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Minocycline in combination with chemotherapy or radiation therapy in vitro and in vivo*

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Summary. In the present study the potential of minocycline, a semisynthetic tetracycline that inhibits collagenase activity in vivo, as an adjuvant to standard anticancer therapies was explored in vitro and in vivo. In EMT-6 cells, minocycline proved to be only minimally cytotoxic, producing a 50% cell kill at concentrations of 132 and 220 μM in normally oxygenated and hypoxic cells, respectively, after 24 h exposure to the drug. In vitro, there appeared to be no interaction between minocycline and cisplatin (CDDP), melphalan, 4-hydroperoxycyclophosphamide, or radiation. In tumor-cell survival studies using the FSaIIC murine fibrosarcoma, short-term treatment with minocycline (5 × 5 mg/kg given over 24 h) was only minimally cytotoxic and did not alter the tumor response to a range of radiation doses. However, when minocycline (5 × 5 mg/kg given over 24 h) was added to treatment with cyclophosphamide, there was a 4-fold increase in FSaIIC tumor-cell killing across the dose range of cyclophosphamide doses tested, whereas the killing of bone marrow granulocyte macrophage colony-forming units (CFU-GM) remained unchanged. The Lewis lung carcinoma was used to assess the response of both the primary tumor and metastatic lung disease to treatment with minocycline (14 × 5 mg/kg) given alone or in combination with several cytotoxic anticancer drugs or with radiation delivered locally to the primary tumor. Of the various therapies tested, minocycline proved to be especially effective as an addition to treatment with cyclophosphamide both in increasing the response of the primary tumor and in reducing the number of lung metastases. The tumor growth delay produced by melphalan, radiation, Adriamycin, and bleomycin was also increased by the addition of minocycline to these therapies. These results indicate that minocycline given in clinically achievable doses may be an effective addition to some standard therapeutic regimens and that the mechanism of modulation by minocycline is likely to involve an effect of the drug on the host and not its direct interaction with other therapeutic modalities at the level of the tumor cell.

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Introduction

Cancer is a systemic problem whose cure requires eradication of both the primary tumor and any metastatic disease. It has long been recognized that tumors, like normal tissues, require the delivery of nutrients and oxygen through the vasculature for growth and that tumors develop a vascular supply by recruitment of normal tissue vasculature and through neovascularization (angiogenesis) [6–9, 11, 12, 41, 58, 61]. The process of angiogenesis enables the growth of primary and metastatic solid tumor masses [6–9, 11, 12, 57, 58]. The utility of an angiostatic agent in cancer therapy would be to inhibit the further growth of tumor masses, although retraction of some neovasculature may also be possible [8, 12].

The search for antiangiogenic substances has primarily led to the discovery of proteins and small molecules that inhibit various steps in the breakdown of the basement membrane [55, 56]. These include naturally occurring proteins such as protamine [50]; interferon-α [17, 60]; platelet factor [50]; tissue inhibitors of metalloproteinases (TIMPs) [47, 59]; peptides derived from cartilages [26, 29], vitreous humor [49], smooth muscle [4], and aorta [5]; as well as synthetic peptides such as synthetic laminin peptide (CDPG) YIGSR-NH_2 [42] and somatostatin analogs such as somatuline [1]. Antiangiogenic small molecules include naturally occurring heparins [12], a variety of steroids [3, 9, 10, 28], several retinoids [20, 25, 31, 43], warfarin [2], and fumagillin [21, 24], as well as synthetic agents such as sulfated chitin derivatives [30], sulfated cyclodextrins [12], SC44463 [35], SC39026 [48], derivatives of fumagillin [21], and minocycline [48]. Radiation also inhibits

blood vessel growth [22, 23, 32, 34]. Many of these agents function by inhibiting enzymes that degrade the basement membrane. Therefore, in addition to being antiangiogenic, these treatments may function as antimetastatic agents by preventing invasion of tumor cells through the basement membrane [55–57].

Collagenase is an enzyme that degrades collagen IV and is secreted by both tumor cells and normal host cells. It has been recognized for some time that the tetracyclines can inhibit tissue collagenase activity, and tetracycline administration has been used in the treatment of periodontal disease [13] and of gingival collagenolytic activity in diabetes [13, 15] and to inhibit joint deterioration in patients with rheumatoid arthritis [16, 62]. Among the tetracyclines, the semisynthetic derivative minocycline is a relatively potent collagenase inhibitor [14]. Minocycline also has a relatively high circulating half-life of about 12 h and is highly lipid-soluble and thus may have a favorable tissue-penetrating ability [14, 48, 62]. Recently, Tamargo et al. [48] reported that minocycline inhibited neovascularization in rabbit corneas implanted with the VX2 carcinoma.

On the basis of these data, our laboratory began investigating the interaction of antiangiogenic agents with various cytotoxic anticancer treatments. In the present report we describe the effect of the addition of minocycline to cancer therapies in vitro and in vivo on both primary and metastatic disease.

Materials and methods

Drugs. Minocycline, melphalan, cyclophosphamide, and 5-fluorouracil were purchased from Sigma Chemical Co. (St. Louis, Mo.). *cis*-diamminedichloroplatinum(II) (CDDP) was a gift from Dr. A. Crosswell, Bristol-Myers-Squibb Co. (Wallingford, Conn.). 4-Hydroperoxycyclophosphamide (4-HC) was a gift from Drs. P. Hilgard and J. Pohl, Asta Pharma (Bielefeld, FRG). All other drugs were purchased from the Dana-Farber Cancer Institute pharmacy.

Cell culture. EMT-6 mouse mammary tumor cells have been widely used for the study of hypoxia [37–39]. Cultures were maintained in exponential growth in Waymouth's medium (I.S.I. Corp., Chicago, Ill.) supplemented with 15% newborn calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml); Grand Island Biological Co., Grand Island, N.Y.). The doubling time of cultures growing at 37° C in a humidified atmosphere of 5% CO_2/95% air was 16–19 h [40]. In vitro plating efficiencies of control cultures ranged from 65% to 80%.

To produce hypoxia, we fitted plastic flasks containing exponentially growing monolayers in complete medium plus serum with sterile rubber septa and exposed them to a continuously flowing 95% N_2/5% CO_2 humidified atmosphere for 4 h at 37° C as previously reported [52, 53]. Parallel flasks were maintained in 95% air/5% CO_2. At the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septum without disturbing the hypoxia. EMT-6 cells were exposed to various concentrations (5, 10, 50, 100, 250, or 500 μM) of minocycline under normally oxygenated conditions for 24 h or under hypoxic conditions for 5 h followed by normally oxygenated conditions for 19 h. In combination-treatment experiments, EMT-6 cells were exposed to 100 μM minocycline for 4 h prior to other treatment, during 1 h exposure to melphalan, 4-hydroperoxycyclophosphamide, or CDDP or during radiation delivery, and for an additional 19 h. In the combination studies, hypoxia was maintained for the first 5 h of drug exposure.

Cell viability was measured by the ability of single cells to form colonies in vitro as described elsewhere [52, 53]. Each experiment was repeated three to five times, and each data point per experiment represented the results of three different dilutions of cells plated in triplicate.

Tumors. The FSaII fibrosarcoma [36] adapted for growth in culture (FSaIIC) [51] was carried in male C3H/He mice (Jackson Laboratory, Bar Harbor, Me.). The Lewis lung tumor [44–46] was carried in male C57BL mice (Taconic Laboratories, Germantown, N.Y.). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted s. c. into the legs of male mice aged 8–10 weeks.

Tumor excision assay. When the tumors had reached a volume of approximately 100 mm^3 (about 1 week after tumor-cell implantation), the animals were treated with various doses of radiation or were given i.p. injections of various doses of minocycline or cyclophosphamide alone, or minocycline (5×5 mg/kg) was given over 24 h, with X-rays or cyclophosphamide being given with the third minocycline dose. Mice were killed at 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised and single-cell suspensions were prepared as previously described [51, 54]. The plating efficiency of untreated tumor-cell suspensions ranged from 10% to 16%. The results were expressed as the surviving fraction (± SE) of cells from treated groups as compared with untreated controls [54].

Bone marrow toxicity. Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle, and a colony-forming unit-granulocyte macrophage (CFU-GM) assay was carried out as previously described [54]. Colonies of at least 50 cells were scored on an Acculite colony counter (Fisher Scientific, Springfield, N.J.). The results of three experiments, in which each group was measured in duplicate at three cell concentrations, were averaged. The results were expressed as the surviving fraction of treated groups as compared with untreated controls.

Tumor growth-delay experiments. By day 4 after tumor-cell implantation, Lewis lung tumors had begun neovascularization [18, 19]. Animals bearing Lewis lung tumors were treated daily with i.p. minocycline (5 mg/kg) on days 4–18 following tumor implantation. When the tumors had reached a volume of approximately 100 mm^3 in volume (day 7 after tumor-cell implantation), cytotoxic therapy was initiated. CDDP (10 mg/kg), melphalan (10 mg/kg), and cyclophosphamide (150 mg/kg) were injected i.p. on day 7. Cyclophosphamide (150 mg/kg) was given on days 7, 9, and 11 following tumor implantation. Radiation was delivered locally to the tumor-bearing limb as 20 Gy given on day 7 or as 3 Gy given daily on days 7–11. 5-Fluorouracil (40 mg/kg) and etoposide (15 mg/kg) were given daily i.p. on days 7–10. Methotrexate (0.8 mg/kg) and Adriamycin (1.75 mg/kg) were injected daily i.p. on days 7–11. Bleomycin (10 mg/kg) was given i.p. on days 6, 10, 13, and 16.

The progress of each tumor was measured thrice weekly until it had reached a volume of 500 mm^3. Tumor growth delay was calculated as the number of days required for each treated tumor to reach a volume of 500 mm^3 as compared with untreated control tumors. Each treatment group comprised six animals, and the experiment was repeated three times. Tumor growth-delay data represented the mean value ± SE calculated for the treatment group as compared with the control group.

Results

Minocycline was not very cytotoxic toward normally oxygenated or hypoxic (5 h hypoxic + 19 h) EMT-6 cells on 24 h exposure to the drug (Fig. 1). The concentration of minocycline producing 50% growth inhibition (IC_{50}) was 132 μM in normally oxygenated EMT-6 cells and 220 μM in hypoxic EMT-6 cells. Radiation was more cytotoxic toward normally oxygenated EMT-6 cells than toward

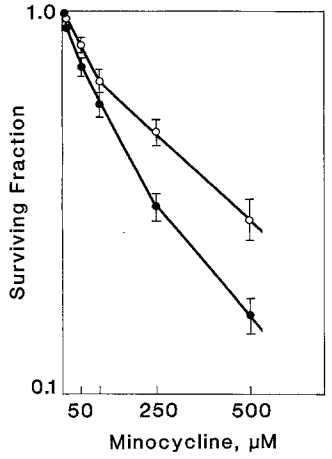

Fig. 1. Survival curves for exponentially growing, normally oxygenated (●) and hypoxic (○ EMT-6 cells exposed to various concentrations of minocycline for 24 h. Hypoxia was maintained for the first 5 h of drug exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

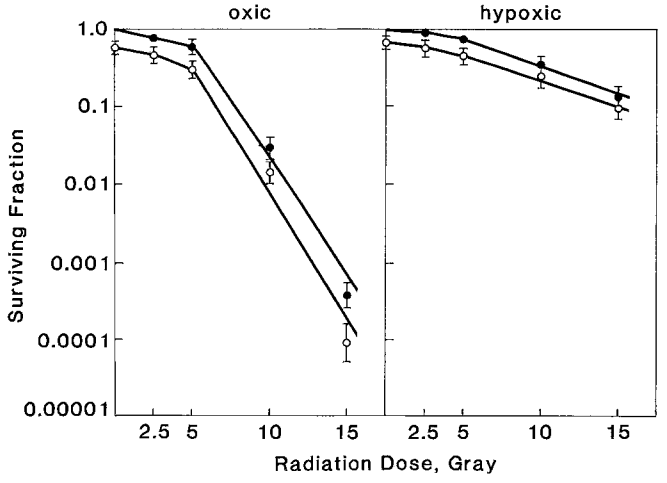

Fig. 2. Survival curves for exponentially growing, normally oxygenated (*left*) and hypoxic (*right*) EMT-6 cells exposed to various doses of X-rays alone (●) or combined with minocycline (100 μM, 24 h; ○), with radiation being delivered under normally oxygenated or hypoxic conditions during the 5th of minocycline exposure. The survival values plotted on the *y*-axis of each panel in *open circles* represent the cytotoxicity of minocycline (100 μM, 24 h) under the conditions indicated. *Points*, Mean values for 3 independent determinations; *bars*, SEM

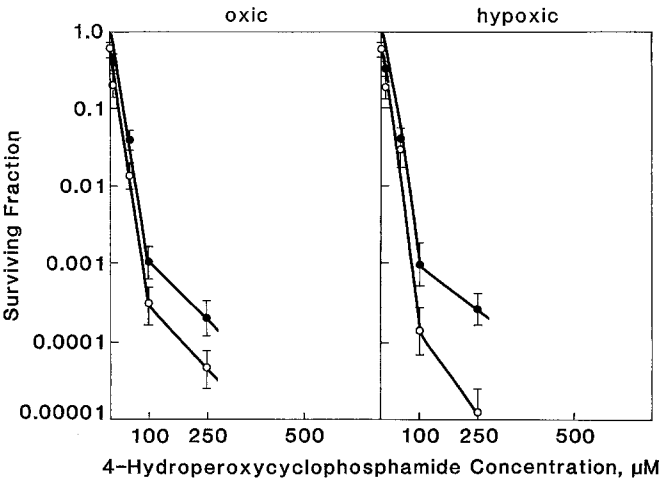

Fig. 3. Survival curves for exponentially growing, normally oxygenated (*left*) and hypoxic (*right*) EMT-6 cells exposed to various concentrations of 4-hydroperoxycyclophosphamide alone (●) or combined with minocycline (100 μM, 24 h; ○), with radiation being delivered under normally oxygenated or hypoxic conditions during the 5th of minocycline exposure. The survival values plotted on the *y*-axis of each panel in *open circles* represent the cytotoxicity of minocycline (100 μM, 24 h) under the conditions indicated. *Points*, Mean values for 3 independent determinations; *bars*, SEM

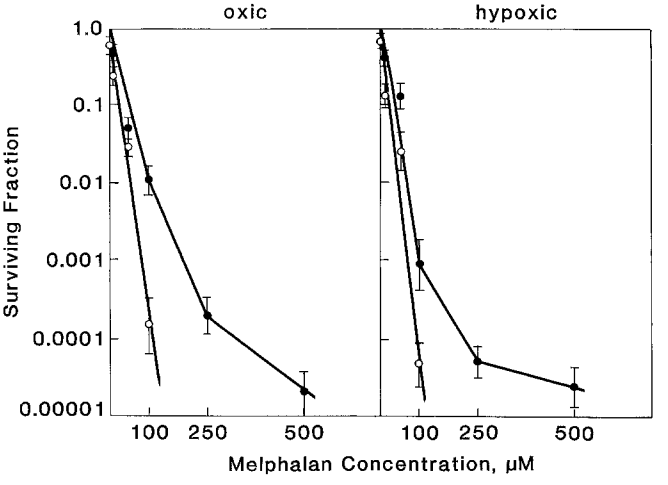

Fig. 4. Survival curves for exponentially growing, normally oxygenated (*left*) and hypoxic (*right*) EMT-6 cells exposed to various concentrations of melphalan alone (●) or combined with minocycline (100 μM, 24 h; ○), with radiation being delivered under normally oxygenated or hypoxic conditions during the 5th h of minocycline exposure. The survival values plotted on the *y*-axis of each panel in *open circles* represent the concentration of minocycline (100 μM, 24 h) under the conditions indicated. *Points*, Mean values for 3 independent determinations; *bars*, SEM

hypoxic EMT-6 cells (Fig. 2). When EMT-6 cells under either oxygenation condition were exposed to 100 μM minocycline for 24 h, with radiation being delivered during the 5th of minocycline exposure, there was no enhancement in the cytotoxicity of the radiation. 4-Hydroperoxycyclophosphamide was equally cytotoxic toward normally oxygenated and hypoxic EMT-6 cells (Fig. 3). Exposure of EMT-6 cells under either oxygenation condition to minocycline (100 μM) for 24 h, with 4-hydroperoxycyclophosphamide being added during the 5th of minocycline exposure, resulted in cytotoxicity that appeared to be additive. The cytotoxicity of melphalan toward normally oxygenated or hypoxic EMT-6 cells was essentially the same (Fig. 4). Treatment with minocycline (100 μM, 24 h) whereby cells were exposed to melphalan during the 5th of minocycline treatment also resulted in additive cytotoxicity for the two agents. No difference was observed in the cytotoxicity of CDDP toward normally oxygenated versus hypoxic EMT-6 cells (Fig. 5). Exposure of the cells to minocycline (100 μM, 24 h), with CDDP being added during the 5th of minocycline exposure, did not significantly alter the cytotoxicity of CDDP.

The effect of minocycline in vivo on the survival of FSaIIC tumor cells and bone marrow CFU-GM is shown in Fig. 6. Minocycline killed increasing numbers of FSaIIC tumor cells with increasing doses of the drug; however,

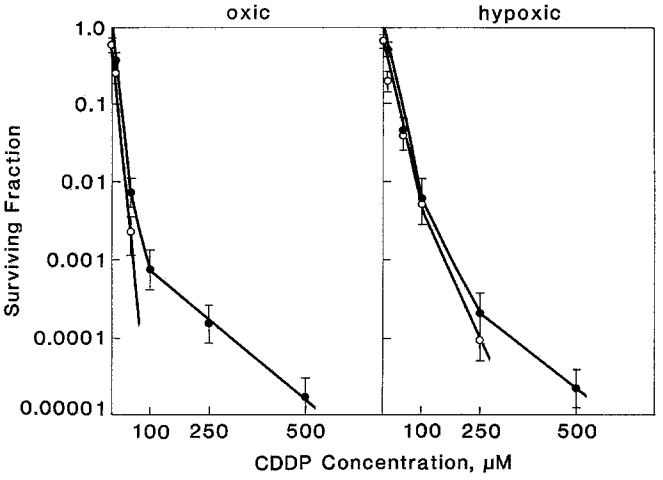

Fig. 5. Survival curves for exponentially growing, normally oxygenated (*left*) and hypoxic (*right*) EMT-6 cells exposed to various concentrations of CDDP alone (●) or combined with minocycline (100 μM, 24 h; ○), with radiation being delivered under normally oxygenated or hypoxic conditions during the 5th h of minocycline exposure. The survival values plotted on the *y*-axis of each panel in *open circles* represent the cytotoxicity of minocycline (100 μM, 24 h) under the conditions indicated. *Points*, Mean values for 3 independent determinations, *bars*, SEM

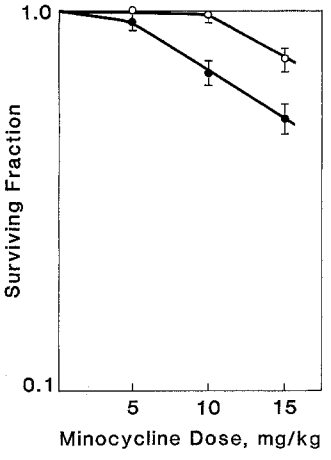

Fig. 6. Survival of FSaIIC cells (●) and bone marrow CFU-GM (○) from animals bearing FSaIIC tumors that were treated with various doses of minocycline. The drug was injected i.p. five times over 24 h at the doses shown. *Points*, Mean values for 3 independent experiments; *bars*, SEM

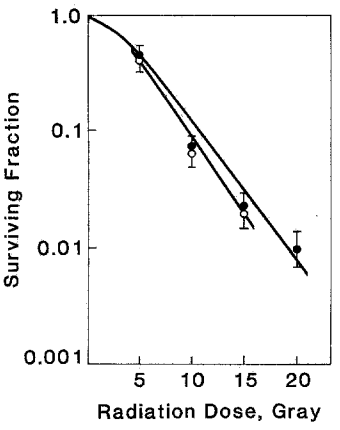

Fig. 7. Survival of FSaIIC cells from FSaIIC tumors exposed to various doses of radiation alone (●) or combined with minocycline (5 × 5 mg/kg given i.p. over 24 h; ○). Radiation was delivered as a single dose immediately after the third minocycline dose. *Points*, Mean values for 3 independent experiments; *bars*, SEM

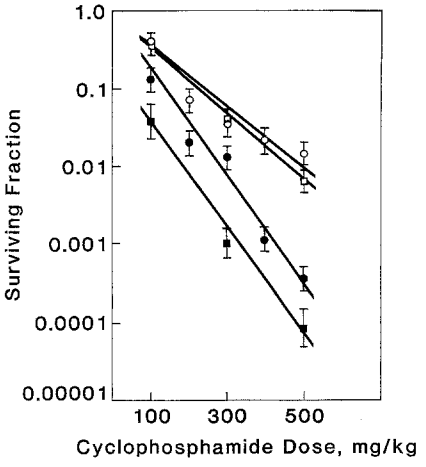

Fig. 8. Survival of FSaIIC cells (●) and bone marrow CFU-GM (○) from animals bearing FSaIIC tumors that were treated with various doses of cyclophosphamide alone (●) or combined with minocycline (5 × 5 mg/kg given i.p. over 24 h; ○). Cyclophosphamide was given in a single i.p. dose immediately after the third minocycline dose. *Points*, Mean values for 3 independent experiments; *bars*, SEM

over the dose range tested, minocycline was not very cytotoxic. Minocycline was less toxic toward bone marrow GFU-GM than toward FSaIIC tumor cells. Single doses of radiation also killed increasing numbers of FSaIIC tumor cells with increasing doses of radiation (Fig. 7). The combination of minocycline (5 × 5 mg/kg) with radiation delivered immediately after the third dose of minocycline did not alter the response of the FSaIIC tumor to radiation. Cyclophosphamide killed increasing numbers of FSaIIC tumor cells with increasing doses of the drug in a log-linear manner as well (Fig. 8). When cells were exposed to minocycline (5 × 5 mg/kg) in combination with cyclophosphamide applied at the time of the third dose of minocycline, there was about a 4-fold increase in the tumor-cell killing obtained for cyclophosphamide over the dose range examined. Cyclophosphamide was less toxic to bone marrow CFU-GM than to FSaIIC tumor cells. The addition of minocycline (5 × 5 mg/kg) to treatment with cyclophosphamide did not change the toxicity of cyclophosphamide to bone marrow CFU-GM.

The Lewis lung carcinoma growing in C57BL mice was chosen for tumor growth-delay studies because this tumor is relatively resistant to many cancer therapies and metastasizes avidly to the lungs from s.c. implants. For these tumor growth-delay studies, administration of minocycline (5 mg/kg) was initiated on day 4 after tumor-cell implantation, by which time neovascularization of the tumors had begun [61, 62]. Daily i.p. injection of minocycline for 2 weeks produced no significant delay in the growth of treated tumors as compared with untreated con-

Table 1. Growth delay of the Lewis lung tumor produced by various anticancer treatments used alone or in combination with minocycline

Treatment group	Dose[a]	Tumor growth delay (days)[b]	
		Alone	+ Minocycline[c]
Minocycline	14× 5 mg/kg	0.6±0.3	
CDDP	1× 10 mg/kg	4.5±0.3	5.0±0.3 (1.1)
Melphalan	1× 10 mg/kg	2.7±0.3	4.3±0.3 (1.6)*
Cyclophosphamide	1×150 mg/kg	7.2±0.4	24.7±2.7 (3.4)**
	3×150 mg/kg	21.5±1.7	45.2±2.9 (2.1)[d,*]
Radiation	1× 20 Gy	6.2±0.5	11.9±1.4 (1.9)*
	5× 3 Gy	4.4±0.3	7.8±0.6 (1.8)*
5-Fluorouracil	4× 40 mg/kg	5.1±0.4	6.2±0.5 (1.2)
Methotrexate	5× 0.8 mg/kg	6.6±0.4	8.2±0.6 (1.3)
Etoposide	4× 15 mg/kg	7.8±0.7	8.3±0.7 (1.1)
Adriamycin	5× 1.75 mg/kg	7.0±0.6	9.8±0.8 (1.4)
Bleomycin	4× 10 mg/kg	8.5±0.6	12.0±1.2 (1.4)

[a] Minocycline (5 mg/kg) was given i.p. on days 4–18 following tumor implantation. CDDP (10 mg/kg), melphalan (10 mg/kg), and cyclophosphamide (150 mg/kg) were injected i.p. on day 7 after tumor implantation. Cyclophosphamide (150 mg/kg) was given i.p. on days 7, 9, and 11 following tumor implantation. Radiation was delivered locally to the tumor-bearing limb as 20 Gy given on day 7 or 3 Gy given daily on days 7–11. 5-Fluorouracil (40 mg/kg) and etoposide (15 mg/kg) were given daily i.p. on days 7–10. Methotrexate (0.8 mg/kg) and Adriamycin (1.75 mg/kg) were injected daily i.p. on days 7–11. Bleomycin (10 mg/kg) was given i.p. on days 6, 10, 13, and 16

[b] Tumor growth delay is the difference in days for treated tumors to reach a volume of 500 mm^3 as compared with untreated control tumors. Untreated control tumors reached 500 mm^3 in about 14 days. Data represent the mean values ± SE for 15 animals

[c] Minocycline (5 mg/kg) was injected i.p. on days 4–18. Numbers in parentheses represent the magnitude of difference in tumor growth delay observed between animals receiving the treatment combination and those receiving the standard therapy alone

[d] Four animals were long-term survivors (>120 days)

* Significantly different from the drug alone ($P < 0.01$ according to the Dunn multiple-comparisons test)

** Significantly different from the drug alone ($P < 0.001$ according to the Dunn multiple-comparisons test)

Table 2. Numbers of lung metastases from s.c. Lewis lung tumors encountered on day 20 after various anticancer treatments given alone or in combination with minocycline

Treatment group	Dose[b]	Mean number of lung metastases[a]	
		Alone	+Minocycline[b]
Untreated controls		15 (10; 66%)	
Minocycline	14× 5 mg/kg	12 (4; 33%)	
CDDP	1× 10 mg/kg	12	10.5 (5; 48%)
Melphalan	1× 10 mg/kg	8	6 (3; 50%)
Cyclophosphamide	1×150 mg/kg	6.5	3 (1; 33%)
	3×150 mg/kg	3.5	0.5 (0; 0)
Radiation	1× 20 Gy	8	8 (1; 25%)
	5× 3 Gy	7	6.5 (2; 30%)
5-Fluorouracil	4× 40 mg/kg	8	8 (3; 38%)
Methotrexate	5× 0.8 mg/kg	7	7 (3; 36%)
Etoposide	4× 15 mg/kg	8	8 (5; 58%)
Adriamycin	5× 1.75 mg/kg	8	7.5 (5; 63%)
Bleomycin	4× 10 mg/kg	7	7 (4.5; 64%)

[a] The external lung metastases encountered on day 20 following tumor implantation were counted manually and scored as measuring ≥3 mm^3 in diameter. Data represent the mean values for 4–12 pairs of lungs. Figures in parentheses indicate the number of large (vascularized) metastases and the percentage of the total number of metastases that were large

[b] For the schedules of drug administration see Table 1

trol tumors (Table 1). Single-agent chemotherapy or radiation therapy was given to the tumor-bearing animals beginning on day 7, by which time the tumors had reached a volume of about 100 mm^3.

Each treatment agent was given at a standard dose and schedule for that specific treatment. The addition of minocycline did not significantly enhance the tumor growth delay produced by CDDP, 5-fluorouracil, methotrexate, or etoposide. However, significant increases in tumor growth delay were observed when tumor-bearing animals were treated with melphalan, cyclophosphamide, radiation, Adriamycin, or bleomycin. Nearly a 2-fold increase in tumor growth delay was observed following the addition of minocycline (14×5 mg/kg) to treatment with single-dose or fractionated radiation. The most marked enhancement in tumor growth delay was obtained using the combination of minocycline and cyclophosphamide. Cyclophosphamide (150 mg/kg) was an effective chemotherapeutic agent in the Lewis lung tumor, producing a tumor growth delay of about 7 days following its single-dose administration and that of about 21 days on its administration in three doses given on alternate days. The addition of minocycline (14×5 mg/kg) to treatment with cyclophosphamide resulted in a 3.4- and 2.1-fold increase in tumor growth delay for the single-dose and multiple-dose regimens of cyclophosphamide, respectively. In the multiple-dose cyclophosphamide plus minocycline group, 4 of 15 animals were long-term survivors (>120 days).

Untreated control animals bearing Lewis lung tumors survive for 21–25 days after tumor implantation and succumb to disease metastatic to the lungs. In the present study, the number and size of lung metastases encountered in untreated animals and in animals treated as described for the tumor growth-delay studies were scored on day 20 following tumor implantation (Table 2). Treatment with minocycline (5 mg/kg) beginning on day 4 and extending through day 18 had little effect on the total number of lung metastases found on day 20; however, only one-third of the metastases encountered were large enough to be vascularized as compared with two-thirds in the untreated control animals. The antitumor treatments reduced the number of lung metastases in many cases to about one-half the number observed in the untreated control animals. The combination of minocycline with the standard therapies resulted in further reductions in the number of lung metastases found after treatment with CDDP, melphalan, and cyclophosphamide. The fraction of metastases that were large enough to be vascularized after the combination treatment were lower in the treated mice than in the untreated control animals. The lowest numbers of large metastases were found in animals that had been treated with cyclophosphamide and minocycline; in fact, those treated with multiple doses of cyclophosphamide in combination

with minocycline displayed no large lung metastasis on day20.

Discussion

Although cytotoxic therapies often make some impact on the treatment of solid tumors, cure is rarely attained. We have been searching for modulators to add to standard therapies, which, by virtue of their effects on the physiological, biological, or biochemical properties of the tumor, would increase the tumor's susceptibility to cytotoxic treatment without enhancing the toxicity of the therapy to the host. Antiangiogenic/antimetastatic agents could be envisioned to act as modulators of other therapies by inhibiting further growth or regrowth of both primary and metastatic disease and suppressing further metastatic invasion. In the ideal case, an angiostatic condition might lead to the death of the tumor cells most distal from the established tumor vasculature, thereby reducing the tumor burden of the host and resulting in a tumor mass that would more easily be permeated by chemotherapy agents and treated by radiation therapy.

Minocycline is a semisynthetic derivative of tetracycline that has a relatively long circulating half-life of about 12 h and is available for human use. The minocycline dose used in the tumor growth-delay studies is readily achievable in humans. The current study did not provide any data indicating that a 2-week course of daily minocycline (5 mg/kg) was significantly angiostatic to the primary Lewis lung tumor implanted s. c. in the legs of C57BL mice or that this treatment significantly reduced the capacity of Lewis lung-cancer cells to metastasize to the lungs of tumor-bearing mice, although the number of vascularized metastases was reduced from a mean of 10 to a mean of 4 per animal. The combination of minocycline with several standard cancer therapies significantly improved the tumor growth delay produced by those therapies, indicating that minocycline treatment significantly altered either the tumor or the host in some way that rendered the tumor more responsive to treatment.

The cell-culture studies indicated that minocycline was minimally cytotoxic toward EMT-6 cells after 24 h exposure, but only at very high concentrations. Combinations of minocycline and radiation or chemotherapy demonstrated only an additive effect but indicating no direct interactions between minocycline and the other treatments. Short-term treatment of established FSaIIC tumors (days 7–8) with minocycline ($5 \times 5 - 5 \times 15$ mg/kg) resulted in little direct tumor-cell killing. Only 50% of the tumor cells were killed at the highest dose of minocycline tested. We found no measurable toxic effect for minocycline on bone marrow CFU-GM except at the highest dose of the drug, which killed about 25% of the CFU-GM. There was no enhancement of tumor-cell killing by X-rays delivered in combination with short-term administration of minocycline. However, we obtained greater-than-additive killing of FSaIIC tumor cells following the addition of short-term minocycline treatment to single injections of cyclophosphamide given over a range of doses, indicating that there may be a direct interaction between minocycline and cyclophosphamide at the level of the tumor or that minocycline may induce changes in normal tissues of the host such as the liver, thereby increasing the efficiency of the conversion of cyclophosphamide to its active alkylating species.

The primary and metastatic tumor growth-delay studies in the Lewis lung carcinoma indicated that long-term (2-week) treatment with minocycline (5 mg/kg) reduced the growth rate of metastatic disease. In these studies, there appeared to be a special interaction between minocycline and cyclophosphamide in terms of the response of both the primary tumor and the metastatic disease to treatment; in fact, long-term disease-free survival or cure was achieved in 4 of 15 animals. This particularly marked improvement in the antitumor effect of cyclophosphamide plus minocycline may have involved an interaction of these drugs at the level of hepatic metabolism. Minocycline exhibits hepatotoxic potential and undergoes hepatic metabolism to several different species. Cyclophosphamide is a prodrug that undergoes metabolism by hepatic microsomal enzymes to a critical intermediate to become an active alkylating agent. Studies are under way to explore this possibility.

The ability of tetracyclines to inhibit tissue collagenase activity was first described by Golub et al. [13, 14] in the early 1980s. At about the same time, Folkman et al. [10] reported that heparin or a heparin fragment in the presence of corticone caused inhibition of angiogenesis and regression of several established murine solid tumors. Because of the side effects of the cortisone treatment, Folkman et al. [10] also treated their tumor-bearing mice with tetracycline and bactrim in the drinking water throughout the heparin/cortisone treatment period and for 2 additional weeks. In a subsequent study, Penhaligon and Camplejohn [33] examined the effect of five different heparin preparations in the presence of cortisone on two transplantable mouse tumors. These investigators treated tumor-bearing animals with oxytetracycline and bactrim beginning on the day of tumor implantation. Lee et al. [27] examined the potential of cortisone acetate to inhibit tumor angiogenesis in C3H mice bearing MBT-2 tumors. Lee et al. [28] also included tetracycline and sulfatrim in the drinking water of the animals throughout the course of treatment with cortisone acetate. In each case, the animals in these three studies probably received a tetracycline dose that was greater than or comparable with the dose of minocycline used in the current study. It is therefore quite likely that the tetracycline treatment was an active component in the antiangiogeneic effects observed in these studies.

In summary, then, inhibition of collagenase by minocycline may have occurred to some extent in vivo at the doses used in the present study, although the effect was not sufficient to slow the growth of the primary Lewis lung tumor. We found no evidence of a direct interaction between minocycline and any of the other anticancer treatments tested in vitro. However, we noted a significant enhancement of the in vivo tumor response to several standard cytotoxic treatments given in combination with minocycline as compared with the cytotoxic treatment alone, especially for the combination of minocycline and cyclophosphamide. The mechanism of this modulation appears to involve effects on the host. We are continuing to

explore the mechanism of action of this interesting modulator.

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