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ORIGINAL ARTICLE

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Host distribution and response to antitumor alkylating agents of EMT-6 tumor cells from subcutaneous tumor implants

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Abstract *Purpose*: Minimal residual tumor or minimal residual metastatic disease is a major clinical problem for detection and treatment. The purpose of the study was to develop a model system to detect the occurrence and response to therapy of minimal residual tumor in distant organs. *Methods*: Animals bearing subcutaneously growing established (day 8) murine EMT-6 mammary carcinoma tumors were treated with single doses of the antitumor alkylating agents, cyclophosphamide, melphalan, *cis*-diamminedichloroplatinum(II) (CDDP) or thiotepa. Tumors, livers, lungs, brain, spleen, blood and bone marrow were collected from the animals 24 h later and single-cell suspensions of these tissues were plated and cultured under conditions suitable for tumor cell colony growth. *Results*: Tumor cell colonies grew from each of the tissues with varying frequency ranging from about 6×10^3 tumor cell colonies per 10^6 cells plated from the liver, to about 2 tumor cell colonies per 10^6 cells plated from the brain. There was a wide range of sensitivity, spanning 2- to 3-log of the tumor cells, to the antitumor alkylating agents depending upon the tissue in which the tumor cells were located. Tumor cells in the circulating blood were most sensitive to the antitumor alkylating agents with no colony growth after treatment of the animals with any of the four drugs tested. The primary tumor growing subcutaneously in the upper hindleg of the animals was also relatively sensitive to each of the four antitumor alkylating agents tested. EMT-6 tumor cells in the spleen were very sensitive to cyclophosphamide, moderately sensitive to melphalan, less sensitive to CDDP and least sensitive to thiotepa. EMT-6 tumor cells in the bone marrow were moderately sensitive to cyclophosphamide, melphalan and thiotepa but less sensitive to CDDP. EMT-6 tumor cells in the lungs were relatively sensitive to thiotepa, moderately sensitive to cyclophosphamide and CDDP and least sensitive to melphalan. EMT-6 tumor cells in the liver or brain were least responsive to treatment of the host with any of the four antitumor alkylating agents tested. *Conclusions*: Treatment of the tumor-bearing animals with the antiangiogenic combination, TNP-470/minocycline, markedly increased EMT-6 tumor cell killing by cyclophosphamide in the liver, lungs and bone marrow. These results indicate that location within the host is an important determinant in the response of tumor cells to therapy.

Key words Detection of micrometastases · Metastatic disease response · Tumor tissue distribution · Tumor/host interaction

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Introduction

Systemic treatment of malignant disease now has a modern history of more than 50 years. Over that time, the greatest successes of chemotherapy in curing cancer have been in leukemia, lymphoma and selected solid tumors such as testis cancer. Progress with chemotherapy in the treatment of many of the most common solid tumors continues to be incremental with increasing numbers of long-term survivors. The administration of very high doses of chemotherapy requiring hematopoietic stem cell support for the treatment of solid tumors, especially breast cancer, has had a history of more than 10-years [7, 9, 13, 20, 23]. Even with this heroic treatment, cure of solid tumors such as breast cancer and small-cell lung cancer remains elusive

[1, 8, 10]. High-dose chemotherapy regimens with hematopoietic stem cell support have allowed the frequency of complete clinical response to be markedly increased; however, during the first 1–2 years after therapy, tumors in most patients recur. These results as well as the predictable times and patterns of recurrence of many solid tumors after standard courses of chemotherapy or standard regimens of radiation therapy have led to the hypothesis that a few viable malignant cells with proliferative capacity remain after therapy and eventually repopulate the primary tumor and/or grow into metastatic lesions. These "minimal residual tumor" cells exist at levels below detectability by standard clinical techniques.

More than 100 years ago Paget recognized from his autopsy studies that malignant diseases had very predictable patterns of growth and spread (metastasis) [19, 22]. In modern times, physicians recognize patterns of response to chemotherapy and patterns of relapse. In breast cancer patients, for example, lymph node and skin metastases tend to respond better to chemotherapy than lung and bone metastases [24]. Preclinical studies have also demonstrated that organ or tissue location can affect tumor growth, gene expression, and response to therapy [2, 4, 5, 11, 15, 17, 25, 31, 36, 37].

Treatment regimens consisting of high-dose combination chemotherapy with hematopoietic stem cell transplantation have been under clinical investigation for more than 10 years [3, 6, 8, 10, 12, 16, 18, 20, 21, 35]. The "efficacy" of this treatment approach, however, remains an open question. Preclinical in vivo modeling allows rigorous examination of many aspects of treatments, treatment combinations, sequences, and so on, that would be impossible to approach by clinical trial because the number of variables involved would require a prohibitively large number of patients. The study of high-dose therapy preclinically has been hampered by the lack of appropriate models [26]. The scientific study of cancer therapy relevant to the high-dose setting has required the development of preclinical models that go beyond the conventional dose endpoints of increase in lifespan and tumor regression/growth delay. High-dose therapy can be modeled using the tumor cell survival assay that allows tumor-bearing animals to be treated with "supralethal" doses of anticancer treatments with a quantitative measure of tumor cell killing [14, 26].

One important difference between excision assays and the in situ assays of increase in life span, tumor regression/growth delay or local tumor control is that excision assays require removal of the tumor from the environment in which it was treated. This difference and the nature of the assay procedure lead to a number of advantages and disadvantages in using excision assays rather than in situ assays. The ability to measure cell survival directly is important because it gives basic information about what is the ultimate definitive cellular effect. Tumor excision assays also allow greater accuracy and finer resolution between various therapeutic regimens than do the in situ assays. Perhaps the greatest disadvantage of excision assays is that extended treatment regimens cannot be used owing to tumor cell loss and tumor cell proliferation over the treatment time. Thus, an excision assay provides a static picture of tumor response at a short time after treatment. The use of excision assays to determine survival curves for tumor cells treated in vivo can provide insights concerning both treatment efficacy and tumor biology. Like the human disease, the murine EMT-6 mammary carcinoma is metastatic when implanted subcutaneously into the female Balb/C syngeneic host. In the study reported here, we examined EMT-6 tumor cell distribution in the host from subcutaneous tumors and the response of the tumor cells in several organs to high-dose antitumor alkylating agent therapy.

Materials and methods

Drugs

Cyclophosphamide (CTX), melphalan (PAM), *cis*-diamminedichloro-platinum II (CDDP) and minocycline were purchased as the pure powders from Sigma Chemical Co. (St. Louis, Mo.). Thiotepa was purchased from the Dana-Farber Cancer Institute pharmacy. TNP-470 was a gift from Takeda Pharmaceuticals (Osaka, Japan).

Tumor

The EMT-6 murine mammary carcinoma was carried in female Balb/C mice (Taconic Farms, Germantown, N.Y.). For experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted subcutaneously into both hindlegs of female, Balb/C mice at 8 to 10 weeks of age [31].

Tumor cell survival assay

For the experiments, cells prepared from a brei of several stock tumors were implanted subcutaneously into both hindlegs of female, Balb/C mice. When the volume of the tumors reached approximately 200 mm^3, the animals were left untreated or were treated with single intraperitoneal doses of the antitumor agents. Animals were killed 24 h after chemotherapy treatment and were then immersed in 95% ethanol. The tumors, livers, lungs, bone marrow, peripheral blood, spleens and brains were collected under sterile conditions, weighed and single-cell suspensions were prepared from each tissue for the colony forming assay [29, 30].

Blood

Blood was collected by cardiac puncture from Metophane-anesthetized animals using a heparinized 26-gauge needle and syringe. A known volume of blood was added to Waymouth's medium (5 ml; Mediatech, Fisher Scientific, Pittsburgh, Pa.). After centrifugation, the pellet was resuspended in 4 ml aqueous 0.17 *M* ammonium

chloride solution, rapidly mixed and centrifuged. After decanting the supernatant, the pellet was resuspended in Waymouth's medium and all of the nucleated cells were counted. Known numbers of cells were plated in a monolayer for colony formation in Waymouth's medium containing 15% newborn calf serum (Hyclone Laboratories, Logan, Utah), penicillin (10 U/ml) and streptomycin (10 μg/ml) (GIBCO, Grand Island, N.Y.) and gentamicin (100 μg/ml; Mediatech, Fisher Scientific, Pittsburgh, Pa.) as described below.

Spleen

After removal from the animals the spleens were placed in a drop of Waymouth's medium on the frosted end of a sterile microscope slide, then disaggregated by compressing the tissue with a second microscope slide. The cells were washed from the slides and filtered through sterile gauze to remove the capsule. After centrifugation, the pellet was resuspended in 4 ml aqueous 0.17 *M* ammonium chloride solution. The cell suspension was rapidly mixed, then centrifuged and the pellet was resuspended in Waymouth's medium. All of the nucleated cells in an aliquot of the cell suspension were counted and known numbers of cells were plated in a monolayer for colony formation in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin as described below.

Bone marrow

A pool of marrow from four femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle and dispersing it into 4 ml Waymouth's medium. After centrifugation, the cells were resuspended in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin. All nucleated cells were counted and known numbers of cells were plated in a monolayer for colony formation in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin as described below.

Brain

The brains were removed and placed with 5 ml Waymouth's medium into a Wheaton Potter-Elvehjem tissue homogenizer (Fisher Scientific). The brains were disaggregated by five passes of the tissue homogenizer without shear. The cell suspension was then centrifuged and the pellet resuspended in 10 ml Waymouth's medium containing 15% newborn calf serum. All of the nucleated cells were counted and known numbers of cells were plated in a monolayer for colony formation in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin as described below.

Lungs

The lungs were removed and minced to a fine brei with crossed scalpels. The lung brei was suspended in 10 ml Waymouth's medium containing 1 ml DNase (0.1 mg/ml; Sigma) and 1 ml collagenase (450 U/ml; Sigma). The suspension was incubated at 37 °C for 3 h in a shaking water bath. The suspension was filtered through sterile gauze. After centrifugation, the pellet was resuspended in Waymouth's medium containing 15% newborn calf serum. All nucleated cells were counted and known numbers of cells were plated in monolayers for colony formation in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin as described below.

Liver

The livers were removed and minced to a fine brei with crossed scalpels. The liver brei was suspended in 10 ml Waymouth's medium containing 1 ml DNase (0.1 mg/ml; Sigma) and 1 ml collagenase (450 U/ml; Sigma). The suspension was incubated at 37 °C for 30 min in a shaking water bath and then filtered through sterile gauze. After centrifugation, the pellet was resuspended in Waymouth's medium containing 15% newborn calf serum. All nucleated cells were counted and known numbers of cells were plated in a monolayer for colony formation in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin as described below.

Tumor

The tumor was removed and minced to a fine brei with crossed scalpels. The tumor brei was suspended in 10 ml Waymouth's medium containing 1 ml DNase (0.1 mg/ml; Sigma) and 1 ml collagenase (450 U/ml; Sigma). The suspension was incubated at 37 °C for 30 min in a shaking water bath and then filtered through sterile gauze. After centrifugation the pellet was resuspended in Waymouth's medium containing 15% newborn calf serum. All nucleated cells were counted and known numbers of cells were plated in a monolayer for colony formation in Waymouth's medium containing 15% newborn calf serum, penicillin and streptomycin, and gentamicin as described below.

Colony formation

Cells were plated at various densities from 10^5 up to 10^8 in 100-mm dishes and placed in a humid incubator at 37 °C in an atmosphere of air containing 5% carbon dioxide. After 2 weeks, the plates were stained with crystal violet and tumor cell colonies of ≥ 50 cells were counted. The results were expressed as the surviving fraction $\pm$ SEM of cells from treated groups as compared with untreated controls $\pm$ SEM.

Results

To determine the "natural" metastatic pattern for the EMT-6 tumor, we excised several target tissues from animals bearing subcutaneous EMT-6 tumors on day 9 post-tumor cell implant when the primary tumors were 200 mm^3 in volume. Conditions for preparation of single-cell suspensions from each organ or tissue were adjusted to maximize cell yield as detailed in the Materials and methods. Table 1 shows the nucleated cell yields from the various tissues. When expressed on a per gram tissue basis, the spleen yielded 359×10^6 cells, the lungs yielded 88×10^6 cells, the tumor yielded 71×10^6 cells, the brain yielded 48×10^6 cells, the liver yielded 9.4×10^6 cells and the blood yielded 2.4×10^6 cells.

When known numbers of nucleated cells from each tissue were plated under growth conditions suitable for the proliferation of EMT-6 cells, tumor cell colonies grew from the tissues with varying frequency ranging from about 6.0 ($\pm$1.5) $\times 10^3$ tumor cell colonies per 10^6 cells plated from the liver, to about 2.0 ($\pm$1.0) tumor

Table 1 Yields of single, nucleated cells from various organs, tissues and the EMT-6 tumor in Balb/C mice (Methods for the disaggregation of the tissues are given in detail in the Methods section)

Organ or tissue	Cell yield	Organ or tissue amount
EMT-6 tumor	106×10^6	1.49 g
Liver	17.2×10^6	1.84 g
Bone marrow	44.4×10^6	Not weighed
Brain	36.3×10^6	0.76 g
Lungs	22.0×10^6	0.25 g
Spleen	176×10^6	0.49 g (enlarged)
Blood	2.4×10^6	1 ml

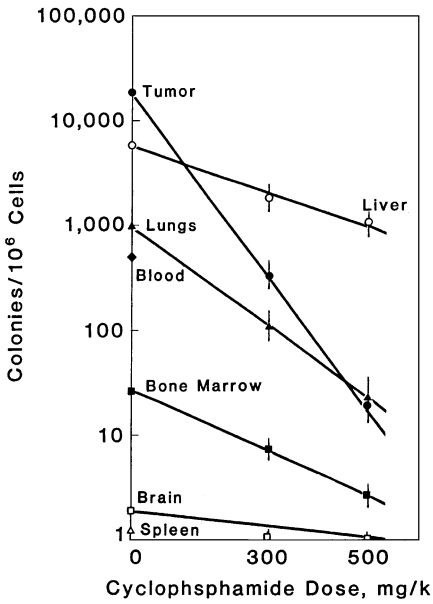

Fig. 1 EMT-6 tumor cell colonies per 10^6 cells plated in culture from various organs and tissues isolated from animals bearing the EMT-6 tumor on day 9 after tumor cell implantation with no treatment (0 mg/kg of cyclophosphamide), or after administration of cyclophosphamide (300 or 500 mg/kg) on day 8

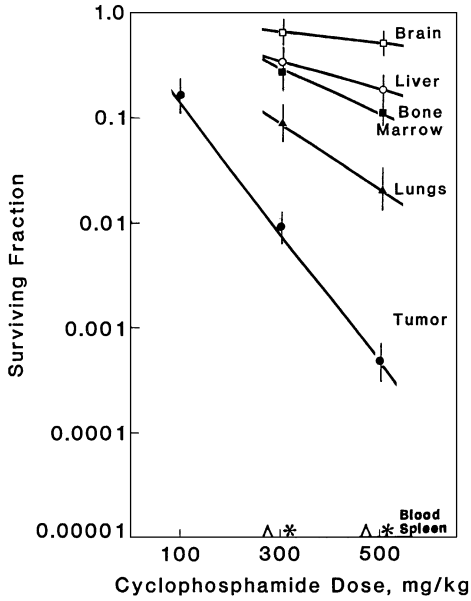

Fig. 2 Survival of EMT-6 tumor cells grown from the various organs and tissues of animals bearing the EMT-6 tumor after treatment of the animals with cyclophosphamide (100, 300 or 500 mg/kg) on day 8 and tissue excision on day 9. Points are the means of three independent determinations

cell colonies per 10^6 cells plated from the brain (Fig. 1, 0 mg/kg CTX). When known numbers of nucleated cells were also plated from nontumor-bearing control animals, cells (but not colonies) were present only on dishes plated with cells from the liver. The background of cells from the liver were readily distinguishable from the colonies of EMT-6 tumor cells. When the animals were treated with CTX, the dose response of the EMT-6 tumor cells varied depending upon the tissue site of the disease (Figs. 1, 2). As can be seen from the data presented traditionally as surviving fraction in Fig. 2, tumor cells located in the brain, liver, lungs and bone marrow were less responsive to CTX than was the primary tumor, while no tumor colonies grew from blood or from the spleen after CTX treatment. Similar experiments were carried out with EMT-6 tumor-bearing animals treated on day 8 with CDDP, PAM or thiotepa followed by tumor and normal tissue excision on day 9 (Figs. 3–5). Tumor cells in the circulating blood were most sensitive to the antitumor alkylating agents, with no colony growth after treatment of the animals with any of the four drugs tested. The primary tumor growing subcutaneously in the upper hindleg of the animals was also relatively sensitive to each of the four antitumor alkylating agents tested. EMT-6 tumor cells in the spleen were very sensitive to CTX (SF < 0.00001), moderately sensitive to PAM and CDDP (SF $=$ 0.03–0.08) and less sensitive to thiotepa (SF $= 0.2$–0.6). EMT-6 tumor cells in the bone marrow were moderately sensitive to CTX, PAM and thiotepa but less sensitive to CDDP. EMT-6 tumor cells in the lungs were relatively sensitive to thiotepa, moderately sensitive to CTX and CDDP and least sensitive to PAM. EMT-6 tumor cells in the liver or brain were least responsive to treatment of the host with any of the four antitumor alkylating agents tested with EMT-6 tumor cells in the brain responding moderately to PAM and EMT-6 tumor cells in the liver responding moderately to CDDP.

The range of tumor cell killing with these four anticancer agents spanned 2- to 3-log depending upon the tissue in which the tumor cells were located. The administration of relatively nontoxic agents described as potentiators or modulators to improve the efficacy of standard cytotoxic anticancer therapies has been explored extensively in preclinical solid tumor models [26–28]. A modulator combination that has shown promise is the antiangiogenic agent TNP-470 along with the collagenase inhibitor minocycline [32–34].

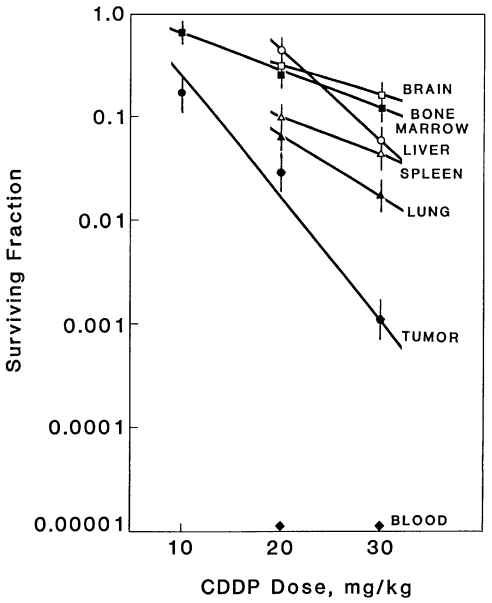

Fig. 3 Survival of EMT-6 tumor cells grown from the various organs and tissues of animals bearing the EMT-6 tumor after treatment of the animals with CDDP (10, 20 or 30 mg/kg) on day 8 and tissue excision on day 9. Points are the means of three independent determinations

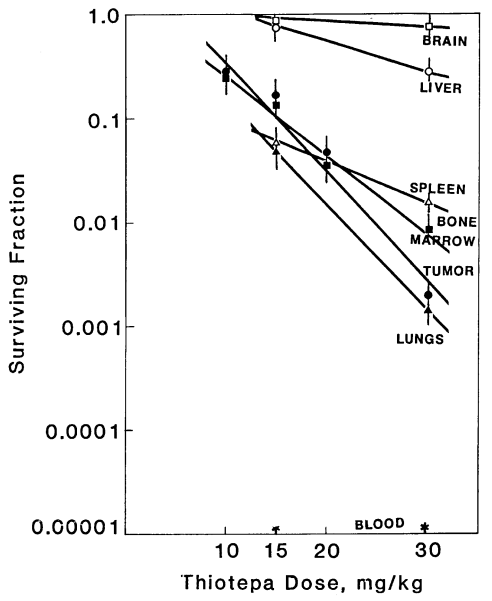

Fig. 5 Survival of EMT-6 tumor cells grown from the various organs and tissues of animals bearing the EMT-6 tumor after treatment of the animals with thiotepa (10, 15, 20 and 30 mg/kg) on day 8 and tissue excision on day 9. Points are the means of three independent determinations

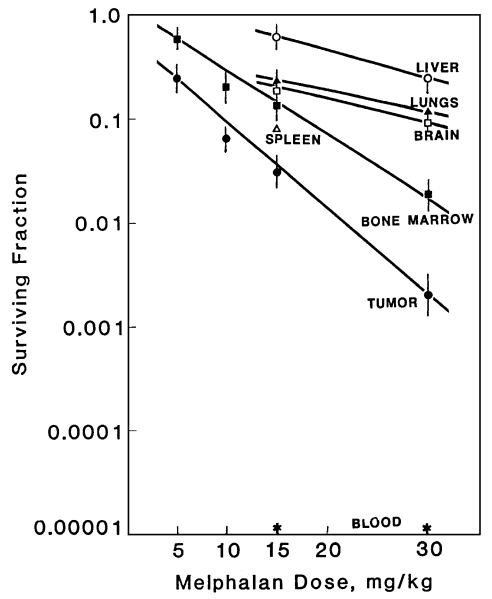

Fig. 4 Survival of EMT-6 tumor cells grown from the various organs and tissues of animals bearing the EMT-6 tumor after treatment of the animals with melphalan (5, 10, 15 or 30 mg/kg) on day 8 and tissue excision on day 9. Points are the means of three independent determinations

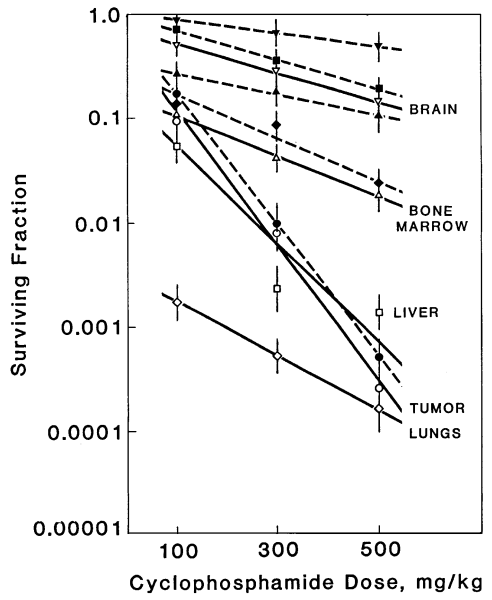

Fig. 6 Survival of EMT-6 tumor cells grown from the various organs and tissues of animals bearing the EMT-6 tumor after treatment of the animals with cyclophosphamide alone (*dotted lines*) or after treatment of the animals with TNP-470 (30 mg/kg) subcutaneously on days 4, 6 and 8 along with minocycline (10 mg/kg) intraperitoneally on days 4 through 8 then cyclophosphamide (100, 300 or 500 mg/kg) on day 8 with tissue excision on day 9 (●, ○ tumor; ■, □ liver; ◆, ◇ lungs; ▲, △ bone marrow; ▼, ▽ brain). Points are the means of three independent determinations

When animals were treated with TNP-470 and minocycline on days 4 through 8 and then with single doses of CTX, the tumor cell killing in the various tissues shown in Fig. 6 was obtained. No tumor cell colonies grew from the circulating blood or spleen of these animals. Treatment with the modulator combination produced no change in the sensitivity of primary tumor to treatment with CTX. However, there was a

1- to 2-log increase in the killing of EMT-6 tumor cells in the liver and lungs, a five- to eightfold increase in the killing of EMT-6 tumor cells in the bone marrow and a two- to fivefold increase in the killing of EMT-6 cells in the brain when the animals were treated with TNP-470 and minocycline prior to CTX.

Discussion

In this study, the tumor cell survival assay was expanded to include not only the response of the primary tumor to therapy but also the response of micrometastatic disease in distal normal tissues to the same therapy. By adjusting the tissue disaggregation technique, cell yields were obtained from each tissue which readily allowed the plating of millions of nucleated cells from the tissues and thus allowed the detection of viable proliferating malignant cells to about one per million cells. Tumor cells were detectable in each of the seven tissues studied. The range of sensitivity of EMT-6 tumor cells distributed through the host to treatment with each of the four antitumor alkylating agents spanned at least 6 log. Tumor cells in circulating blood were the most sensitive and, generally, tumor cells in the brain and liver were the least sensitive.

Although viable tumor cells could be isolated from each of the six normal tissues studied, it is not known whether these tumor cells would have grown into clinically important metastatic disease. While, clearly, the EMT-6 tumor cells were sensitive to each of the antitumor alkylating agents studied, the dominant variable in controlling the degree of tumor cell killing was location within the host. In other words, to varying degrees the normal tissues protected the malignant cells from the cytotoxic therapies. The capacity of the normal tissue to detoxify or exclude the cytotoxic agents was the critical therapeutic variable. Treatment with the antiangiogenic combination of TNP-470 and minocycline has been shown to increase the uptake of [^{14}C] CTX, Pt from CDDP, Hoechst 33342 and oxygen into Lewis lung tumors and into many of the normal tissues of mice bearing Lewis lung tumors [27, 32–34]. In this study, animals bearing the EMT-6 mammary carcinoma showed markedly increased tumor cell killing in the lungs and liver and lesser changes in the killing of tumor cells in other tissues after 5 days of treatment with TNP-470 and minocycline. It is likely that both the tumor, especially its angiogenic profile, and the normal tissue were variables in this response. It may be possible to select and/or develop modulators to target increased cytotoxic drug sensitivity depending upon the site(s) of metastatic disease.

This preclinical model demonstrates the wide variability in tumor response to therapy within the host and may also help to elucidate clinically relevant means of increasing therapeutic sensitivity in specific tissues prone to metastatic dissemination.

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