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"Two-route chemotherapy" using high-dose intra-arterial neocarzinostatin and systemic tiopronin, its antidote, for rat limb tumor*

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Summary. We studied the effect of "two-route chemotherapy" (TRC) with intra-arterial (IA) neocarzinostatin (NCS) and IV *N*-(2-mercaptopropionyl)-glycine (tiopronin), its antidote, on rat limb tumors. Chemotherapy experiments were carried out on day 9 after the inoculation of 10^6 syngeneic transitional carcinoma cells into the hind limb in female Wistar King A rats. In the group given TRC, 3500 units/kg NCS and 800 mg/kg tiopronin were given via the femoral artery and the femoral vein, respectively. The antitumor effect was evaluated by the tumor weight on day 12 after the treatment. Compared with the weight of tumors in untreated controls, TRC reduced tumor weight to one-tenth, while 700 units/kg IA NCS alone reduced tumor weight to one-third and 700 units/kg systemic NCS alone reduced tumor weight to three-fourths of the control weight. In the group given TRC, WBC and nucleated bone marrow cells were completely protected and loss of body weight was slight.

Introduction

Intra-arterial (IA) infusion of an anticancer drug is often superior to systemic administration, since a high concentration of the drug can be maintained at the tumor site. Though IA infusion has been widely prescribed since it was reported by Klopp et al. [14], the dose that can be given is limited because of unavoidable side effects after the drug enters the systemic circulation.

We have devised a method of combination chemotherapy, termed "two-route chemotherapy" (TRC), in which an anticancer drug and its antidote are given respectively, locally at the tumor site and systemically. This therapy can reduce the side effects of anticancer drugs, and we used it to investigate the effectiveness of anticancer drugs given in elevated doses. The first trial, with a combination of melphalan (L-phenylalanine mustard) or L-phenylalanine mustard *N*-oxide and its antidote cysteine, led to an improvement in the therapeutic efficacy of IA chemotherapy for rat liver tumors [1]. These drugs were given via the hepatic artery and the tail vein, respectively. The next series of studies with a combination of *cis*-diamminedichloroplatinum (II) (DDP) and sodium thiosulfate (STS) also produced remarkable antitumor effects in peritoneal dissemination, bladder tumors, and liver and lung metastases in laboratory animals [11, 12, 19, 21, 22]. The drug combination was determined on the basis of evidence that STS inactivates the toxicity of DDP [6, 9, 13]. TRC with DDP and STS is now under clinical trial [7, 15]. In the present study, we selected a new combination of NCS and tiopronin, since the latter has been reported to have a potent antagonizing activity against NCS [4, 10]. We found that treatment of rat limb tumor with TRC using NCS and tiopronin led to an antitumor effect superior to treatment with NCS alone and provided some protection against the side effects of NCS.

Materials and methods

Animals. ddY Female mice (Kyudo Co., Ltd, Fukuoka, Japan) weighing 23–30 g at 7 weeks of age were used for in vivo toxicity tests. Wistar King A (WKA) female rats (Animal Center of Kyushu University) weighing 156–210 g at 8 weeks of age and used for chemotherapy experiments were maintained on a standard laboratory diet and tapwater ad libitum.

Test drugs. Original NCS solution (liquid form, provided by Kayaku Antibiotic Research Co., Ltd, Tokyo, Japan) was diluted to the desired concentration with 0.9% NaCl solution before use. Original tiopronin solution (liquid form, 50 mg/ml, provided by Santen Pharmaceutical Co., Ltd, Tokyo, Japan) was used undiluted.

Lethal toxicity tests. Various doses of NCS were given IV immediately after IV administration of tiopronin (800 mg/kg) or saline, and LD_{50} values were determined by Probit analysis [3, 16] 30 days after the drug administration.

Chemotherapy experiments. A transitional cell carcinoma (RBT-1) [11, 16] induced in WKA rats with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosoamine has been maintained by serial transplantation into the hind limb of syngeneic WKA rats. The tumor was excised, minced, suspended in

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Abbreviations used: NCS, Neocarzinostatin; TRC, two-route chemotherapy

phsophate-buffered saline and passed through a metal sieve to obtain a single-cell suspension. After a trypan blue exclusion test, 10^6 viable cells per rat were inoculated IM into the left hind limb of each WKA rat.

On day 9 after the tumor inoculation, at which time each tumor was as large as the tip of the little finger, rats bearing tumors were separated at random into six groups for chemotherapy experiments. Rats anesthetized with ether were treated under an operation microscope (Konan Camera, R&I, K-280, Nishinomiya, Japan) as follows: In the group given IA (femoral artery) infusions of NCS, the NCS solution was administered in a volume of 0.35 ml/-100 g body weight; an infusion pump (Model 975E, Harvard Apparatus, Boston, USA) was used to give a flowrate of approximately 0.9 ml/min through a hypodermic needle; after IA NCS infusion the catheter was removed and the femoral artery was ligated to avoid bleeding; tiopronin or saline in a volume of 1.6 ml/100 g body weight was given, simultaneously with the initiation of NCS infusion, into the right femoral vein at a flowrate of approximately 4.2 ml/min. In the group given TRC, 3500 U/kg NCS ($3.1 \times LD_{50}$ of IV NCS in rats [15]) and 800 mg/kg IV tiopronin were given. Controls for TRC received IA and IV administration of 700 U/kg NCS, without tiopronin, and femoral artery ligation. NCS was given IV via the tail vein in a volume of 1.95 ml/100 g body weight.

Evaluation of antitumor effects. Antitumor effects were evaluated by regression of the tumor growth. All rats were killed 12 days after the treatment and the tumors were enucleated and weighed.

Evaluation of side effects. The tumor-bearing rats were weighed on various days after the treatments. To ascertain any hematologic disorders, rats without tumor were killed on day 3 after administration of the drugs and bled by decapitation for the determination of WBC count. The blood was then added to a known amount of Isoton, and following the addition of six drops of Zap-Oglobin II, the cell number was determined with a Coulter counter. Nucleated bone marrow cells from the femur were obtained by flushing approximately 3 ml phosphate-buffered saline though the end of the femur. This cell suspension was diluted 1 : 20 with Turk's solution and the number of nucleated cells per femur was determined with a hemocytometer [2].

Results

As shown in Fig. 1, the LD_{50} of IV NCS was 2566 units/kg when the drug was given with IV saline, whereas it was increased to 67896 units/kg when the drug was given with IV tiopronin. The results indicate that tiopronin provides protection against lethal toxicity of NCS in vivo.

Antitumor effects of various treatments on the growth of transitional cell carcinoma (RBT-1) in rat limb are summarized in Table 1. The average tumor weight was lowest in rats given TRC with NCS and tiopronin, and in these animals it was significantly lower than in rats given IA or IV infusions of NCS without tiopronin. In the group given TRC none of the rats died, even when they were given a large dose of NCS ($3.1 \times LD_{50}$ of IV NCS in rats [17], and necrosis in the treated limb never occurred. Although the tumor weight in animals that received intra-arterial infusion of 700 units/kg NCS was one-third that of the untreated contols ($P < 0.05$), there were no statistically significant differences in tumor weight among the groups given IV infusions of 700 units/kg NCS, femoral artery ligation alone, and no treatment (controls).

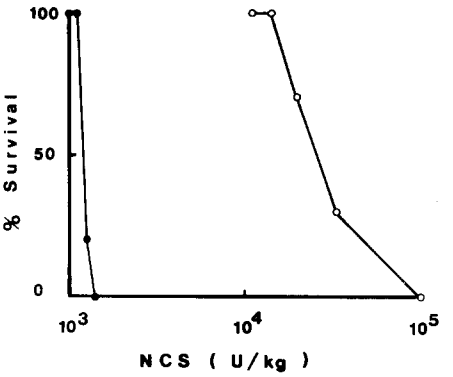

Fig. 1. Toxicity of NCS IV in mice also given 800 mg/kg tiopronin IV. ●, NCS+Saline; ○, NCS+tiopronin. Each *point* indicates percentage survival among ten mice 30 days after treatments

WBC and nucleated bone marrow cells were measured as indexes of hematologic disorders, a major dose-limiting factor of NCS [17, 20] (Table 2). In the group given TRC there were no decreases in either WBC or the nucleated bone marrow cells. In contrast, IA or IV infusion of 700 units/kg NCS alone produced a significant decrease both in WBC and nucleated bone marrow cells.

Figure 2 shows changes in average body weights of tumor-bearing rats after various treatments. Among all the treatments, IA administration of 3500 units/kg NCS without tiopronin produced the most severe loss of body weight, and all rats died of toxicity within 4 days after the treatment (Fig. 2). A dosage of 700 units/kg NCS was the limit for safety in single NCS treatments since all rats receiving 1000 units/kg NCS alone IV and four of five rats given receiving 1000 units/kg NCS alone IA died with severe loss of body weight (data not shown). In contrast, in the group given TRC using intra-arterial 3500 U/kg of NCS with tiopronin, there was only a slight loss of body weight and the degree was less than in the group given NCS 700 units/kg IV.

Table 1. Effects of treatments on the growth of RBT-1 in the rat limb

Treatment[a]	No. of rats	Mean tumor weight g±SEM	*P* value[c]
Untreated control	7	25.9±3.1	
NCS 3500 units/kg IA+ tiopronin 800 mg/kg IV	7	2.7±0.8	<0.05*
NCS 3500 units/kg IA+ saline IV	5	All rats died of toxicity	
NCS 700 units/kg IA+ saline IV	7	7.9±3.0	<0.05**
NCS 700 units/kg IV	6[b]	19.7±2.0	NS
Femoral artery ligation	5	17.4±4.2	NS

[a] Treatments were given on day 9 after inoculation of 10^6 viable RBT-1 cells into the hind limb
[b] One of seven rats died of toxicity
[c] Mann-Whitney test: * VS; ** $P < 0.05$; NS, not significant

Table 2. Changes in WBC and nucleated bone marrow cells 3 days after NCS treatments, with or without tiopronin

Treatment[a]	No. of rats	WBC (mean ± SEM)	*P* value[b]	Nucleated bone marrow cells per femur (X10⁴) (mean ± SEM)	*P* value[b]
Untreated control	5	6211 ± 426		1032 ± 462	
NCS 3500 units/kg IA + tiopronin 800 mg/kg IV	5	6753 ± 544	NS	1032 ± 461	NS
NCS 700 units/kg IA + saline IV	5	3471 ± 863	< 0.05	412 ± 184	< 0.05
NCS 700 units/kg IV	5	2670 ± 379	< 0.05	372 ± 166	< 0.05

[a] Rats without tumor were treated. All rats were killed 3 days after the treatment
[b] Probability was evaluated by Mann-Whitney test; NS, not significant

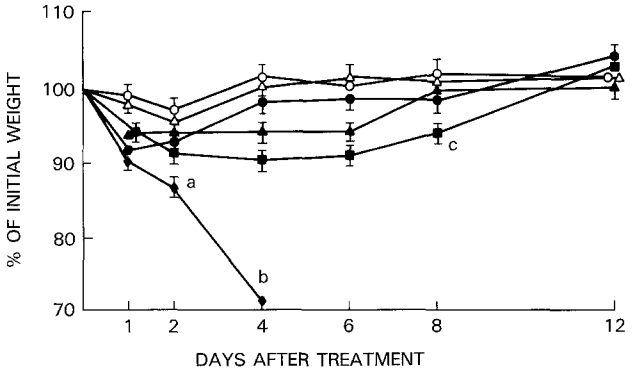

Fig. 2. Changes in average body weight of rats after NCS treatments with or without tiopronin. *Bar* represents SE of the mean. ○, untreated control; ●, 3500 units/kg NCS by IA route + 800 mg/kg tiopronin by IV route (TRC); ◆, 3500 units/kg NCS by IA route + saline IV (a = 2 of 5 rats died of toxicity, b = 3 of 5 rats died of toxicity). ▲, 700 units/kg NCS by IA route + saline IV; ■, 700 units/kg NCS by IV route (c, 1 of 7 rats died of toxicity); △, femoral artery ligation alone

Discussion

NCS is an antitumor antibiotic with activity against leukemia and against bladder, liver, stomach, and pancreas cancers [17]. However, the therapeutic usefulness of this drug is often limited, as it has severe bone marrow toxicity [17, 20].

The present study revealed that TRC with a combination of high-dose IA NCS and IV tiopronin, the antidote to NCS, produced a remarkable therapeutic effect on the growth of rat limb tumor without bone marrow toxicity. In this therapy, tiopronin reduced the adverse effects of NCS, and the dose of NCS could then be increased. Although tiopronin has been used clinically to treat mercury poisoning and hepatic disorders [5, 8], the safety of tiopronin administration in high doses has not been ascertained. When 800 mg/kg tiopronin was given alone IV in a volume of 3.2 ml to rats without tumors ($n = 5$), loss of body weight and toxicity-related deaths were not observed (unpublished results). It is likely that a higher dose of tiopronin would provide even greater protection against NCS toxicity [10], but we were unable to administer a higher dose because we felt it would be unphysiological for rats if the injection volume were increased above 3.2 ml. The dose of 800 mg/kg tiopronin in 3.2 ml was therefore used in the chemotherapy experiment, and NCS-induced bone marrow toxicity and loss of body weight were consequently avoided. Although the mode of interaction occurring between NCS and tiopronin remains uncertain, we speculate that the rat limb tumor cells can be attacked directly by a high concentration of NCS administered IA, while the drug entering the bloodstream from the tumor area is neutralized by the tiopronin administered systemically.

It has been reported that the nonprotein chromophore of NCS has DNA-degrading activity comparable to that of the native NCS and that the activity of nonprotein chromophore is lost immediately when the chromophore is dissociated from its stabilizer, apoprotein of NCS [18]. It may be speculated that the scission of disulfide bonds of apoprotein by tiopronin, a sulfhydryl compound, may cause the dissociation of chromophore from apoprotein and chromophore may therefore be inactivated.

The TRC presented here using a new combination of NCS by the IA route and IV tiopronin, may be applicable to regionally confined malignancies seen clinically. In addition, this TRC may be useful for cancers which are resistant to TRC with DDP and STS, since the spectrum of antitumor activity of NCS differs from that of DDP.

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