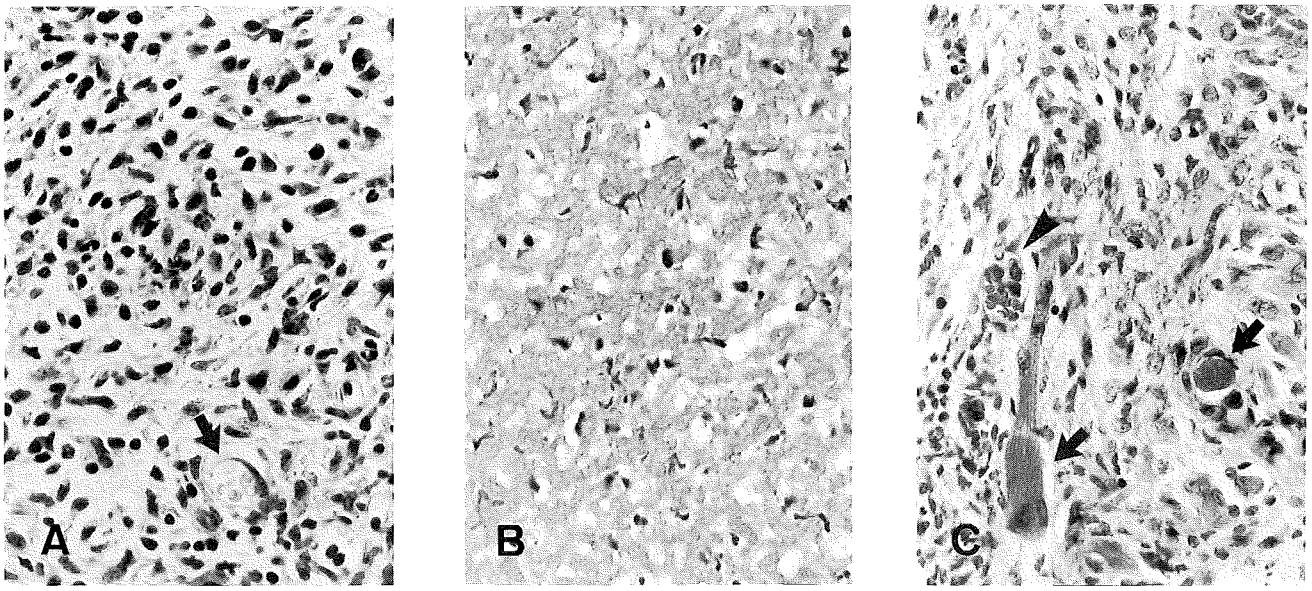

Fig. 2. Photomicrographs of the tumors in the control group (A), it group (B) and iv group (C). The tumor in the control group (A) is highly cellular, and the tumor cells show pleomorphism with irregular hyperchromatic nuclei. The lumen of tumor vessel (arrow) is not thrombosed, and necrosis is not seen. In the central area of the tumor in the it group (B), massive necrosis with nuclear debris is evident. In the iv group (C), lumens of tumor vessels are thrombosed by a coagulum of fibrin (arrow) and erythrocytes (arrow head). HE stain, ×400.

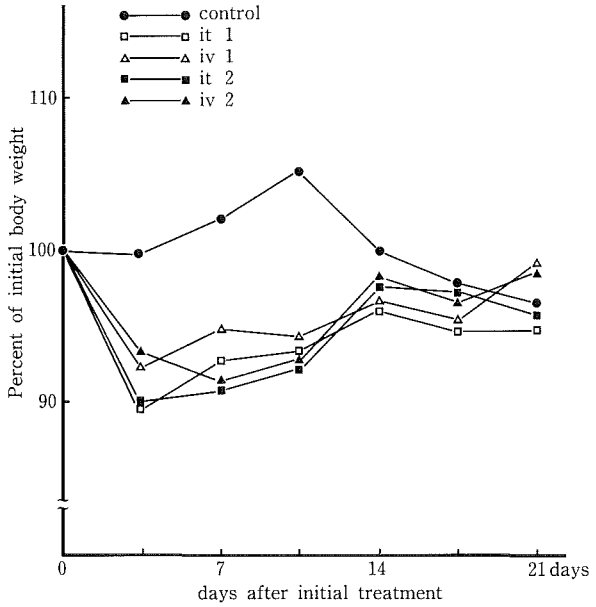

Fig. 3. Changes of body weight during rHuLT administration.
it1, intratumoral injection of rHuLT 20,000 units once a week; it2, twice a week; iv1, intravenous injection once a week; iv2, twice a week.

cell growth *in vitro*[5,8,12] and *in vivo*[9,16]. Most of these biological studies have been carried out with relatively crude LT preparations, so progress has been limited primarily because of the small amount of active material.

In 1984, Gray et al[9] reported the cloning and expression of DNA sequences encoding human LT, and succeeded in producing of recombinant human LT (rHuLT) from *Escherichia coli*. Furthermore, Kakutani et al[11] reported the purification of rHuLT produced by transformed Chinese hamster ovary cells in 1987. Thus, it has now become possible for us to obtain a large amount of highly purified LT by recombinant DNA technology.

Pennica et al[17] have indicated that tumor necrosis factor (TNF) has about 30% homology in its amino acid sequence with LT. In studies *in vitro*, in addition to their direct cytotoxicity, TNF and LT have a similar and wide range of biological activities among these: expression of class I MHC antigens[13], induction of interleukin-1 production[2], inhibition of viral replication (interferon-like activity)[23], stimulation of fibroblasts[3,25], inhibition of angiogenesis[21], injury to endothelial cells[18], effect on vascular smooth muscle cells[22], stimulation of the adherence of neutrophils to endothelium[6], enhancement on coagulant properties of endothelial cell[15], activation of tumoricidal macrophages[4], chemotaxis of monocytes and polymorphonuclear leukocytes[14], stimulation of T cell proliferation[19].

Concerning rHuLT or rHuTNF, some studies have been reported biological activity *in vivo*[20,24]. However, the antitumor mechanism is still unknown. Palladino et al[16] examined the antitumor activities of rHuLT *in vivo*, and observed thrombosed tumor vessels in microscopic examinations. Higuchi et al[10] reported the effects of rHuLT on macrophages *in vivo* and *in vitro*, and emphasized that rHuLT could induce the migration of macrophages into the tumor and activate macrophages *in situ* to kill tumor cells.

In our histological examination, massive necrosis

was seen in the central area of the tumor in the it group. This necrosis was observed macroscopically two or three days after initial treatment. Although we have not examined the antitumor effect of rHuLT on this tumor *in vitro*, it was considered that the main antitumor mechanism in the it group might be direct citotoxicity. On the other hand, many thrombotic and obstructive changes of tumor vessels were seen around the necrotic lesions in the iv group. This evidence strongly suggests that in addition to the direct cytotoxicity, thrombo-obstructive changes of tumor vessels played an important role in antitumor effect and induction of necrosis *in vivo*. We also observed neutrophils and mononuclear cells infiltrating around the necrosis, the functions of which remain obscure.

In this study, rHuLT was shown to have significant antitumor effects. There was no difference in these effects between the various administration, presumably because of high dose administration of rHuLT. LD_{50} of this rHuLT with one shot intravenous administration was 5×10^5—1×10^6 units/nude mouse in our preliminary study. Although the dose of rHuLT in this study was less than one tenth of LD_{50}, the obvious side effect was a decrease in body weights in all treated mice. Fortunately, none of the mice died as a result from these side effects. Further studies will be necessary to ascertain the optimal dose of rHuLT.

It was concluded that rHuLT had strong antitumor effect on a tumor line of human malignant glioma transplanted subcutaneously into nude mice. In addition to the direct cytotoxicity, histological evaluation suggested that thrombo-obstructive changes of tumor vessels played an important part in antitumor effect of rHuLT *in vivo*. Although more investigations is needed, we expect that LT will be used as a new agent against malignant brain tumors in the future.

ACKNOWLEDGMENTS

We thank Drs. K. Watanabe, T. Kakutani and T. Kajikawa (Biochemical Res. Lab., Kanegafuchi Chemical Ind. Co.) for the supply of rHuLT and for their kind advice regarding this study.

(Received March 1, 1989)
(Accepted May 29, 1989)

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