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Characteristics of the Inhibitory Effect of Mitoxantrone and Pirarubicin on Lung Metastases of Colon Carcinoma 26

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This study was performed to evaluate the antimetastatic activity of antitumor agents against metastatic colon carcinoma 26 (Co 26Lu), and to investigate their mechanisms of action. Pirarubicin demonstrated the most striking antitumor activity in mice bearing intravenously injected Co 26Lu cells. Etoposide and mitoxantrone also showed marked antitumor activity. Pirarubicin and mitoxantrone also exerted remarkable inhibitory effect on spontaneous lung metastases from subcutaneously implanted Co 26Lu. Pirarubicin showed marked inhibition of both primary tumor growth and lung metastases. Mitoxantrone was effective in preventing lung metastases even at doses that did not exhibit an antitumor effect on the primary tumor. Moreover, mitoxantrone administered two days after intravenous injection of tumor cells obviously reduced the number of lung colonies, while simultaneous injection of the drug did not inhibit colony formation. Mitoxantrone effectively inhibited angiogenesis on the chorioallantoic membrane at doses that did not affect the growth rate of embryos. These results suggest that mitoxantrone, besides its direct antitumor effect on tumor cells, may inhibit lung metastases by inhibiting angiogenesis.

Key words: Lung metastasis — Colon carcinoma 26 — Mitoxantrone — Pirarubicin — Chorioallantoic membrane assay

It is generally held that many patients diagnosed as having cancer will acquire disseminated disease at some point during their clinical course.[1)] Therefore, the development of metastases is one of the most critical problems for cancer patients. The research presented here was done as part of a program to discover drugs and methods to treat and/or prevent cancer metastases. In previous studies we attempted to identify antitumor agents effective on hepatic metastases and compared them with antitumor agents effective on subcutaneously implanted colon carcinoma 26 (Co 26) and the same tumor in the liver.[2)] Mitomycin-C was considerably more effective on Co 26 in the liver than on the subcutaneously implanted tumor.[2)] In this study, we attempted to find antitumor agents which are effective on lung metastases. Lung metastases were induced artificially in CDF_1 mice by intravenous injection of cells from the original Co 26 tumor and from highly metastatic colon carcinoma 26 (Co 26Lu) which was derived from Co 26. The antitumor activities of fifteen antitumor agents currently used in clinical practice in Japan were assayed using both colon carcinomas. In addition, the antitumor agents found to be effective were assayed using a spontaneous lung metastasis system in which Co 26Lu was implanted subcutaneously. The mechanisms of their antimetastatic activity were investigated.

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MATERIALS AND METHODS

Chemicals Actinomycin-D (Banyu Pharmaceutical Co., Ltd., Tokyo), bleomycin, cisplatin and etoposide (Nippon Kayaku Co., Ltd., Tokyo), carboplatin (Bristol-Myers-Squibb K.K., Tokyo), vincristine and vindesine (Shionogi & Co., Ltd., Osaka), doxorubicin, epirubicin, 5-fluorouracil and mitomycin-C (Kyowa Hakko Kogyo Co., Ltd., Tokyo), mitoxantrone and methotrexate (Lederle (Japan), Ltd., Tokyo), and daunorubicin and pirarubicin (Meiji Seika Kaisha, Tokyo) were used. Acetylsalicylic acid and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals Inbred, 5-week-old, male BALB/c and CDF_1 mice weighing approximately 22 g each were obtained from Japan SLC (Hamamatsu). Six-week-old mice were used in this study and were maintained in plastic cages with woodchip bedding under specific-pathogen-free conditions in an animal laboratory with controlled temperature (24 ± 2°C). Animals were given CE-2 pellet diet (CLEA Japan, Inc., Tokyo) and water *ad libitum*.

***In vivo* selection of a metastatic variant of Co 26 cells** The Co 26 tumor was maintained *in vivo* by serial subcutaneous transplantation into BALB/c male mice. The original tumor cells were of low metastatic potential. Metastatic tumor cells were obtained by sequential selection of lung metastases formed following intravenous injection of single Co 26 cells. Namely, a freshly excised original tumor was minced in Hanks' balanced salt solu-

tion (Life Technologies, Inc., Grand Island, NY) and strained through a 120 stainless-steel mesh. Viability was determined by trypan blue dye exclusion and the cell suspension was diluted to the desired cell concentration (5×10^4 cells/0.1 ml). One hundred μl of this cell suspension was injected via the tail vein, and the mice were killed when moribund at about 2 weeks later. Lung metastases were excised and serially implanted into the back of new mice. This procedure was repeated and lung metastases from the subcutaneously implanted tumor were obtained after five cycles. Lung metastases from the subcutaneously implanted tumor (about 3 weeks after implantation) were implanted into the back of new mice and this procedure was repeated through forty cycles. The frequency of lung metastases increased as compared with Co 26, and all mice had many macroscopic lung metastases 4 weeks after subcutaneous implantation of the tumor.

Treatment of mice bearing artificial lung metastases Six CDF_1 mice were used for each drug tested. Tumor cells were suspended in 0.9% NaCl solution (5×10^5 cells/ml) and an aliquot (0.1 ml) was injected via the tail vein of each mouse on day 0. These mice were then randomized and allocated to the various groups. Antitumor agents were dissolved in 5% glucose in water. These drugs were injected intravenously on days 7 and 14 at their maximally tolerated doses. Mice were observed daily. The antitumor effect was determined by comparing the mean survival time of each treated group with that of the control group, and was expressed as the increase in life-span (ILS). All dead mice were autopsied; numerous metastases confined to the lungs were observed in these animals.

Spontaneous metastasis model and treatment The following procedure was used to evaluate the effect of drugs on spontaneous lung metastases. On day 0, 1×10^5 Co 26Lu cells (0.1 ml) were implanted subcutaneously into the back of mice and the animals were then randomly allocated to the control and treatment groups (6–9 animals per group). On day 10, all mice had micrometastases in the lungs. The drugs were injected intravenously on days 10, 17 and 24. The longest (a) and shortest (b) diameters of the primary tumor were measured twice a week using calipers, and the volume was calculated using the formula: $ab^2/2$ (mm^3). All the animals that survived were killed on day 32, and the lungs were removed, rinsed in 0.9% NaCl solution containing heparin and fixed for one day in acetone to determine the number of macroscopic lung metastases.

Effect of mitoxantrone on the formation of colonies of Co 26Lu cells in the lungs On day 0, 5×10^3 Co 26Lu cells were injected via the tail vein (7 animals per group). Mitoxantrone (0.63 mg/kg) was injected intravenously 60 min before the injection of tumor cells, or 5 min, 60 min or 2 days after the injection of tumor cells. All the animals that survived were killed by cervical dislocation on day 12. The lungs were resected, rinsed in 0.9% NaCl solution containing heparin and fixed for one day in acetone to determine the number of macroscopic lung colonies.

Chorioallantoic membrane (CAM) assay to determine the effect of antitumor agents on angiogenesis The effect of mitoxantrone on angiogenesis was assayed using the CAM of chicken (Leghorn Hybecobovans) embryos in the shell according to a method previously described but with some modifications.[3] First 2 ml of albumin was removed from a 3-day-old fertilized egg, then the air chamber side was opened and covered with a cap, and the egg was incubated for one day. Ten μl of an appropriate concentration of mitoxantrone in 0.9% NaCl solution containing 1% methylcellulose was put in a silicone ring, which was placed on the CAM of 4-day-fertilized embryos. After further incubation for 48 h, a fat emulsion was injected into the CAM to visualize clearly the blood vessels and a photograph of the CAM was taken. Antiangiogenic activity was indicated by an avascular zone of 3 mm or more in diameter. The results were expressed as the percentage of embryos showing inhibition.

Culture of endothelial cells Rat lung endothelial cells were kindly provided by Dr. Aoyagi (Showa Pharmaceutical University) and were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics. The cells (3×10^4) were plated onto 24-well culture plates and incubated at 37°C. After 24 h, the medium was replaced with MEM containing 3% fetal calf serum; mitoxantrone or pirarubicin was then added and the cells were further cultured for 48 h. Cultured cells were treated with 0.25% trypsin (Gibco BRL, Grand Island, NY) to disperse them and viable cells were counted.

Culture of Co 26Lu cells The Co 26Lu cell line has been maintained in our laboratory. The cells were cultured in RPMI-1640 medium (IBL, Fujioka, Gumma) supplemented with 10% fetal calf serum (Gibco BRL). For the survival assays, 1×10^4 cells were seeded into 96-well plates and incubated for 24 h at 37°C to allow complete attachment of viable cells. The wells were then individually treated with the desired concentrations of mitoxantrone and pirarubicin. After incubation for 48 h, the medium was removed and adherent cells were fixed to the plate with 5% formaldehyde in phosphate-buffered saline. The cells were then stained with a 0.5% aqueous solution of crystal violet followed by elution of the dye with 33% acetic acid.[4] Absorbance at 570 nm was determined with an ELISA analyzer (ETY-96R, Oriental Instruments Ltd., Tokyo).

Statistical analysis Results obtained for the various experimental groups were compared using Student's t test

(survival and tumor volume) or the Mann-Whitney U test (number of lung metastases).

RESULTS

Effects of various antitumor agents on survival in mice bearing lung colonies of Co 26 and Co 26Lu (i.v.) Cells from the nonselected tumor (Co 26) had little ability to metastasize when implanted subcutaneously. On the other hand, the cells of the successively selected tumor (Co 26Lu) did form multiple metastatic nodules in the lungs. When these cells (Co 26 and Co 26Lu) were injected via the tail vein, all animals developed numerous macroscopic colonies in the lungs (the weight of the lungs at death was approximately 1 g each while that of lungs from normal CDF_1 mice was 0.16 ± 0.03 g).

In order to determine whether the sensitivity of Co 26 and Co 26Lu to antitumor agents was different, the effects of fifteen antitumor agents on the survival of mice bearing Co 26 and Co 26Lu were investigated (Table I). There was little difference in antitumor effects, as expressed by ILS (%), between mice bearing Co 26 and Co 26Lu. In both tumor systems, pirarubicin showed the best ILS (125 and 139%) among the four anthracycline derivatives employed, and was more effective than doxorubicin (85 and 74%). The ILS values of the groups treated with mitoxantrone and etoposide were 57–67%. Other drugs tested showed ILS values of less than 40%.

Effects of pirarubicin, mitoxantrone and etoposide on metastasis formation in mice bearing Co 26Lu (s.c.) When the volume of the primary tumor was $\geqslant 100$ mm^3 (days 10–14), 100% of the animals had microscopic lung metastases. The effects of pirarubicin, etoposide, mitoxantrone, 5-fluorouracil and cisplatin on metastasis formation in mice bearing a Co 26Lu tumor were investigated. These drugs were administered on days 10, 17 and 24. There was a significant decrease in the number of lung metastases in animals treated with pirarubicin and mitoxantrone, together with marked inhibition of the primary tumor growth (Fig. 1). Some mice had no lung metastases. Furthermore, mitoxantrone inhibited formation of lung metastases even at doses lower than those required to inhibit growth of the primary tumor (Fig. 1). Etoposide (Fig. 2), 5-fluorouracil and cisplatin (data not shown) had little inhibitory effect on the growth of the primary tumor or on the formation of metastatic lesions, even at the maximum doses employed in this tumor system.

Effects of mitoxantrone on the number of tumor colonies in the lungs Mitoxantrone markedly inhibited the development of lung colonies. The influence of time elapsed between intravenous injection of Co 26Lu cells and that

Table I. Increase in Lifespan after Treatment with Various Antitumor Agents in Mice Bearing Artificial Metastases

Antitumor agent	Dose (mg/kg)	Co 26		Co 26Lu	
		MST[a] (days)	ILS (%)	MST[a] (days)	ILS (%)
Control		19.0±0.5[b]	–	15.7±0.7[b]	–
Daunorubicin	12.5	28.2±1.4***	48	21.5±2.6	37
Doxorubicin	12.5	35.2±1.6***	85	27.3±1.2***	74
Epirubicin	12.5	31.2±1.6***	64	27.8±1.1***	77
Pirarubicin	12.5	42.7±3.2***	125	37.5±4.2***	139
Mitoxantrone	2.5	30.5±0.7***	61	24.7±0.2***	57
Vincristine	1.0	22.0±0.4***	16	16.3±0.3	4
Vindesine	2.5	21.3±0.7*	12	18.5±0.2**	18
Etoposide	50	25.6±0.8***	63	33.0±2.1***	67
Bleomycin	100	24.2±1.4*	27	17.3±0.8	10
Actinomycin-D	0.5	21.7±1.7	14	17.6±0.7	12
Mitomycin-C	5.0	24.5±1.1***	29	17.5±0.7	11
5-Fluorouracil	200	25.5±1.0***	34	21.5±0.4***	37
Methotrexate	50	20.5±0.5*	8	16.8±0.5	7
Cisplatin	5.0	24.1±0.8***	27	20.8±0.4***	33
Carboplatin	100	25.4±0.7***	34	21.0±2.0*	34

Co 26 (original tumor) or Co 26Lu (metastatic cells from colon carcinoma 26) cells were injected via the tail vein (5×10^4 cells). All antitumor agents were administered i.v. at the maximum tolerated dose on days 7 and 14.

a) MST = mean survival time.

b) Mean ± SE of dead mice (six mice/group).

*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared with control.

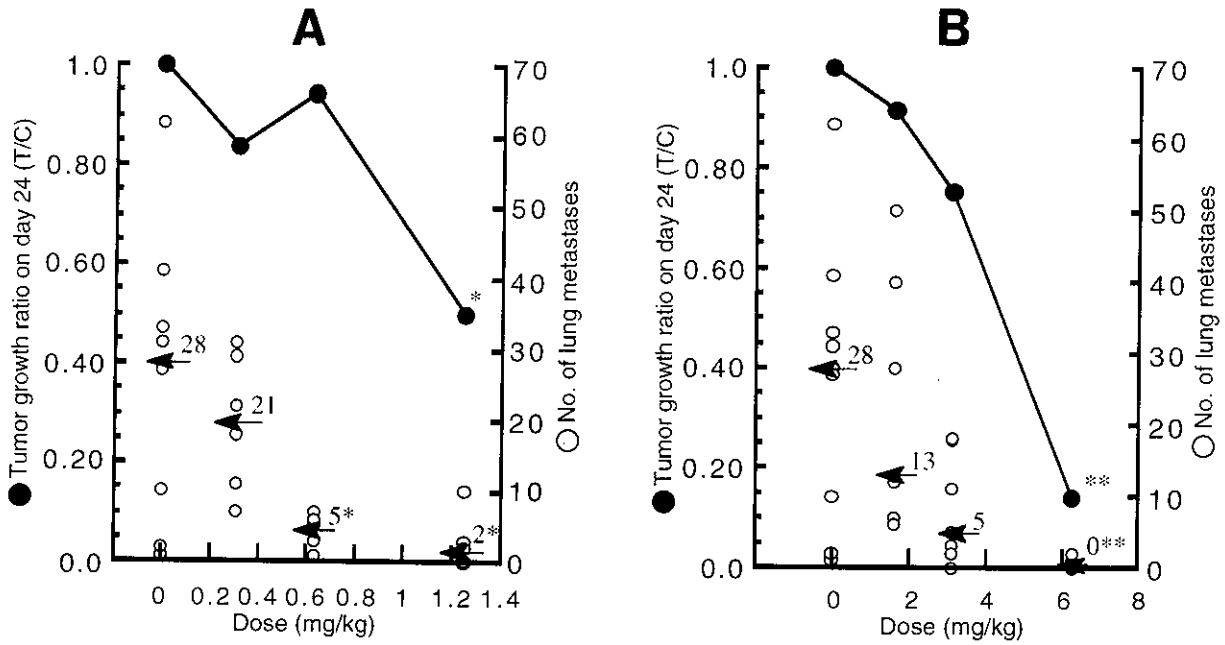

Fig. 1. Effects of mitoxantrone (A) and pirarubicin (B) on subcutaneous implants and lung metastases in mice bearing Co 26Lu tumor. Co 26Lu cells (1×10^5 cells/mouse) were implanted s.c. into the back of mice on day 0. Mitoxantrone and pirarubicin were administered i.v. on days 10, 17 and 24. Tumor volume was determined on day 24 and the ratio of average tumor volume in the treated group (T) to that of the untreated group (C) was calculated. The number of lung metastases was determined on day 32. Arrows with figures represent the median number of metastases (6–9 mice/group). *, $P<0.05$; **, $P<0.01$ compared with untreated group.

of mitoxantrone was investigated (Table II). Although the administration of mitoxantrone 5 min or 1 h after intravenous injection of the tumor cells did not inhibit colony formation in the lungs, treatment with mitoxantrone two days after the injection of tumor cells remarkably reduced the number of lung colonies.

Effects of mitoxantrone on angiogenesis in CAM Mitoxantrone inhibited angiogenesis in CAM in a dose-dependent manner. At a dose of 1000 ng/egg of mitoxantrone the drug strongly inhibited the growth of blood vessels and one of the 5 embryos died. The ED_{50} of mitoxantrone was about 10 ng/egg, and those of pirarubicin and doxorubicin were about 700 and 550 ng/egg, respectively.

Effects of mitoxantrone and pirarubicin on endothelial cells and Co 26Lu cells New capillary blood vessels are essential for the continuous growth of a tumor. Inhibition of angiogenesis may inhibit tumor growth. Therefore, it seemed important to investigate the sensitivity of endothelial cells and tumor cells to mitoxantrone and pirarubicin. Rat lung endothelial cells were used. Mitoxantrone and pirarubicin inhibited the growth of endothelial cells at IC_{50} (50% inhibition concentration) values of 5.5 and 15 ng/ml, respectively (Fig. 3A). On the other hand, the IC_{50} values of mitoxantrone and pirarubicin

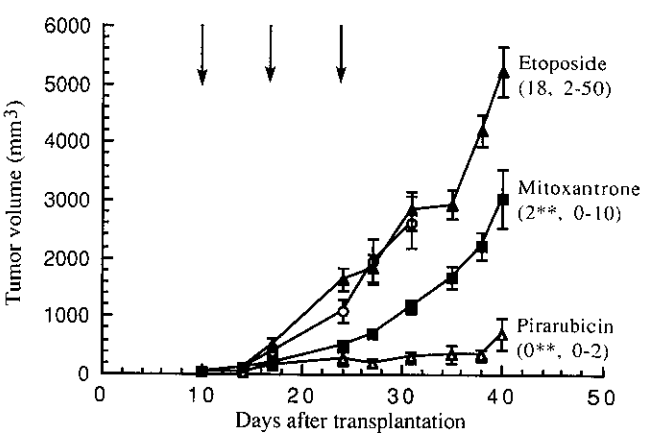

Fig. 2. Effects of mitoxantrone, pirarubicin and etoposide at maximum doses on primary tumor growth and number of metastases in the lung. The number of lung metastases was determined on day 31 (control group) and day 40 (treatment groups). The median number of metastases in the untreated control group (○) was 23 (range, 14–31). The doses of mitoxantrone (■), pirarubicin (△) and etoposide (▲) were 1.25, 6.25 and 25 mg/kg, respectively. Arrows indicate the days of i.v. treatment with each drug. ( ) = (median number of metastases, range). **, $P<0.01$ compared with untreated group.

Table II. Effects of the Time Elapsed between Tumor Cells Injection (i.v.) and Administration of Mitoxantrone on the Number of Lung Colonies of Co 26Lu

Time elapsed between tumor inoculation and administration of mitoxantrone[a]	Median no. of lung colonies (no. of colonies in each animal)[b]
Control (untreated)	5 (3, 3, 5, 5, 8, 9, 11)
−60 min	4 (3, 4, 4, 4, 6, 9, 10)
5 min	3 (0, 0, 2, 3, 7, 12, 23)
60 min	5 (0, 2, 3, 5, 5, 5, 5)
2 days	1** (1, 1, 1, 1, 1, 2, 5)

a) Mitoxantrone was administered i.v. at a dose of 0.63 mg/kg before (−60 min) and after (5 min, 60 min and 2 days) i.v. injection of Co 26Lu cells (5×10^3 cells).
b) The number of lung colonies was determined on day 12.
**, $P<0.01$, compared with control.

against Co 26Lu cells were 38 and 33 ng/ml, respectively (Fig. 3B). Mitoxantrone showed marked inhibition of endothelial cell growth at any concentration above 5 ng/ml compared with its effect on tumor cells. Pirarubicin markedly inhibited the growth of endothelial cells at concentrations of 15–100 ng/ml and inhibited the growth of both endothelial cells and Co 26Lu cells at concentrations above 100 ng/ml.

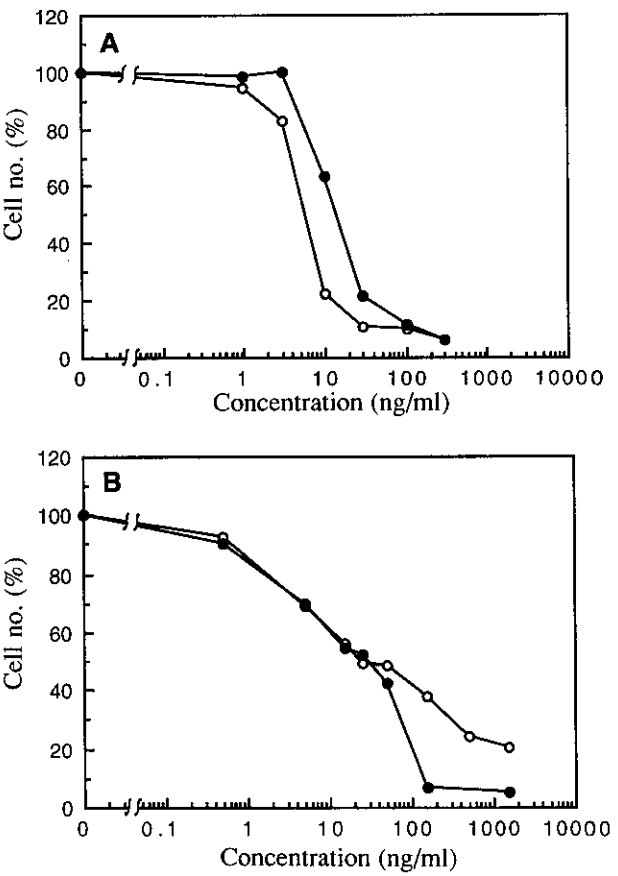

Fig. 3. Effect of increasing concentrations of mitoxantrone (○) and pirarubicin (●) on the growth of rat lung endothelial cells (A) and Co 26Lu cells (B). The number of viable cells is expressed as the percentage of control values (without drugs). Each point represents the mean of at least three separate experiments.

DISCUSSION

We have successfully established a highly metastatic tumor (Co 26Lu) derived from Co 26 and have maintained it *in vivo* in our laboratory. Many micrometastases can be seen in the lungs in mice bearing subcutaneously implanted Co 26Lu. No visible tumors are detected in organs other than the lungs. When Co 26Lu cells are injected via the tail vein, tumor colonies in the lungs and survival times are almost the same as those obtained with Co 26 cells. When injected intravenously, both tumor cell lines have almost the same ability to form colonies in the lungs. Co 26Lu cells may be able to detach more easily from the primary tumor mass and penetrate into the blood circulation compared with Co 26 cells. We used these two tumors (Co 26 and Co 26Lu) to investigate the effect of antitumor agents on lung colonies. We have already examined the effects of various antitumor agents on liver metastases of Co 26.[2] Among the 17 antitumor agents used in clinical practice in Japan, mitomycin-C was found to be quite potent.[2] In this study, however, mitomycin-C showed only a weak effect on lung metastases even though these metastases had originated from Co 26. Among the anthracyclines, doxorubicin was the most effective agent in mice bearing Co 26 in the liver (138% ILS), and pirarubicin (55%) was significantly less effective than doxorubicin ($P<0.05$).[2] On the other hand, in mice bearing Co 26 in the lungs, pirarubicin showed the highest ILS%; it was significantly more active in this regard than doxorubicin ($P<0.05$). It is of interest that the activity of anthracycline derivatives against Co 26 differed depending upon the site (organ) of tumor growth. The difference in activity between the lungs and liver may be due to different distributions of pirarubicin and doxorubicin within the body. When the same doses of pirarubicin and doxorubicin are administered, pirarubicin levels in the lungs are higher than those of doxorubicin, while doxorubicin levels in the liver are higher than those of pirarubicin.[5] These findings suggest that, if the tumor is sensitive to anthracyclines, doxorubicin may be the best therapeutic choice in the case of metastases in the liver, and pirarubicin for metastases in the lung.

On the other hand, mitoxantrone has been found to be ineffective against Co 26 in the liver (19%) and subcutaneously implanted Co 26 (23%),[2] but in the present study it demonstrated a marked effect against lung metastases of this tumor. Distribution of mitoxantrone to the lungs is fairly low compared to the liver.[6] Therefore, the different effect of this drug against metastases in the lungs and liver cannot be explained only in terms of the distribution of the drug. Mitoxantrone showed stronger inhibition of lung colonization when administered on day 2 than when administered on day 0. In the case of subcutaneously implanted Co 26Lu, new capillary blood vessels were formed vigorously two days after tumor implantation but were markedly inhibited by treatment with mitoxantrone (data not shown). Moreover, mitoxantrone markedly inhibited angiogenesis on the chorioallantoic membrane at very low doses without affecting the growth rate of the embryos. Polverini and Navak also reported inhibition of angiogenesis by mitoxantrone using the avascular cornea of rat eyes.[7] Formation of new capillary blood vessels is essential for the continuous growth of lung colonies. Inhibition of metastases by mitoxantrone may be due to inhibition of angiogenesis in the lung colonies of the tumor. Mitoxantrone effectively inhibits prostaglandins (PGs) production.[8] PGs are known to play a key role during tumor development. PGE1 and PGE2 have been reported to induce vascular endothelial growth factor expression[9] and to stimulate capillary formation *in vivo*.[10] However, in our tumor system, indomethacin and acetylsalicylic acid, inhibitors of PGs, did not inhibit the number of lung metastases (data not shown). Metastases of this tumor may not be influenced by PGs levels. Mitoxantrone directly affects endothelial cells and inhibits angiogenesis and thereby the formation of metastases. Based on the results presented here we think that combination therapy with mitoxantrone and pirarubicin may prove effective on primary tumor and lung metastases. Moreover, mitoxantrone may be useful as an adjuvant chemotherapy to prevent lung metastases, even if the primary tumor is not sensitive to this drug.

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