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Potentiation of Antitumor Effect of Aclarubicin on Rat Hepatoma Model by Hepatic Arterially Administered Oily Dosage Forms

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The antitumor effect of aclarubicin (ACR) in an oily dosage formulation composed of Lipiodol (lymphographic oil), or with linoleic acid and 1-dodecylazacycloheptan-2-one (Azone), was investigated by hepatic intra-arterial administration against Walker 256 carcinosarcoma implanted in the rat liver. Evaluation based on the growth rate of the tumor indicated that ACR was more effective on administration as Lipiodol solution or oil-in-water emulsion compared to saline solution, but the addition of linoleic acid or Azone to Lipiodol solution did not further improve the antitumor activity of ACR. A clear correlation between the depression of the hepatic tumor growth rate and the levels of ACR and its active metabolites in the tumor tissue 24 h after the hepatic intra-arterial administration was also observed.

Keywords——aclarubicin; potentiated antitumor effect; hepatic intra-arterial administration; oily dosage form; Lipiodol; Walker 256 carcinosarcoma; rat hepatoma model

In cancer chemotherapy, recent efforts have been directed towards enhancing the selective delivery of anticancer drugs to a target organ, and arterial administration of drugs to the tumor sites *via* a catheter has been used as a surgical method for this purpose.[1] Since the drugs are usually not retained in the arteries of tumor tissue for a long time when arterially administered as a plain aqueous solution, combined use with an embolic substance has been suggested to potentiate the therapeutic activity.[2-4] The encapsulation of a drug in alubumin microspheres[5,6] or poly-lactic acid microspheres[7] was demonstrated to be effective as an arterial embolus and a reservoir of the drugs. Drugs dissolved in lymphographic oils have been shown to be capable of embolization in the tumor-feeding arteries and selective accumulation in the tumor tissues.[8,9]

On the other hand, we have reported that fusogenic lipids improved the bioavailability of poorly absorbable drugs from the gastrointestinal tract,[10-12] and also that fusogenic lipids such as linoleic acid (LA) potentiated the antitumor effect of bleomycin by increasing the uptake of bleomycin into the tumor cells.[13] 1-Dodecylazacycloheptan-2-one (Azone), a transdermal absorption enhancer,[14] had a similar effect on the absorption from the alimentary canal.[15] Therefore, the use of these lipid-soluble absorption enhancers could be useful in cancer chemotherapy from the viewpoint of modification of tumor cell membrane permeability.

In this paper, we describe the potentiation of the antitumor activity of aclarubicin (ACR), a lipid-soluble drug, against Walker 256 carcinosarcoma as a rat hepatoma model. Dosage formulation was performed with lymphographic oil (Lipiodol), LA or Azone, and hepatic intra-arterial administration was employed.

Experimental

Materials——ACR was kindly supplied by Sanraku Inc. (Tokyo, Japan). LA, Azone and Lipiodol were

supplied by Nippon Oil & Fats Co. (Tokyo, Japan), Teikoku Pharmaceutical Co. (Kagawa, Japan) and Kodama Co. (Tokyo, Japan), respectively. All other chemicals and solvents were commercial products of reagent grade.

Preparation of Test Solutions——ACR–Lipiodol solution (ACR-LPD) was prepared by dissolving 1 mg of ACR in 50 mg of Lipiodol containing 5 mg of ethyl alcohol. LA or Azone (10 mg) was added to 56 mg of ACR-LPD (abbreviated as ACR-LPD (LA) or ACR-LPD (AZ), respectively). Emulsions of these oily solutions were prepared by emulsifying 56 mg of ACR-LPD or 66 mg of ACR-LPD (LA or AZ) and 0.2 ml of phosphate-buffered saline containing 20% (w/w) bovine serum albumin, followed by sonication at 50 w for 2 min with a sonicator (model 5202, Ohtake Works Co. Tokyo, Japan). These emulsions were abbreviated as ACR-EM, ACR-EM (LA) and ACR-EM (AZ), respectively. The 0.6% (w/w) aqueous solution of ACR (ACR-AQ) was prepared by dissolving ACR in saline solution.

Animal Experiments——Male Wistar rats (Shizuoka Laboratory Animal Center) weighing 280—300 g were used. Animals were not fasted and were anesthetized intraperitoneally with sodium pentobarbital (32 mg/kg) during laparotomy performed through a midline abdominal incision for the tumor cell implantation and for arterial injection of the test solutions.

Tumor Cell Implantation——Walker 256 carcinosarcoma was kindly supplied by Shionogi Pharmaceutical Co., Ltd. (Osaka, Japan). This carcinosarcoma was minced with ophthalmologic scissors, and the tumor cells were suspended in Hanks' balanced salt solution. A 50 μl aliquot of the suspension containing 5.0×10^7 cells was injected *via* a 26-gauge needle into the subcapsular parenchyma of the left lateral lobe of the rat liver.

Drug Administration——The abdomen of the rat was re-opened by a midline incision 4 d after tumor implantation and fixed with a retractor in order to expose the hepatic portal and the duodenum. Two pairs of arterial clamps were applied to the right gastroepiploic artery and the celiac artery. With the aid of magnification ($\times$ 10) by stereoscopic microscopy, each test solution was injected into the lumen of the cranial pancreaticoduodenal artery *via* a handmade glass needle (o.d., 0.2 mm) connected to vinyl tubing. The injected liquid flows into the hepatic artery *via* the cranial pancreaticoduodenal artery and the gastroduodenal artery. The injected position was tied and the arterial clamps were removed to restore the circulation of the hepatic artery.

Evaluation of Anticancer Effect——Three days after the tumor implantation, the size of each tumor was measured and a test solution (dose of ACR: 5 mg/kg) was injected once *via* the hepatic artery of the rat. A mock operation group was treated with saline solution. One week after the injections, rats were sacrificed and the size and weight of each tumor were measured. The tumor size was calculated as the product of the length of the major and the minor axes. Growth rate was calculated as follows: growth rate=(tumor size at autopsy−tumor size at treatment)/(tumor size at treatment).

Distribution of ACR in Rat Liver——ACR levels in the liver of rats implanted with tumor cells 7 d before were evaluated 3 and 24 h after the hepatic arterial injection of test solution. The blood was collected and the liver was excised, and tumor or normal tissue samples were analyzed for ACR and its metabolites.

Drug Assay——The reversed-phase high-performance liquid chromatographic method was applied to the determination of ACR and its metabolites by using a slight modification of the procedure described by Ogasawara *et al.*[16] The reversed-phase column (Cosmosil 5C18 packed column, 4.6 i.d. $\times$ 250 mm, Nakarai Chemicals Co., Kyoto, Japan) was eluted with acetonitrile–0.04 M ammonium formate-buffered solution (60:40, v/v, pH 5.0). The extraction procedure for the hepatic tissue was as follows: 0.5 g of the hepatic tissue was homogenized with a mixture of 0.5 ml of 0.25 N HCl, 0.01 ml of saturated solution of potassium salicylate and 0.5 ml of 0.1 M phosphate-buffered solution (pH 7.0). Next, the supernatant was obtained by the centrifugation of this homogenate for 10 min at $5000 \times g$; the extraction from the supernatant was performed by the same procedure as in the case of plasma after adding 23 μl of 1 N NaOH. ACR and its glycoside-type metabolites (MA144 Ml and -Nl) were measured by use of a standard method with fluorescence detector (model RF-530, Shimadzu Co., Kyoto, Japan) at the excitation wavelength of 435 nm and emission of 505 nm. Aclacinomycin B, which has not been detected among the mammalian metabolites of ACR, was supplied by Sanraku Inc., and was used as an internal standard. The standard preparations of metabolites (MA144 Ml and -Nl) were also supplied by Sanraku Inc.

Statistics——Experimental results were analyzed statistically by means of Student's *t*-test.

Results

Antitumor Activity

The individual tumor sizes and weights are illustrated in Fig. 1 and summarized in Table I. Experiment 1 was performed to evaluate the effectiveness of ACR-LPD and ACR-EM. The growth rate of the tumor in the ACR-AQ treated group was about two-fifths of that in the mock operation group, but the difference was not significant. The growth rate of the tumor in the ACR-LPD or ACR-EM treated group was about one-fifth of that in the mock operation group, and the difference was statistically significant ($p < 0.05$). The difference in the tumor

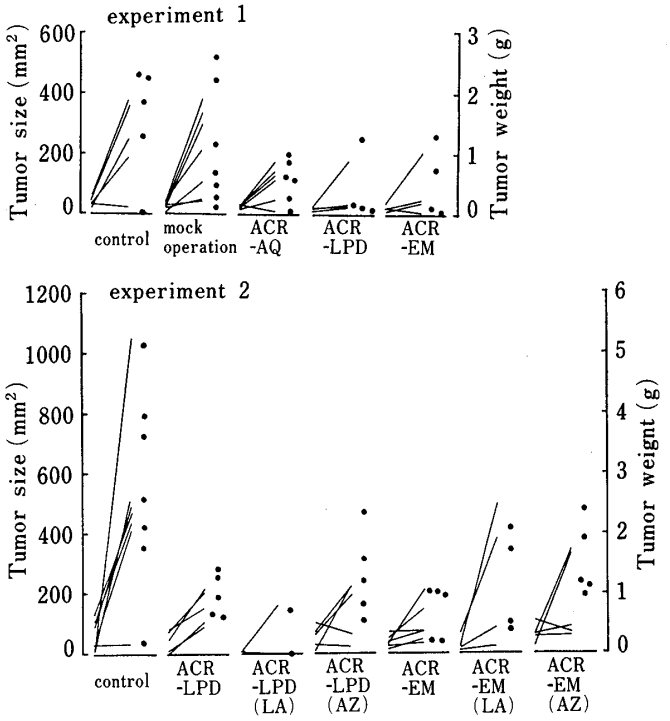

Fig. 1. Tumor Size and Weight of Walker 256 Carcinosarcoma Implanted in Rat Liver 7d after the Injection of Test Solution *via* the Hepatic Artery

Dots in each group indicate individual tumor weight data. The left and right ends of lines in each group are mean tumor sizes at treatment and autopsy, respectively.

TABLE I. Effect of Dosage Form on Antitumor Activity of ACR

Treatment	Growth rate[a,c]	% of control[b]	Tumor weight (g)[a]	% of control[b]
Experiment 1				
Control	22.7± 9.8 (5)	100	1.54±0.38 (5)	100
Mock operation	16.3± 4.9 (7)	71.8	1.07±0.37 (7)	69.5
ACR-AQ	6.6± 2.0 (6)	29.1	0.56±0.15 (6)	36.4
ACR-LPD	3.3± 1.2 (4)[d]	14.5	0.40±0.25 (4)	26.0
ACR-EM	2.8± 1.4 (4)[d]	12.3	0.54±0.26 (4)	35.1
Experiment 2				
Control	79.4±64.6 (7)	100	2.77±0.57 (7)	100
ACR-LPD	14.1± 7.9 (5)	17.8	0.98±0.14 (5)	35.4
ACR-LPD (LA)	18.1±13.5 (2)	22.8	0.36±0.26 (2)	13.1
ACR-LPD (AZ)	6.8± 5.9 (5)	8.6	1.28±0.31 (5)	46.2
ACR-EM	2.3± 0.8 (6)	2.9	0.64±0.15 (5)	23.1
ACR-EM (LA)	9.9± 5.2 (4)	12.5	1.19±0.42 (4)	43.0
ACR-EM (AZ)	6.2± 4.8 (5)	7.8	1.51±0.27 (5)	54.5

a) Each value represents the mean±S.E. of 2—7 experiments. *b*) Percentage with respect to the control group in each experiment. *c*) Value of growth rate was calculated as described in the experimental section. *d*) Statistically significant ($p<0.05$) as compared with the mock operation group. Figures in parentheses refer to the number of animals.

weight, however, was not obvious. Experiment 2 was performed to evaluate the effect of LA or Azone added to ACR-LPD or ACR-EM. However, there was no significant depressive effect of the growth rate of the tumor or on the tumor weight compared to the ACR-LPD or ACR-EM treated group.

Distribution of ACR in Liver

Figure 2 shows the levels of ACR and its antitumor-active metabolites (MA144Ml and -Nl) in the liver 3 and 24 h after the hepatic arterial injection of each test solution. The total concentrations of ACR and antitumor-active metabolites in the tumor tissue at 3 h differed

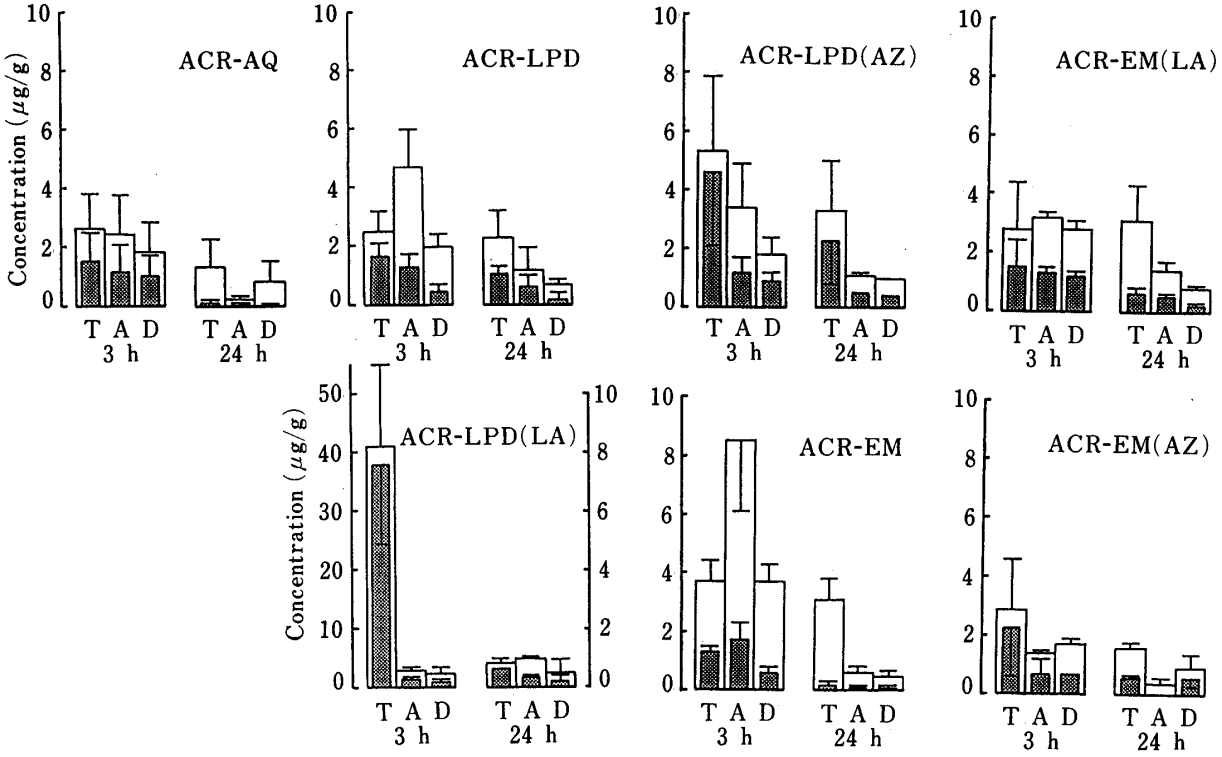

Fig. 2. Concentrations of ACR and Its Active Metabolites in Rat Liver Bearing Walker 256 Carcinosarcoma 3 and 24 h after Injection *via* the Hepatic Artery

Outer open columns show total concentrations of ACR and active metabolites. Inner dotted columns show concentration of ACR. T, tumor tissue; A, normal liver tissue adjacent to tumor; D, normal liver tissue distant from tumor. Results are expressed as the mean values ± S.E. of 3 experiments.

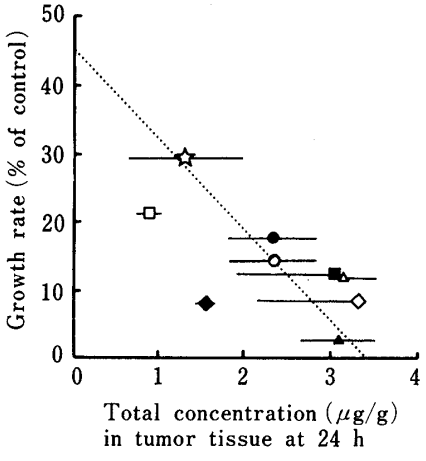

Fig. 3. Relationship between the Growth Rate of Tumor in the Liver (Percent of Control) and the Total Concentrations of ACR and Its Active Metabolites in the Tumor Tissue 24 h after Drug Administration *via* the Hepatic Artery

Correlation coefficient = −0.686 ($p < 0.05$). A dotted line indicates the regression line. ACR-AQ (☆), ACR-LPD (○) and ACR-EM (△) in experiment 1. ACR-LPD (●), ACR-LPD (LA) (□), ACR-LPD (AZ) (◇), ACR-EM (▲), ACR-EM (■) and ACR-EM (AZ) (◆) in experiment 2.

only slightly among the test preparations except ACR-LPD (LA). The total drug levels in the tumor tissue at 24 h after treatment with each only dosage form were higher than that with ACR-AQ. Figure 3 indicates the linear regression and correlation between the growth rate of the tumor relative to the control and the total drug level in the tumor tissue at 24 h. This correlation was statistically significant ($p < 0.05$), but no significant correlation of other combinations (growth rate-ACR level at 3 h in the tumor tissue, or growth rate-ACR level at 3 or 24 h in the normal liver tissue) was observed.

Discussion

The results of the therapeutic experiments in this work indicated that ACR-LPD or ACR-EM clearly improved the antitumor effect of ACR as compared with ACR-AQ (exp. 1 in Table I). The basic mechanism of the enhancement of the therapeutic effect of anticancer drugs by Lipiodol was explained by Iwai *et al.*[8] as follows: Lipiodol–anticancer drug is embolized selectively at the neovasculature in the tumor, and the drug is delivered slowly from Lipiodol to the tumor cells. However, although it is considered that Walker 256 carcinosarcoma forms neovasculature as it grows, the tumor may not form such neovasculature within the short experimental period used in this study.[17] Therefore, it is likely that a mechanism other than neovasculature embolization by oily dosage forms may be concerned in this enhanced therapeutic effect. The vasculature in the normal tissue around the tumor may be apt to be embolized by ACR-LPD or ACR-EM. The intra-arterial administration of ACR-LPD caused liver parenchyma to undergo necrosis by embolization, but that of ACR-EM did not cause necrosis as judged by microscopic observation (not shown). We think that not only embolization but also some other factors participates in the enhanced antitumor effect of ACR.

The therapeutic effect of ACR-LPD or ACR-EM could not be potentiated by addition of LA or Azone (exp. 2 in Table I). These additives are permeation enhancers for the absorption of poorly absorbable drugs, mainly *via* the enteral route,[10-12] but they do not usually accelerate the absorption of readily absorbable drugs. ACR, a lipid-soluble drug, is thought to permeate readily through the membrane of tumor cells, and was not considered to be greatly affected by these permeation enhancers. The metabolites of ACR include glycoside and aglycone types.[18] The MA144 Ml and -Nl, which were detected in the liver (Fig. 2), are glycoside type metabolites and it was reported that there is little difference of antitumor activity among ACR, MA144 Ml and -Nl.[18] A significant correlation between the total concentrations of drug in the tumor tissue at 24 h after administration *via* the hepatic artery and the growth rate of the tumor was observed (Fig. 3). There was, however, no significant correlation between the total levels of drug in the tumor tissue at 3 h and the growth rate of the tumor. Consequently, it is considered that the retention of ACR and its active metabolites in the tumor tissue for a longer period is important to depress the growth of the tumor. This result suggests that a sustained-release type dosage form of ACR would be more effective to suppress the tumor growth than ACR-AQ. The precise drug delivery mechanism of ACR-LPD or ACR-EM is now under study.

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