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Enhancement of therapeutic efficacy of aclarubicin against lymph node metastases using a new dosage form: aclarubicin adsorbed on activated carbon particles

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Seven days after a subcutaneous inoculation of 5×10^5 P388 leukemia cells into the foot pad of the left hind paw of donor mouse, aclarubicin (0.2 mg/kg body weight) was injected subcutaneously into the hind paw of the opposite foot pad in the form of ACR-CH or aclarubicin aqueous solution. On day 10, the left popliteal and the lower para-aortic lymph nodes taken from each donor were transferred intraperitoneally to a normal recipient mouse. The combined survival time of recipients and the viable P388 leukemia cell number in popliteal and para-aortic lymph nodes were estimated with a calibration formula. Our results showed that the survival curve of recipients given ACR-CH was statistically improved compared with that of other treatment groups.

Key words: Activated carbon, aclarubicin, ACR-CH, drug delivery system, lymph node metastases, P388 leukemia cell.

Introduction

Aclarubicin is one of several anthracyclines which is used as an anticancer drug and is reported to show less cardiotoxicity than adriamycin or daunomycin.[1] Clinical trials of aclarubicin began in 1977 and promising responses were observed in various types of malignancies.[2] It is well known that water soluble anticancer agents are rapidly adsorbed into the circulating blood from the injection site and that high levels of the agents cannot be maintained in the regional lymph nodes for a long period of time when they are injected into tissues in the form of an aqueous solution.[3]

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Our recent studies have shown that a new dosage form of aclarubicin (ACR-CH), which is adsorbed to small activated carbon particles in the form of a suspension, results in the distribution of high levels of aclarubicin to regional lymph nodes, where a fixed concentration of aclarubicin is released continuously for a long period of time.[4,5] In our previous studies, we developed a quantitative method of evaluating the therapeutic effects for lymph node metastases.[6] We have now extended these studies, and report here the enhancement and efficacy of anticancer therapy against metastases in the regional lymph nodes in mice using this novel regimen.

Materials and methods

Agents

Activated carbon (Mitsubishi 1500AA®, Mitsubishi Chemical Co., Tokyo, Japan), with primary particles 20 nm in diameter and a specific surface area of 1480 m^2/g was used as the carrier. One of the anthracyclines, aclarubicin[1] (Aclacynon®, Sanraku Co., Tokyo),[1] which has anticancer effects on various types of malignancies,[2] was used as the anticancer drug.

Preparation of dosage forms

To prepare the carrier for the drugs, activated carbon (50 mg/ml) and polyvinylpyrrolidone (polyvinylpyrrolidone K-30®, PVP, Nakarai Chemical

Co., Kyoto, Japan, 20 mg/ml) were mixed in saline and kneaded with three rollers to form a suspension of carbon particles, which were measured by photon correlation spectroscopy to average 157 nm in diameter.[7] The activated carbon suspension was sealed in a glass tube and sterilized at 120°C for 10 min. Aclarubicin (5 mg/ml) was added to the activated carbon suspension. The mixture was diluted with saline and shaken at 120 cycles/min for 1 h at 37°C to maintain equilibrium. In this way, the new form of aclarubicin combined with carbon and PVP (ACR-CH) consisted of 0.1 mg/ml of aclarubicin, 0.5 mg/ml of activated carbon and 0.2 mg/ml of PVP in saline. In addition, an aqueous solution of aclarubicin (ACR-sol), comprising 0.1 mg/ml of aclarubicin in saline, together with an activated carbon suspension without aclarubicin and 0.5 mg/ml of activated carbon and 0.2 mg/ml of PVP in saline were prepared (Table 1).

Properties of ACR-CH

It is known that ACR-CH has a strong affinity for the lymphatic system.[5] In a previous experiment aclarubicin activity was measured in the regional lymph nodes after injection of aclarubicin into the right forefoot pad of rat in the form of ACR-CH or aclarubicin aqueous solution. Aclarubicin activity was found to be significantly higher, 3 to 5 times than that in rats given aclarubicin aqueous solution, for up to 24 h after injection.[8] A second advantage of using activated carbon as a drug carrier is its ability to release aclarubicin in a free state at a designated fixed level for a long period of time. There is a state of dynamic equilibrium between aclarubicin on activated carbon particles and aclarubicin in a free state around the activated carbon particles. Thus, when the concentration of free aclarubicin decreases around the activated carbon particles, the particles release more aclarubicin. Since a large amount of aclarubicin is adsorbed onto the activated carbon, its slow release at elevated levels continues for a long period of time. Finally, the adsorption isotherm of aclarubicin on activated carbon at 37°C in saline can be expressed as $Q = 51.2C^{0.478}$, where Q is the amount of aclarubicin adsorbed onto the activated carbon (μg/ml) and C is the concentration of free aclarubicin (μg/ml).[4]

Table 1. Dosage forms of aclarubicin

Dosage form	Composition	
ACR-CH	aclarubicin	0.1 mg/ml
	activated carbon	0.5 mg/ml
	PVP in saline	0.2 mg/ml
ACR-sol	aclarubicin in saline	0.1 mg/ml
Activated carbon suspension	activated carbon	0.5 mg/ml
	PVP in saline	0.2 mg/ml

Animals and tumor development

Six week old male CDF_1 mice, weighing 23–25 g, were purchased from Shizuoka Laboratory Animal Center, (Hamamatsu, Japan). They were kept under standard conditions (specific pathogen free, 22°C, 100% relative humidity and a 12 h day–night cycle). Mouse leukemia cell P388 (P388 tumor) was used as the experimental tumor and was maintained through intraperitoneal implantation in carrier mice. This tumor was chosen due to its facility to metastasize to regional lymph nodes when inoculated subcutaneously in mice.[9] The ascites containing P388 tumor cells were collected from carrier mice by abdominal paracentasis and were dispersed into Hank's solution containing a final dilution of 1×10^7 P388 tumor cells/ml. The viability of tumor cells was determined by the Trypan blue exclusion test and was shown to be greater than 95%. This tumor cell suspension was used within 2 h after preparation.

Therapeutic experiment

A total of 100 mice received a subcutaneous transplantation of 0.05 ml/mouse (5×10^5 cells/mouse) of the P388 tumor cell suspension into the footpad of the left hind paw using a 27 gauge needle and syringe. Seven days after injection, 100 mice with tumors of about 6.5 mm in diameter were selected for the experiment and divided into five groups, comprising 20 mice per group. Drugs were administered on day 7, since early studies showed that metastasis was established in the popliteal lymph node and para-aortic lymph node 7 days after inoculation of tumor cells. Accordingly, four groups of up to 20 mice received a subcutaneous bolus injection of 0.05 ml of ACR-CH (ACR-CH group) or aqueous aclarubicin solution (ACR-sol group), activated carbon suspension without aclacinomycin (activated carbon group, 19 mice) or saline (saline group). The last group was not given any drugs (non-treatment group).

On day 10, the number of viable P388 tumor cells in the metastatic lesion in the popliteal and para-aortic lymph nodes was measured by the procedure reported by Hagiwara *et al.*[11] Mice were sacrificed on day 10 and the left popliteal and para-aortic lymph nodes were extirpated and minced with scissors. A suspension of tissue was taken from each mouse and was transferred intraperitoneally to a normal recipient mouse under aseptic conditions for the estimation of the number of viable P388 tumor cells in the lymph node.

The tumor recipients were observed daily for 60 days following transfer and the mean survival time in each group was calculated in order to compare the therapeutic efficacy of the drugs on lymph node metastases. Statistical analysis of these examples was performed by a Student's *t*-test. In addition, the therapeutic efficacy in each group was also expressed as follows:

$$\text{T/C\%} = \frac{\text{mean survival time in each group}}{\text{mean survival time in the non-treated group}}$$

Finally, the number of viable P388 leukemia cells in the left popliteal and para-aortic lymph nodes in each treatment group was estimated using a calibration curve and formula from the mean survival time of the recipient:[6] $Y = -2.5 \log_{10} X + 22.5$, where Y is the mean survival time of recipients and X is the cell number of P388 leukemia cells.

Results

Tables 2 and 3 show the results of administration of anticancer agents. As shown in Table 2, the mean survival time of the popliteal lymph node group was prolonged following administration of ACR-CH (12.55 ± 3.94 days, mean ± SD) and the therapeutic efficacy of the drugs was 146.78% in T/C%. In contrast, the remaining four treatments extended the survival times of animals from 8.55 to 10.10 days (therapeutic efficiencies of 94–132%). Thus, the mean survival time for the ACR-CH treatment group was longer than that for the other four groups. Statistical comparisons of results showed significant differences ($p < 0.01$–0.05). No significant differences were found in the survival times of the remaining four groups. In the para-aortic lymph node transferred group (Table 3), the mean survival time of the recipients was also prolonged following treatment with ACR-CH (12.90 ± 3.70 days) and the efficacy of the drugs was calculated to be 134% in T/C%. Mean survival times of the remaining groups were found to range from 9.60 to 11.5 days (therapeutic efficiencies 101–120%). As shown previously, the mean survival time in the ACR-CH treated group was longer than that in the other four groups and statistical differences were found following comparison with the saline, activated carbon and non-treatment groups ($p < 0.01$). However, significant differences were not found when comparing ACR-CH animals with the ACR-sol group.

The number of viable P388 leukemia cells in the popliteal lymph nodes and para-aortic lymph nodes was estimated from the mean survival time of the recipients using the calibration curve and formula described above. Thus, the number of tumor cells in the popliteal lymph nodes of the ACR-CH treated group was estimated to be less than 1.6×10^4 cells. However, the other four groups showed estimated numbers ranging from 5.1×10^4 to 1.0×10^5 cells, which were considerably larger than that in the ACR-CH treated group. Moreover, in the ACR-CH treated group, the number of tumor cells in the

Table 2. Therapeutic efficacy of ACR-CH on popliteal lymph node metastasis

Drug treatment (no. of mice)	MST ± SD[a] (days)	T/C%	No. of cells[b]
ACR-CH (20)	12.55 ± 3.94	147.6	1.6×10^4
ACR-sol (20)	10.1 ± 3.78[c]	121.0	1.0×10^5
Saline (20)	8.55 ± 2.50[d]	102.4	4.9×10^5
Activated carbon (19)	8.05 ± 3.45[d]	96.4	7.0×10^5
Non-treatment (20)	8.35 ± 2.19[d]	100	5.1×10^5

[a] Mean survival time of mice ± standard deviation.
[b] Number of cancer cells estimated with the calibration formula.
[c] The MST was statistically significantly shorter ($p < 0.01$), as compared with that of the ACR-CH treated group.
[d] The MST was significantly shorter ($p < 0.05$), as compared with that of the ACR-CH treated group.

Table 3. Therapeutic efficacy of ACR-CH on para-aortic lymph node metastasis

Drug treatment (no. of mice)	MST ± SD[a] (days)	T/C%	No. of cells[b]
ACR-CH (20)	12.9 ± 3.7	134	7.8×10^3
ACR-sol (20)	11.5 ± 2.99	120	6.5×10^4
Saline (20)	9.65 ± 3.45[c]	101	1.6×10^5
Activated carbon (19)	9.89 ± 3.19[c]	103	1.7×10^5
Non-treatment (20)	9.60 ± 3.04[c]	100	1.6×10^5

[a] Mean survival time of mice ± standard deviation.
[b] Number of cancer cells estimated with the calibration formula.
[c] The MST was statistically significantly shorter ($p < 0.01$), as compared with that of the ACR-CH treated group.

para-aortic lymph nodes was estimated to be less than 7.8×10^3 cells. However, the remaining four groups showed estimated ranges of 6.5×10^4 to 1.6×10^5 cells, which again were larger than that observed in the ACR-CH treated group.

Discussion

The purpose of cancer chemotherapy is to selectively concentrate anticancer drugs for long periods of time within cancerous lesions, such as lymph node metastases. We have developed a novel form of administering one such drug, aclarubicin, following its adsorption to a suspension of activated carbon particles (ACR-CH). In our previous studies, we have reported the selective distribution of ACR-CH to regional lymph nodes.[5,6,11] We have examined an experimental model in mice in which the anticancer effects of anticancer drugs on lymph node metastases could be quantified.[6] We have now extended this work and present results of therapeutic efficacy of ACR-CH on lymph node metastases using this method.

The therpeutic effects on the metastatic lesions in the popliteal and para-aortic lymph nodes were evaluated quantitatively by examination of the survival time of the recipients, and the number of tumor cells estimated from the calibration curve and formula. Our results showed that the survival time of the recipients was significantly extended in the ACR-CH treatment group compared with the other four treatment groups. In addition, the calculated number of viable P388 leukemia cells in the popliteal and para-aortic lymph nodes in the ACR-CH treated group was much smaller than in the other groups.

We conclude that local injection of ACR-CH provides excellent therapeutic efficacy on metastases within the regional lymph nodes as compared with treatment with aclarubicin aqueous solution. We feel that the therapeutic effects of this drug are related to the extended maintenance of high concentrations within regional lymph nodes following local injection of ACR-CH. We feel that this novel form of the drug, i.e. combined with carbon, is clinically relevant, and is an anticancer agent with enhanced therapeutic efficacy and lower toxicity as compared with the aqueous form of aclarubicin.

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