原 著

吉田肉腫に対する抗腫瘍薬と超音波の併用治療

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The Combination Treatment of Ultrasound and Antitumor

Drugs on Yoshida Sarcoma

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Summary

The antitumor effect ultrasound (ULS) and/or antitumor drugs such as daunomycin (daunorubicin, DNR) and adriamycin (ADM) were determined in the rats bearing Yoshida sarcoma. The ULS transducer that was placed on the tumor was cooled to keep the temperature of the tissue within the nonthermal range. For the choice of the timing of the ULS irradiation after the administration of the drug, the drug concentrations in the tumor and blood were determined and were analyzed pharmacokinetically.

The drugs or ULS showed antitumor effect judged by the growth of the tumor size or the survival time of the rat. The combination treatment of ULS and the drug marked an additional or synergistic effects on Yoshida sarcoma.

Key Words:

超音波効果	Ultrasound irradiation
非温熱効果	Nonthermal effect
アドリアマイシン	Adriamycin
ダウノマイシン	Daunomycin
吉田肉腫	Yoshida sarcoma

要 旨

超音波(ULS)単独又は、抗腫瘍薬 DNR と ADM との併用の抗腫瘍効果を吉田肉腫ラットで判定した。腫瘍に接触させたトランスジューサーは冷却し、組織の温度を高温域にならないようにした。ULS の薬物投与後の照射時間を決定するために血中と腫瘍中薬物濃度を測定し、薬物動力学的に解析した。腫瘍の大きさの成長とラットの生存期間から判断して薬物も ULS も抗腫瘍効果を示した。ULS と薬物の併用治療は、吉田肉腫に相加的又は相乗的な効果を示した。

INTRODUCTION

Ultrasound (ULS) is widely used in the diagnoses of various diseases and in the treatment of tumors.^{1,2)} The hyperthermia effects of ULS on tumors were expected, but recently the nonthermal effects of ULS on several tumors have been reported.³⁾ ULS is thought to have various physical and chemical effects in its nature of being an energy beam. ULS can raise the temperature, destroy materials even the tissues and cells, cause chemical reactions, and generate the oxygen radicals by cavitation.^{4,5,6)} It can be focused to predetermined position and can be converted to the energy, so it is reasonable to use ULS to activate the chemical compounds in the target spot. Some attempts have been made to potentiate the antitumor activities of drugs by ULS.⁷⁾ Akimoto reported the combined use of nonthermal ULS with the antitumor drug, Mitomycin C, to the rat AH 109 tumor and said the efficacy was synergistic.⁸⁾ In this study, the effect of ULS in combination with adriamycin (ADM) or daunomycin (daunorubicin, DNR) on Yoshida sarcoma were tested. These drugs have potent antitumor effects but are accompanied by inevitable adverse effects, and so it is desirable to use them in small doses.

The ULS is best applied to the tumor when the

drug concentration in the tumor is maximum. The time course of the drugs in the tumor and blood were determined to know when the maximum tumor concentration appeared. In the studies to estimate the treatment effect, the Donryu rats, inoculated Yoshida sarcoma subcutaneously, were exposed to ULS in the non-thermal range, in combination with the antitumor drugs. The antitumor effects were evaluated by observing the growth of tumor and the survival time of the tumor bearing rats.

MATERIALS AND METHODS

Materials

Daunomycin (daunorubicin, DNR) and adriamycin (ADM) were supplied by Meiji Seika Kaisha Ltd., Tokyo, Japan.

Animal studies

Male Donryu rats (8 to 10 weeks old) were given a pellet diet (CE-7, Kurea Japan Co. Ltd., Tokyo, Japan) and were allowed water *ad libitum*. Yoshida sarcoma were initially supplied by Meiji Seika and were maintained serially in our laboratory in ascites forms. Yoshida sarcoma cells suspended in physiological saline solution (5×10^6 cells / 0.5 ml) were inoculated subcutaneously on the left dorsal scapula region of the rat. When the tumor grew to about a 15 mm diameter 8 days after inoculation, the pharmacokinetic study, or the treatment study, was started.

The behavior of drugs in the rat

DNR or ADM were dissolved in sterilized saline solution and administered to the tumor bearing rat by intraperitoneal injection at a dose of 10 mg/kg of body weight. Blood samples were withdrawn from the femoral artery through the cannula to heparinized tubes. The rats were sacrificed by the depletion of blood at scheduled time. The tumors were then quickly

removed, excised, and rinsed with ice-cold saline. The samples were kept at -20°C until they were assayed. The assay was done within 24 hrs after freezing.

Analytical method

DNR or ADM in the physiological samples was determined by high performance liquid chromatography (HPLC) according to the method reported by Iguchi and Tone.⁹⁾ Blood (0.5 ml) was mixed with 1.5 ml 0.1 M NH_4Cl - NH_4OH buffer (pH 9). A portion of the tumor (0.3 g) was added to 1.5 ml of the same buffer and homogenized. The pH of the homogenate was adjusted to nine by the addition of 0.5 N NaOH solution, when necessary. The sample was then shaken with 6 ml chloroform / methanol mixture (2 : 1 v/v) for extraction. After centrifugation at 3000 g for 10 min, the chloroform layer was removed and the aqueous layers was shaken with 4 ml chloroform for the second extraction. Both the first and second chloroform layer were combined and evaporated to dryness in a water bath at 30°C . The residue was dissolved in an adequate volume of chloroform / methanol. Then a 1 ml methanol solution containing known amount of DNR was added for the internal standard of HPLC. The solution was re-evaporated and the residue was dissolved in 0.1–1.0 ml acetonitril / water (35 : 65 v/v). After centrifugation at 2500 g for 5 min, a 10–100 μl portion of the supernatant was injected into the HPLC (Hitachi 655, Hitachi, Co. Ltd., Tokyo, Japan). The column was μ -Bondapack phenyl (Waters Assoc., U.S.A.), the detector was the fluorescence monitor (Hitachi 650–10 LC, excitation 470 nm, emission 550 nm) and the mobile phase was acetonitrile / 0.035 M HCOOH - HCOONH_4 buffer of pH 3 (35 : 65 v/v).

Pharmacokinetic analysis

The behavior of the drugs after intraperitoneal administration was assumed to be described by a one compartment model with a first order transport rate as shown in Fig. 1 A, where k_a is the transport rate constant from the peritoneal cavity to the blood, k_{el} is the elimination rate constant, D is the dose, and V_d is the distribution volume. The blood concentrations of the drug ($B(t)$) are described by equation 1.

$$B(t) = [\exp(-k_{el} \cdot t) - \exp(-k_a \cdot t)] \cdot D \cdot k_a / V_d / (k_a - k_{el}) \quad \text{eq. 1}$$

Fig. 1 A) Pharmacokinetic Model for Intraperitoneal Administration

B) The Model for drug Transport between Blood and Tumor

The observed blood concentrations were fitted to equation 1 and the pharmacokinetic parameters k_{el} , k_a , and V_d were estimated by the nonlinear least squares method.

The tumor tissue is distinguished from the blood compartment as shown in Fig. 1 B, where V_t is the volume of the tumor, and k_1 , k_2 , and k_3 are the first order rate constants. The drug concentration in the tumor ($C(t)$) is given by the following convolution integral equation :

$C(t) = \int_0^t B(t-\theta) \cdot K_1 \cdot \exp(-K_2 \cdot \theta) d\theta$ eq. 2
 where $K_1 = k_1 \cdot V_d / V_t$ and $K_2 = k_2 + k_3$. The parameters of k_1 and k_2 were obtained by the numerical deconvolution and curve fitting to equation 2 using the nonlinear least squares method. The observed drug concentrations in the tumor and the blood concentrations simulated by equation 1 were put into $C(t)$ and $B(t)$ of equation 2, respectively.

ULS irradiation system

A schematic diagram of the ULS irradiation system was shown in Fig. 2. The piezoelectric ceramic polycrystalline transducer (25 mm diameter) with a copper shell and pipes through which water was circulated to keep the shell and tumor temperature below the hyperthermia level. The transducer was driven at 1.92 MHz by a multifrequency ULS generator (Hitachi, Japan).

Fig. 2 Schematic Diagram of the ULS Irradiation System

Treatment methods

The rats bearing a tumor on the right shoulder were divided into four groups of nine rats each as following; 1) Control, and treated with 2) Drug, 3) ULS, or 4) ULS + Drug. For the drug treatment, DNR or ADM were administered to the rat by intraperitoneal injection at a

dose of 1 mg/kg. For the ULS treatment, the rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The hair over the tumors was shaved and ULS gel was applied on the naked skin. The rats were fixed on a board with the tumor side up. The thermistor probe (Anritsu Keiki Co. Ltd., Tokyo, Japan) was inserted into the tumor to monitor the temperature. The ULS transducer was placed tightly on the tumor and ULS was irradiated for ten minutes at the intensity of 0.66 W/cm². The transducer was cooled by circulating water (25 °C) during the irradiation to keep the temperature of the tumor below 35 °C. For the combination treatment, ULS irradiation was started 120 min after the drug administration.

Evaluation of tumor size

The long and short diameters (a and b mm) of the tumor were measured daily using a slide caliper. The tumor size (mm) is calculated by $(a + b) / 2$.

Statistical analysis

The mean and standard error (S.E) was calculated for each group. The values were compared by the Student's t -test with $P < 0.05$ as the minimum level of significance. In the survival studies, the test of outlier was applied to detect and omit the extreme values from the analysis.

RESULTS

The time courses of blood concentrations after intraperitoneal administration of DNR and ADM are shown in Fig. 3. The parameters that represent the distribution of drugs to the tumor calculated using equation 2 are shown in Table 1. The simulation lines using these parameters are shown with the observed data in Fig. 4. The concentrations of DNR and ADM in the tumor reached the maximum 120 min

after the drug administration.

Fig. 3 Time Courses of Drug Concentrations in Blood after Intraperitoneal Administration.

Points and bars represent the means $\pm$ S.E. of 3 to 5 rats.

Lines were simulated using the parameters shown in Table 1.

Table 1 Pharmacokinetic Parameters^{a)} of DNR and ADM after Intraperitoneal Administration

Drug	k_a (min^{-1})	k_{el} (10^{-3}min^{-1})	V_d (l/kg)	K_1 (10^{-2}min^{-1})	K_2 (10min^{-1})
DNR	0.141	0.264	28.2	9.97	3.26
ADM	0.341	7.17	6.96	1.35	0.43

a) Parameters were calculated using the mean blood concentrations of 6 rats.

Fig. 4 Time Courses of Drug Concentration in Tumors after Intraperitoneal Administration.

Each point represents a single individual rat.

Lines were simulated using the parameters shown in Table 1.

ULS and/or DNR

Tumor growth

Fig. 5 shows the effect of treatment on the growth of Yoshida sarcoma. DNR alone had no effect on tumor growth. Both treatments of ULS and of ULS + DNR showed inhibitory effect on growth as compared to the control.

Survival time

The treatment of ULS + DNR was most effective and the survival time was significantly longer than that of the control (Table 2). The ULS group showed the tendency of prolongation

of the survival time but there was no significant difference. The percentage of mean survival time of the treated groups to that of the control group (T/C %) were 126.3, 169.5, and 188.1 % for the DNR group, the ULS group, and the ULS + DNR group, respectively (Table 2).

Fig. 5 Effect of ULS Irradiation on Growth of Yoshida Sarcoma

Each point and bar represents the mean $\pm$ S. E. of 4 to 9 rats.

Key:
Control; $\square$
Drug; $\blacksquare$
ULS; $\circ$
ULS + Drug $\bullet$

Table 2 Effect of ULS and / or DNR on Yoshida Sarcoma

Treatment	N ^{a)}	Survival time	T/C ^{b)}
		(day)	(%)
Control	8	23.6 $\pm$ 2.6	100.
DNR	9	29.8 $\pm$ 5.7	126.3
ULS	9	40.0 $\pm$ 5.3	169.5
ULS + DNR	9	44.4 ^{c)} $\pm$ 4.0	188.1

- a) Number of rats used after the test of outlier.
b) The percentage of mean survival time of the treated group to that of the control group.
c) Significantly different ($p < 0.05$) from the control.

Table 3 Effect of ULS and / or ADM on Yoshida Sarcoma

Treatment	N ^{a)}	Survival time	T/C ^{b)}
		(day)	(%)
Control	8	33.1 $\pm$ 2.4	100
ADM	9	37.7 $\pm$ 5.0	113.9
ULS	9	45.6 $\pm$ 2.2	137.8
ULS + ADM	9	47.6 ^{c)} $\pm$ 2.4	143.8

- a) Number of rats used after the test of outlier.
b) The percentage of mean survival time of the treated group to that of the control group.
c) Significantly different ($p < 0.05$) from the control.

ULS and / or ADM

Tumor growth

Tumor growth was inhibited in all the three treatment (Fig. 5 B). The combination treatment of ULS + ADM was most effective and ULS alone was the next. The combination treatment showed rather an increasing effect on the tumor size.

Survival time

As shown in Table 3, the ULS group and the ULS + ADM group showed prolongations of

survival time of 137.8 and 143.8 % compared to the control group, respectively. The combination treatment showed a significant prolongation of over 18 days.

DISCUSSION

The blood concentration of drugs were simulated by the one compartment model with first order transport rate constant. The profiles of the time course of the two drugs differed. The DNR appeared in the blood as if it was infused, and gave a very small elimination rate constant (k_{el}) of $0.000264 \text{ min}^{-1}$ and a large distribution volume (V_d) of 28.2 l/kg . Though DNR is a close analogue of ADM, they differed markedly in the abilities to bind to the tissue components. The rate and extent of their distribution in tissue may cause the difference in k_{el} and V_d . On the other hand, the time courses of the two drugs in tumor were similar. The drug concentrations increased gradually for 120 min after the administration and remained at the maximum value of about $1 \text{ } \mu\text{g/min}$. From these results, ULS was irradiated from 120 min after the drug administration.

In the DNR studies, DNR alone showed a slight increase in the survival time of the rats as compared to the control (Table 2) but showed rather an effect than an inhibitory effect on the tumor growth (Fig. 5), though there were no significant differences. The treatment of ULS alone showed inhibitory effects on the growth of Yoshida sarcoma and prolonged the survival time. The effects of the combination treatment of ULS + DNR on both the tumor size and the survival time were more potent than those of ULS alone. Judging from the tumor growth data, ULS exposed the latent antitumor activity of DNR in the combination treatment, though DNR itself has no antitumor activity on Yoshida

sarcoma at the dose used in this study.

In the ADM studies, the treatment of ADM showed an antitumor effect on the growth of Yoshida but little effect on the survival time. ULS inhibited the growth of Yoshida sarcoma and prolonged the survival time. The combination treatment of ULS + ADM inhibited the tumor growth and significantly prolonged the survival time as compared to the control group. These facts suggested that the combination treatment of ULS and antitumor drugs (DNR or ADM) was more effective than the other treatments.

The antitumor effect of ULS was not thermal because the tumor temperature was kept below the hyperthermia level (35°C). Though the precise mechanism of the synergistic interaction of ULS on DNR is not clear, it may be due to an increased membrane permeability for the drug, and/or the chemical activation of drugs by the energy generated by ULS cavitation. This study suggested that ULS has *per se* antitumor effects as well as is a useful adjuvant in the tumor chemotherapy. In conclusion, ULS may be focused to the tumor to damage it directly and to active the antitumor drugs that are administered in minimum doses to avoid the potent adverse effects.

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