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Enhanced anti-cancer effects of intralymphatic aclarubicin on distal lymph node metastases: quantitative evaluation using a new experimental model in mice

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The anti-cancer drug aclarubicin (2.0 mg/kg body weight) was injected into the left popliteal lymph node (the primary draining node of the foot-pad region) or into the tail vein, 8 days after a subcutaneous inoculation of 5×10^5 P388 leukemia cells/mouse in the left hind paw foot-pad of mouse (donor). During this time, metastases were established in the lower para-aortic nodes (the secondary draining nodes of this region). On day 10, the lower para-aortic nodes taken from each donor were transferred intraperitoneally to a normal mouse (recipient). From the recipients' survival time, the viable P388 leukemia cell number in the para-aortic nodes per donor mouse was estimated with a calibration line. The recipients' survival curve in the intralymphatic chemotherapy group was statistically significantly better than that in the intravenous chemotherapy group.

Key words: Animal model, intralymphatic chemotherapy, lymphatic metastases.

Introduction

Intralymphatic chemotherapies for cancer lesions of lymph nodal metastases have been developed as a method for site-specific drug delivery.[1–3] Drug distribution has been reported with this method. However, quantitative evaluation of the therapeutic effects for distal lymph nodes metastases has not been possible because an animal cancer metastases model has not been developed. This paper reports an animal model with which the therapeutic effects of intralymphatic cancer chemotherapy on metastatic lesions in the distal lymph nodes may be evaluated quantitatively. Using this model, we found an enhanced therapeutic effect of intralymphatic chemotherapy employing aclarubicin.

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Materials and methods

Animals, tumor cells and the anti-cancer drug

Female CD_2F_1 mice (5 weeks old; The Shimizu Laboratory Animal Center, Hamamatsu) were used as experimental animals. The mice were kept under standard conditions (specific pathogen free, temperature of 22°C, relative humidity of 60%, day–night cycle of 12 h).

P388 leukemia cells were provided by Dr H Sato of The Sasaki Institute (Tokyo) and maintained through intraperitoneal implantation in DBA_2Cr mice (The Shimizu Laboratory Animal Center). The ascites containing P388 leukemia cells were taken from the carrier mice and suspended into Hanks' solution containing 10^7 P388 leukemia cells/ml. The tumor cell viability, as determined by the trypan blue exclusion test, was higher than 95%. The tumor cell suspension was used within 4 h of preparation.

Aclarubicin (Aclacinomycin Inj.[R], Sanraku Co., Ltd, Tokyo), which is one of the anthracyclines,[4] was used as the anti-cancer drug. Aclarubicin was dissolved in saline (1 mg/ml).

Calibration line for the P388 leukemia cell numbers

The following experiment was carried out to obtain a calibration line with which the number of viable P388 leukemia cells could be calculated.[5] One hundred mice were divided into five groups, each composed of 20 mice. A P388 leukemia cell suspension, prepared as described above, was diluted with Hanks' solution to produce suspensions with five tumor cell concentrations: 10^6, 10^5, 10^4, 10^3 and 10^2 cells/ml. The 20 mice in each group received an intraperitoneal injection of 1 ml/mouse of one of the five P388 leukemia cell suspensions. For 60 days after inoculation, the mice were checked daily for deaths and the mean survival time in each group was calculated. The mean survival time was plotted against the number of intraperitoneally inoculated P388 cells on a semi-log abscissa. A calibration line was drawn along these points.

Lymph node metastasis experiment

An experiment was performed to determine how many cancer cells had metastasized into the lymph nodes 8, 10 and 12 days after receiving a subcutaneous inoculation of the P388 leukemia cell suspension (prepared as described above). Sixty mice received a subcutaneous inoculation with 0.05 ml of the P388 leukemia cell suspension (equal to 5×10^5 cells) in the foot-pad of the left hind paw with a 27 gauge needle microsyringe. On days 8, 10 and 12, 20 of the 60 mice (donors) were killed. We extirpated the lymph nodes located at the left popliteal fossa (the left popliteal node; there is one such node per mouse in most mice) and the nodes located along the left iliac artery and along the aorta distal than renal artery (the lower para-aortic nodes; there were two to four nodes per mouse). It is difficult to differentiate the nodes along the left iliac artery and the nodes along the distal aorta. The left popliteal nodes taken from each mouse were individually minced with scissors into tissue fraction suspension in 1 ml Hanks' solution under aseptic conditions. Twenty other normal mice (recipients) were prepared. The suspension of tissue fractions of the popliteal lymph node taken from each donor was individually injected intraperitoneally into each recipient. Through the same procedures the lower para-aortic nodes taken from individual donor were transferred to each recipient.

Twenty more mice were killed on day 10 and the final 20 mice on day 12. The lymph nodes were transferred using the same procedures as described above.

The recipients were checked for deaths daily for 60 days after the transfers. Autopsies were performed on the dead mice to determine the cause of death. The mean number of viable P388 leukemia cells in the donors' lymph nodes was estimated from the recipients' mean survival time using the calibration line obtained previously. The mean number of viable P388 leukemia cells transferred to the recipient was calculated in each of the six groups.

Therapeutic experiment

We tested the possible therapeutic effects of an aclarubicin solution (1 mg/ml in saline) on lymph nodal metastases. P388 leukemia cell suspension at 0.05 ml/mouse (equal to 5×10^5 cells/mouse) was inoculated subcutaneously into the left hind foot-pad of 90 mice on day 0. On day 8, the mice, whose body weight was 25 g/mouse on average, were divided into four treatment groups each composed of 21–23 mice. Aclarubicin was given to the mice under general anesthesia (achieved by an intraperitoneal injection of 2 mg/mouse of pentobarbital sodium). One group received an injection of 0.05 ml/mouse of aclarubicin aqueous solution (equal to 2.0 mg/kg body weight) into the left popliteal node with a 30 gauge butterfly-shaped needle and microsyringe (the ACR-ly group). The second group received 0.05 ml/mouse of saline using the same procedure (the saline group). The third group received an intravenous injection of 0.05 ml/mouse of aclarubicin solution (equal to 2.0 mg/kg body weight) into the tail vein (the ACR-iv group). The last group received no treatment (the non-treatment group). After treatment, 90 mice underwent amputation of the left hind paw at the ankle in order to remove the primary cancer lesion.

On day 10, the mice were killed and the lower para-aortic lymph nodes were extirpated. The lymph nodes taken from an individual mouse were minced with scissors into a tissue fraction suspension in 1 ml of Hanks' solution. The tissue fraction suspensions were then individually transferred intraperitoneally to the normal recipient mice.

The recipients were checked for deaths daily for 60 days after the transfer. The survival curves of the recipients, which represented the number of viable P388 leukemia cells in the lower para-aortic

nodes of the donors in each treatment group, were compared by generalized Wilcoxon's test. The number of viable P388 leukemia cells in the donors' lower para-aortic nodes was estimated from the recipients' survival times using the calibration line obtained from the previous experiment.

Results

Calibration line for the P388 leukemia cell numbers

The calibration line with which we estimated the number of intraperitoneally injected P388 leukemia cells is shown in Figure 1. There was a linear relationship between the survival time of the mice and the logarithm of the number of P388 leukemia cells.

Lymph node metastasis experiment

All recipients who received an injection of the tissue fraction suspension of the donor's popliteal node or lower para-aortic nodes died during the observation period. The results are shown in Table 1. In the group of recipients given the left popliteal node suspension produced on day 8 (the day 8 popliteal group), the survival time was 13.5 ± 2.6 days (mean ± SD), corresponding to 3.5×10^3 cells/popliteal node of a mouse. In the group of recipients given the lower para-aortic nodes suspension produced on day 8 (the day 8 para-aortic group), the survival time was 16.1 × 4.4 days, corresponding to 3.6×10^2 cells/para-aortic nodes of a mouse. In the group of recipients given the left popliteal node suspension produced on day 10 (the day 10 popliteal group), the survival time and the P388 cell number were 10.9 ± 3.1 days and 3.7×10^4 cells/nodes of a mouse, respectively. In the recipients given the lower para-aortic nodes suspension produced on day 10 (the day 10 para-aortic group), the survival time and the P388 cell number were 13.3 ± 3.7 days and 4.0×10^3 cells/nodes of a mouse, respectively. In the recipients given the left popliteal node suspension produced on day 12 (the day 12 popliteal group), the survival time and the P388 cell number were 7.9 ± 1.9 days and 5.3×10^4 cells/nodes of a mouse, respectively. In the recipients given the lower para-aortic nodes suspension produced on day 12 (the day 12 para-aortic group), the survival time and the P388 cell number were 9.6 ± 3.0 days and 1.2×10^5 cells/nodes of a mouse, respectively.

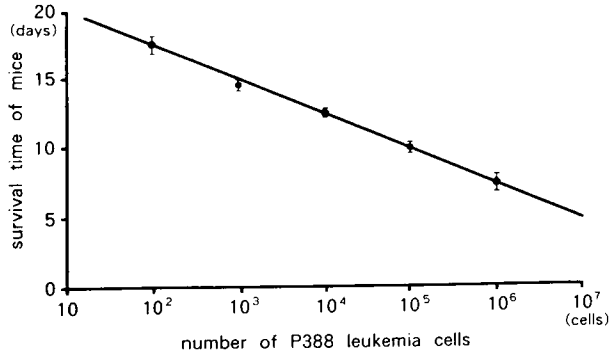

Figure 1. Calibration line for the number of P388 leukemia cells. There is a linear relationship between the number of P388 leukemia cells and the survival time of the mice, thus the cell number may be obtained from the survival time of mice.

Table 1. The number of P388 leukemia cells metastasized to the lymph node and the survival time of the recipient group

Day and lymph node[a]	Survival time (days, mean ± SD)	P388 leukemia cell number (cells/mouse)
Day 8; left popliteal node	13.5 ± 2.6	3.5×10^3
Day 10; left popliteal node	10.9 ± 3.1	3.7×10^4
Day 12; left popliteal node	7.9 ± 1.9	5.3×10^5
Day 8; lower para-aortic nodes	16.1 ± 4.4	3.6×10^2
Day 10; lower para-aortic nodes	13.3 ± 3.7	4.0×10^3
Day 12; lower para-aortic nodes	9.6 ± 3.0	1.2×10^5

[a] Name of lymph node(s) and day of transfer after inoculation

Therapeutic experiment

The results are shown in Figure 2 and Table 2. For the non-treatment group, the survival time of the recipients was 14.0 ± 3.3 days ($N = 23$), which corresponded to 2.1×10^3 P388 leukemia cells/mouse. For the ACR-ly group, five of 23 mice survived up to day 60 and the mean survival time of the other 18 mice was 15.9 ± 2.1 days ($N = 18$). The survival curve of the ACR-ly group was

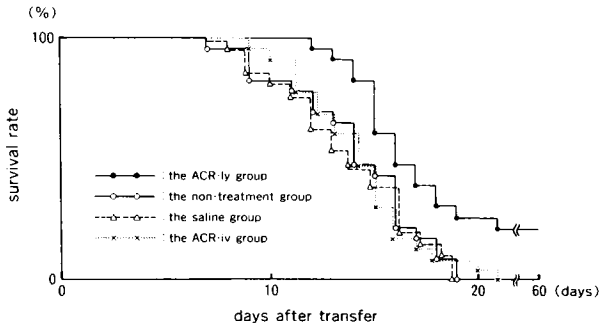

Figure 2. Survival curves of the recipients in the therapeutic experiment. The survival curve of the ACR-iv group was statistically significantly better (by generalized Wilcoxon's test, $p < 0.01$, Z value > 2.95) than those of the other three treatment groups.

statistically significantly better (generalized Wilcoxon's test, $p < 0.01$, Z value > 2.95) than those of the other three groups. The viable P388 leukemia cell number estimated from the survival time of the 23 mice in the ACR-ly group was 3.0×10^2 cells/mouse. For the saline group and the ACR-iv group, the mean survival times were 13.8 ± 3.2 days ($N = 21$), corresponding to 2.5×10^3 P388 leukemia cells/mouse, and 14.3 ± 3.0 days ($N = 23$), corresponding to 1.7×0^3 P388 leukemia cells/mouse, respectively. There were no statistical differences between the survival curves of the saline group, the ACR-iv group and the non-treatment group.

Table 2. Therapeutic results of intralymphatic chemotherapy

Treatment group (N = number of recipients)	Survival time of dead recipients (mean ± SD, N = number of dead recipients)	Number of recipients surviving to day 60	Viable cancer cells in para-aortic nodes of donors (cells/mouse)
ACR-ly group $N = 23$	15.9 ± 2.1 $N = 18$	5	3.0×10^2
Saline group $N = 21$	13.8 ± 3.2 $N = 21$	0	2.5×10^3
ACR-iv group $N = 23$	14.3 ± 3.0 $N = 23$	0	1.7×10^3
Non-treatment group $N = 23$	14.0 ± 3.3 $N = 23$	0	2.1×10^3

Discussion

Anti-cancer drugs administered systemically or subcutaneously may be evaluated for possible therapeutic effects on lymph node metastases using some established experimental animal models.[5,6] For intralymphatically injected anti-cancer drugs, however, there are no established animal models allowing quantitative evaluation. In the present model, P388 leukemia cells were used as the experimental tumor, because (1) with P388 leukemia cells the viable cancer cell number may be bioassayed quantitatively through an intraperitoneal inoculation and (2) P388 leukemia cells, which are used in the first step of anti-cancer drug screening, are sensitive to many kinds of anti-cancer drugs.

In the calibration line experiment, there was a linear relationship between the survival time of mice and the logarithm of the P388 leukemia cell number. This means that the viable P388 leukemia cell number in the donor's lymph nodes can be estimated from the recipient's survival time. In addition, the P388 leukemia cell number in two groups can be compared through a statistical comparison of the recipients' survival curve or time between the two groups.

The popliteal lymph node is the primary draining node and the lower para-aortic nodes are the secondary draining nodes of the foot-pad region of the ipsilo-lateral hind paw.[7]

The lymph node metastasis experiment indicated that 8 days after a subcutaneous inoculation of P388 leukemia cells (at 5×10^5 cells/mouse) in the foot-pad of hind paw, lymph node metastases were established not only in the popliteal node (the primary draining node) but also in the lower para-aortic nodes (the secondary draining nodes). Therefore, in the present therapeutic experiment, we administered the drug (aclarubicin or saline) into the popliteal node or intravenously, 8 days after the cancer inoculation, by which time metastases had been established even in the secondary draining nodes (the lower para-aortic nodes) of all mice. After treatment, the left hind foot including the primary lesion was amputated at the ankle to place the lymph nodes metastases beyond the effects of the primary lesion. Two days after treatment, the viable P388 leukemia cell number in the secondary draining nodes was bioassayed using the intraperitoneal transfer method, through which the precise cell number in the nodes could be obtained. The viable P388 leukemia cell numbers in the treatment groups were compared through a

statistical comparison of the survival curves of the recipients.

The above comparison shows that intralymphatic aclarubicin treatment produces superior therapeutic effects on the metastatic lesions in the distal lymph nodes when compared with intravenous aclarubicin.

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