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ORIGINAL ARTICLE

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Transforming growth factor-β in *in vivo* resistance

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Abstract The potential role of transforming growth factor-β in *in vivo* resistance was examined by administration of transforming growth factor-β-neutralizing antibodies to animals bearing the EMT-6/Parent tumor or the antitumor alkylating resistant tumors, EMT-6/CTX or EMT-6/CDDP. Treatment of tumor-bearing animals with anti-TGF-β antibodies by intraperitoneal injection daily on days 0–8 post-tumor cell implantation increased the sensitivity of the EMT-6/Parent tumor to cyclophosphamide (CTX) and cisplatin (CDDP) and markedly increased the sensitivity of the EMT-6/CTX tumor to CTX and the EMT-6/CDDP tumor to CDDP, as determined by tumor cell survival assay. Bone marrow granulocyte-macrophage colony-forming units (CFU-GM) survival was determined from these same animals. The increase in the sensitivity in the tumors upon treatment with the anti-TGF-β antibodies was also observed in increased sensitivity of the bone marrow CFU-GM to CTX and CDDP. Treatment of non-tumor-bearing animals with the anti-TGF-β regimen did not alter blood ATP or serum glucose level but did decrease serum lactate levels. This treatment also decreased hepatic glutathione, glutathione S-transferase, glutathione reductase, and glutathione peroxidase in non-tumor-bearing animals by 40–60% but increased hepatic cytochrome P450 reductase in these normal animals. Animals bearing the EMT-6/CTX and EMT-6/CDDP tumors had higher serum lactate levels than normal or EMT-6/Parent tumor-bearing animals; these were decreased by the anti-TGF-β regimen. Treatment of animals bearing any of the three tumors with the anti-TGF-β regimen decreased by 30–50% the activity of hepatic glutathione S-transferase and glutathione peroxidase, and increased by 35–80% the activity of hepatic cytochrome P450 reductase. In conclusion, treatment with transforming growth factor-β-neutralizing antibodies restored drug sensitivity in the alkylating agent-resistant tumors, altering both the tumor and host metabolic states.

Key words Transforming growth factor β · Drug resistance · In vivo resistance · Cisplatin resistance · Cyclophosphamide resistance

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Introduction

Relative resistance to cytotoxic therapies, existing prior to therapy or acquired over the course of therapy, is a major clinical problem [1–3]. Preclinical models of various types have been applied to the elucidation of the mechanisms of resistance to therapy and have been used to develop clinically relevant approaches to overcoming such resistance [4–6]. Breast cancer, among the major solid tumors, is an example of a malignancy where resistance to cytotoxic therapies, such as the antitumor alkylating agents, can develop [7, 8]. The EMT-6 mammary carcinoma sublines resistant to the antitumor alkylating agents cyclophosphamide (CTX), thiotepa (Thio), cisplatin (CDDP), and carboplatin (Carbo) were produced by repeated exposure of fresh tumor-bearing hosts to each drug [9]. After ten treatments, metastable resistant tumors were produced. Although the tumors were resistant to drug treatment, the tumor cells in monolayer culture were not [9, 10]. As determined by the tumor cell survival assay from tumors treated *in vivo* at a level of 1 log (90%) of cell killing, the EMT-6/CDDP tumor is fourfold resistant to CDDP and the EMT-6/CTX tumor is threefold resistant to CTX as compared with the EMT-6/Parent tumor [9].

When the survival of bone marrow granulocyte-macrophage colony-forming units (CFU-GM), an

alkylating agent-sensitive normal tissue, was assessed in mice bearing the EMT-6 parental tumor or the *in vivo* resistant EMT-6/CDDP, EMT-6/CTX, EMT-6/Thio and EMT-6/Carbo tumors, the survival pattern of the bone marrow CFU-GM recapitulated the survival of the tumor cells, mimicking the development of resistance and reversion to sensitivity upon removal of the selection pressure for each of the four alkylating agents [9, 10]. When the EMT-6 parental tumor was implanted in the opposite hind limb of animals bearing the EMT-6/CDDP or EMT-6/CTX tumor, the survival of the parental tumor cells after treatment of the animals with the appropriate antitumor alkylating agent was enhanced. The EMT-6/CDDP tumor was cross-resistant to CTX and melphalan, whereas the EMT-6/CTX tumor was somewhat resistant to CDDP and markedly sensitive to etoposide. In each case, the survival pattern of the bone marrow CFU-GM reflected the survival of the tumor cells. Thus, the presence of an alkylating agent-resistant tumor in an animal altered the drug response of tissues distal to the resistant tumor [9, 10].

When the expression of several early response genes and genes associated with malignant disease was assessed in the EMT-6/Parent tumor and the EMT-6/CTX and EMT-6/CDDP *in vivo* resistant lines growing as tumors, it was found that, in the absence of treatment, the levels of mRNA for the genes c-*jun*, c-*fos*, c-*myc*, Ha-*ras* and p53 were increased in the EMT-6/CTX and EMT-6/CDDP tumors as compared with the EMT-6/Parent tumor, whereas the expression of *erb*-2 was similar in all three tumors [9, 11]. There was increased expression of both c-*jun* and *erb*-2 in the livers of tumor-bearing animals. The highest expression of both c-*jun* and *erb*-2 occurred in the livers of animals bearing the EMT-6/CDDP tumor. Treatment of the animals with CDDP or CTX, in general, resulted in increased expression of both genes at 6 h post-treatment. The increased expression of these genes may impart metabolic changes in the tumors and/or hosts that contribute to the resistance of these tumors to specific antitumor alkylating agents [11].

Several observations, including the fibrous nature of the resistant tumors, the increased metastatic potential of the resistant tumors, and the altered pharmacokinetics of the drugs in the resistant tumor-bearing hosts, led to the hypothesis that transforming growth factor-β (TGF-β) might be integrally involved in *in vivo* antitumor alkylating agent resistance in the EMT-6 tumor lines. Because it is difficult to maintain increased systemic levels of TGF-β by administering the protein to mimic the resistance phenotype, administration of TGF-β-neutralizing antibodies to animals bearing the resistant tumors in an attempt to reverse the resistance was chosen as the experimental design to address the hypothesis.

Materials and methods

Drugs and antibodies

Cyclophosphamide (CTX) and *cis*-diamminedichloroplatinum(II) (cisplatin; CDDP) were purchased from Sigma Chemical (St. Louis, Mo.). Transforming growth factor-β (TGF-β)-neutralizing antibodies 2G7 and 4A11 were a most generous gift from Drs Brian M. Fendly and Ed Amento, Genentech (San Francisco, Calif.).

Tumor system

The EMT-6/Parent mouse mammary carcinoma grown as a solid tumor s.c. in the flanks of female Balb/C mice (Taconic Farms, Germantown, N.Y.) has been used widely in radiobiology and chemotherapy studies. We have established alkylating agent-resistant EMT-6 tumor lines by repeated treatment of tumor-bearing animals with CDDP (20 mg/kg) or CTX (300 mg/kg) injected i.p. 24 h before passage of each tumor line into fresh host animals 10 times [9]. The parent tumor line was passaged in the same manner in the absence of drug treatment. The alkylating agent sublines designated EMT-6/CDDP (resistant to CDDP) and EMT-6/CTX (resistant to CTX) were maintained as frozen tumor brei in liquid nitrogen and used for experiments during the second and third tumor passages [9, 10].

Tumor cell survival assay

The EMT-6 murine mammary carcinoma is an *in vivo-in vitro* tumor system [9]. The EMT-6/Parent and alkylating agent-resistant tumors were grown in Balb/C mice. For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted s.c. into the hind legs of Balb/C mice aged 8–10 weeks. Tumor cell survival was also performed when the tumors had reached a volume of approximately 150 mm^3 (day 9 after tumor implantation). Animals were untreated, treated with anti-TGF-β 2G7 (1 mg/kg, i.p.) on days 4–8 or days 0–8, treated with anti-TGF-β 4A11 (1 mg/kg, i.p.) on days 4–8 or days 0–8, or treated with anti-TGF-β 2G7 (1 mg/kg, i.p.) plus anti-TGF-β 4A11 (1 mg/kg, i.p.) on days 4–8 or days 0–8. On day 8 some animals were injected i.p. with CTX (300 or 500 mg/kg) or with CDDP (20 or 50 mg/kg). A 24-h interval was incorporated before the mice were killed to allow for the full expression of drug cytotoxicity and repair of potentially lethal damage. Mice were immersed briefly in 95% ethanol and the tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with two scalpels. Four tumors were pooled to make each treatment group. Approximately 400 mg tumor brei was used to make each single cell suspension. All reagents were sterilized with 0.22-µm Millipore filters and were added aseptically to the tumor cells.

Each sample was washed in 20 ml Waymouth's medium (Mediatech, Pittsburgh, Pa.) after which the liquid was gently decanted and discarded. The samples were resuspended in 450 U collagenase/ml (Sigma, St. Louis, Mo.) and 0.1 mg DNase/ml (Sigma, St. Louis, Mo.) and incubated for 10 min at 37°C in a shaking water bath. The samples were resuspended as described above and incubated for another 15 min at 37°C. Next, 1 ml of 1 mg/ml DNase was added and incubation was continued for 5 min at 37°C. The samples were then filtered through a 70-µm cell strainer (Fisher, Pittsburgh, Pa.) The samples were washed twice, then resuspended in Waymouth's medium supplemented with 15% newborn calf serum. These single-cell suspensions were counted and plated at six different cell concentrations for the colony forming assay. No significant difference was observed in the total cell yield from the pooled tumors in any treatment group. After 1 week, the plates were stained

with crystal violet and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 8–14%. The results were expressed as the surviving fraction (±SE) of cells from the treated groups as compared with untreated controls.

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 the CFU-GM assay was carried out as described previously [12]. Bone marrow cells were suspended in supplemented McCoy's 5A medium containing 15% fetal bovine serum (FBS), 0.3% agar (Difco, Detroit, Mich.), and 10% conditioned medium as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating L-929 mouse fibroblasts (2500 cells/ml; Microbiological Associates, Bethesda, Md.) with 30% FBS in McCoy's 5A medium for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. The supernatant containing the colony-stimulating activity was obtained by centrifugation of the medium at 10 000 $\times g$ for 10 min at 4°C followed by filtration under sterile conditions. The bone marrow cell cultures were incubated for 7 days at 37°C and were fixed with 10% glutaraldehyde. Colonies of at least 50 cells were scored. The results of three experiments, in which each group was measured at six cell concentrations, were averaged. The results were expressed as the surviving fraction (±SE) of treated groups as compared with untreated controls.

Metabolic determinations

The EMT-6/Parent, EMT-6/CTX, and EMT-6/CDDP tumors were implanted and grown as described above. The animals were treated with anti-TGF-β 2G7 (1 mg/kg) and anti-TGF-β 4A11 (1 mg/kg) by i.p. injection once daily on days 0–8. Animals were sacrificed on day 9 and blood (cardiac puncture), tumors, and livers were collected. Each assay was performed on tissue from two animals per experiment and each study was done 3 times.

ATP, lactate, glucose

For determination of ATP, 800 µl of whole blood was immediately added to 1 ml trichloroacetic acid (12%) on ice, vortex-mixed and kept on ice for 10 min. The mixture was then centrifuged to obtain a clear supernatant. The supernatant (0.5 ml) was assayed for ATP using Sigma kit 366-UV. Decrease in absorbance at 340 nm was followed for 10 min. ATP concentration (ΔA) was calculated as:

$$\frac{3.04 \times 100(\text{initial absorbance} - \text{final absorbance})}{6.22 \times 0.25} = 195 \times \Delta A$$

(i.e. mol ATP/dl)

For the serum lactate assay, 200 µl of blood was centrifuged to obtain serum. The serum (10 µl) was assayed for lactate using Sigma kit 735. Each cuvette contained 1 ml of lactate reagent with 10 µl water for the blank or 10 µl of sample. The cuvettes were incubated at room temperature for 10 min, then the absorbance of the standard (40 mg/dl lactate) or sample was recorded at 540 nm with the blank as a reference. Lactate concentration was calculated as:

$$\text{Lactate (mg/dl)} = \frac{\text{Absorbance test}}{\text{Absorbance standard}} \times 40$$

Serum glucose was assayed using 10 µl of serum and Sigma kit 16-UV. The glucose reagent (1.5 ml) was added to each cuvette with 10 µl deionized water for the blank, a glucose standard, or a sample. Absorbances (A) at 340 nm of blank, standards, and samples were recorded against water. Glucose concentration was calculated as:

$$\text{Glucose (mg/dl)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times \text{concentration of standard}$$

For tissue measurements immediately after tumor excision, tumors were washed with phosphate-buffered 0.9% saline (PBS) at 4°C, minced with crossed scalpels and homogenized in 2–3 ml cold PBS using a hand homogenizer. An aliquot of the homogenate (800 µl) was added to 1 ml of 12% trichloroacetic acid. The homogenate mixture was centrifuged and 0.5 ml of supernatant was assayed for ATP, lactate, and glucose as described above. The protein content of the homogenate was determined using Sigma Protein assay kit 541-2 and protein standard solution catalog number 540-10. ATP, lactate, and glucose in tumor homogenate were expressed in terms of mg protein.

Glutathione and phase II enzymes

Glutathione, glutathione S-transferase activity, glutathione reductase activity, and glutathione peroxidase activity were measured in tissue cytosolic preparations, while cytochrome P450 reductase activity was assayed in the tissue microsomal fraction. To prepare the cytosol and microsomes, freshly excised liver and tumor samples were quick-frozen in liquid nitrogen and stored at −80°C before assay. Tissues were minced with scissors and then homogenized in buffer containing 0.25 M sucrose, 50 mM TRIS-HCl, 150 mM KC1, pH 7.4, using a mechanical homogenizer, 3 times for 3 min on ice. Following centrifugation at 10 000 $\times g$ for 30 min, the supernatant was subjected to ultracentrifugation at 100 000 $\times g$ for 1 h to yield the cytosol (supernatant) and microsomes (pellet).

Total intracellular glutathione was measured by an enzyme recycling assay [13]. Glutathione S-transferase activity was assayed according to the method of Habig and Jakoby [14] and glutathione reductase activity and glutathione peroxidase activity were determined by the methods of Goldberg and Spooner [15] and Paglia and Valentine [16], respectively.

Cytochrome P450 reductase activity in microsomes was assayed as reported earlier [17]. Protein content was measured as described above. Results were expressed as nmol glutathione/mg protein (±SE). The enzymatic activities were expressed as nmol substrate converted/min per mg protein (±SE).

Results

The EMT-6 *in vivo* alkylating agent-resistant tumors are markedly less responsive to the drugs to which resistance was developed than is the EMT-6/Parent tumor when grown as solid tumors subcutaneously in the hind legs of female Balb/C mice [9–11]. When animals bearing the EMT-6/Parent tumor or the EMT-6/CTX tumor were treated with a single dose of CTX, the tumor cell killing shown in Fig. 1 was obtained. CTX at a dose of 300 mg/kg killed 2 logs of EMT-6/Parent tumor cells and less than 1 log of EMT-6/CTX tumor cells. CTX at a dose of 500 mg/kg killed 3.5 logs of EMT-6/Parent tumor cells but only 1.5 logs of EMT-6/CTX tumor cells. To assess the possibility that TGF-β might have a role in *in vivo* alkylating agent-resistance, animals bearing the EMT-6/Parent or EMT-6/CTX tumor were treated with anti-TGF-β

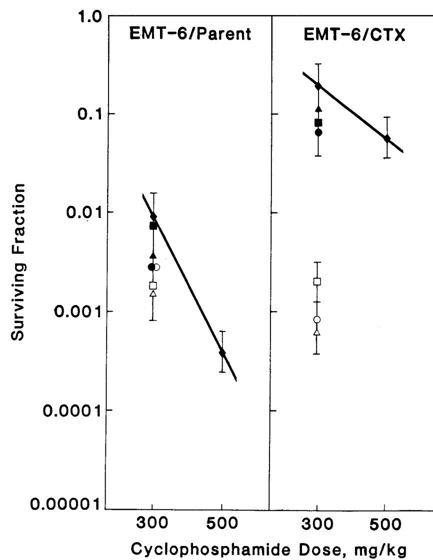

Fig. 1 Survival of EMT-6/Parent tumor cells and EMT-6/CTX tumor cells from tumors treated *in vivo* with cyclophosphamide (*CTX*) alone on day 8 (◆), anti-transforming growth factor-β (TGF-β) 2G7 (1 mg/kg, i.p.) for days 4–8 then CTX on day 8 (■), anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 4–8 then CTX on day 8 (▲), anti-TGF-β 2G7 (1 mg/kg, i.p.) + anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 4–8 then CTX on day 8 (●), anti-TGF-β 2G7 (1 mg/kg, i.p.) for days 0–8 then CTX on day 8 (□), anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 0–8 then CTX on day 8 (△), anti-TGF-β 2G7 (1 mg/kg, i.p.) + anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 0–8 then CTX on day 8 (○). *Points* are the means of three independent experiments; *bars* are the SEM

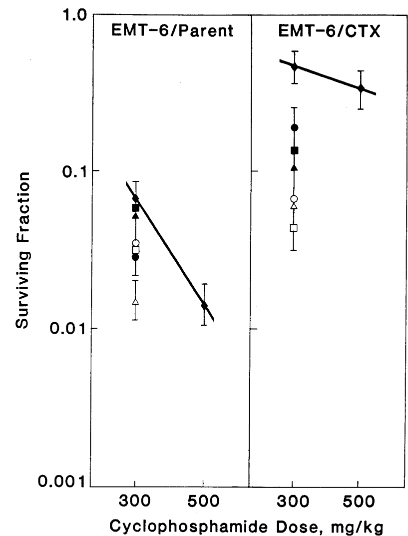

Fig. 2 Survival of bone marrow granulocyte-macrophage colony-forming units (*CFU-GM*) from the same animals shown in Fig. 1. Bone marrow was taken from the femurs of the animals at the time of tumor excision. *Points* are the means of three independent experiments; *bars* are the SEM

2G7, anti-TGF-β 4A11, or anti-TGF-β 2G7 and anti-TGF-β 4A11 daily by i.p. injection on days 4–8 or daily on days 0–8 post-tumor cell implantation. No significant difference was observed in tumor growth rate by administration of these antibody treatments. There was no significant effect of the administration of anti-TGF-β 2G7 or anti-TGF-β 4A11 on days 4–8 on tumor cell killing by CTX (300 mg/kg) in animals bearing either the EMT-6/Parent or EMT-6/CTX tumor; however, treatment with the combination of anti-TGF-β 2G7 and anti-TGF-β 4A11 on days 4–8 post-tumor cell implantation resulted in increased tumor cell killing of both EMT-6/Parent and EMT-6/CTX tumors by CTX (300 mg/kg). When treatment with antibodies to TGF-β was extended to the full period of tumor growth, days 0–8, a significant increase in the tumor cell killing of the EMT-6/Parent tumor by CTX (300 mg/kg) was observed with each of the three antibody regimens ($P < 0.01$). A much greater effect on tumor cell killing by CTX was observed when animals bearing the EMT-6/CTX tumor were treated with any of the anti-TGF-β regimens on days 0–8. The effect of anti-TGF-β treatment on EMT-6/CTX tumor cell killing was not only highly significant ($P < 0.001$) but also resulted in the restoration of drug sensitivity to the level of the parent tumor in the EMT-6/CTX tumor cells.

The survival of bone marrow CFU-GM in bone marrow taken from the same animals described above was assessed (Fig. 2). The bone marrow CFU-GM from animals bearing the EMT-6/Parent tumor was much more sensitive to treatment with CTX than the bone marrow CFU-GM from the animals bearing the EMT-6/CTX tumor, such that 1 log and 2 logs greater killing of bone marrow CFU-GM occurred in animals bearing the EMT-6/Parent tumor than in animals bearing the EMT-6/CTX tumor after treatment with 300 mg/kg or 500 mg/kg of CTX, respectively. Upon treatment of the tumor-bearing animals with antibodies to TGF-β on days 4–8 there was a significant increase in the killing of bone marrow CFU-GM by CTX (300 mg/kg) in animals bearing the EMT-6/CTX tumors. When the anti-TGF-β regimens were extended to days 0–8 there was a significant increase in the killing of bone marrow CFU-GM from animals bearing the EMT-6/CTX tumor ($P < 0.001$) by CTX. The increase in the killing of bone marrow CFU-GM in the EMT-6/CTX tumor-bearing animals was sufficient to restore the sensitivity of the bone marrow CFU-GM in these animals to the level of the bone marrow CFU-GM in animals bearing the EMT-6/Parent tumor.

A similar study was carried out with animals bearing the EMT-6/Parent tumor and animals bearing the EMT-6/CDDP tumor treated with CDDP (Fig. 3). Treatment of animals bearing the EMT-6/Parent tumor or the EMT-6/CDDP tumor with a dose of CDDP of 20 mg/kg resulted in 1 log less killing of the EMT-6/CDDP tumor cells than of the EMT-6/Parent tumor cells. Treatment of animals bearing the EMT-6/Parent tumor or the EMT-6/CDDP tumor with 50 mg/kg of

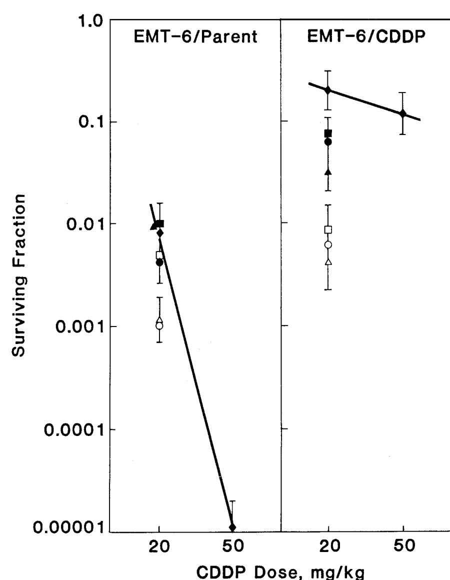

Fig. 3 Survival of EMT-6/Parent tumor cells and EMT-6/CDDP tumor cells from tumors treated *in vivo* with cisplatin (*CDDP*) alone on day 8 (◆), anti-TGF-β 2G7 (1 mg/kg, i.p.) for days 4–8 then CDDP on day 8 (■), anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 4–8 then CDDP on day 8 (▲), anti-TGF-β 2G7 (1 mg/kg, i.p.) + anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 4–8 then CDDP on day 8 (●), anti-TGF-β 2G7 (1 mg/kg, i.p.) for days 0–8 then CDDP on day 8 (□), anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 0–8 then CDDP on day 8 (△), anti-TGF-β 2G7 (1 mg/kg, i.p.) + anti-TGF-β 4A11 (1 mg/kg, i.p.) for days 0–8 then CDDP on day 8 (○). *Points* are the means of three independent experiments; *bars* are the SEM

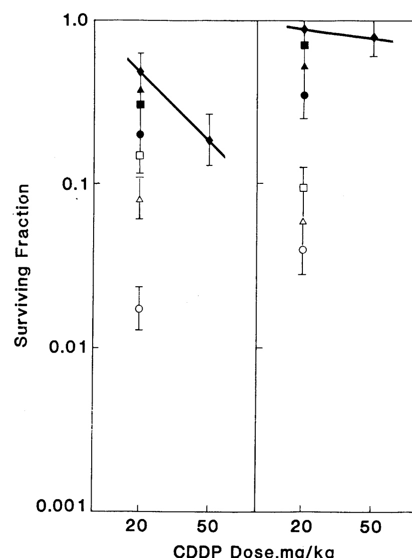

Fig. 4 Survival of bone marrow CFU-GM from the same animals shown in Fig. 3. Bone marrow was taken from the femurs of the animals at the time of tumor excision. *Points* are the means of three independent experiments; *bars* are the SEM

CDDP produced 4 logs less killing of the EMT-6/CDDP tumor cells than of the EMT-6/Parent tumor cells. The anti-TGF-β treatment regimens were the same as those described above. Administration of the antibodies to TGF-β on days 4–8 did not alter the response of the EMT-6/Parent tumor to treatment with CDDP (20 mg/kg) but significantly increased the killing of the EMT-6/CDDP tumor cells by CDDP (20 mg/kg) ($P < 0.01$). Administration of the antibodies to TGF-β on days 0–8 increased the tumor cell killing of the EMT-6/Parent tumor cells by CDDP but increased the tumor cell killing of the EMT-6/CDDP tumor cells by CDDP to a much greater degree ($P < 0.001$). The increase in the tumor cell killing in the EMT-6/CDDP tumor by treatment with anti-TGF-β on days 0–8 in addition to CDDP (20 mg/kg) was sufficient to produce cell killing of the EMT-6/CDDP tumor cells that was equivalent to that of the EMT-6/Parent tumor.

The bone marrow was taken from the femurs of the same animals shown in Fig. 3 and the survival of bone marrow CFU-GM was determined (Fig. 4). Bone marrow CFU-GM are, in general, not very sensitive to CDDP; however, the bone marrow CFU-GM in the animals bearing the EMT-6/CDDP tumors was less sensitive to CDDP than the bone marrow CFU-GM in animals bearing the EMT-6/Parent tumor. Treatment of the EMT-6/Parent and EMT-6/CDDP tumor-bearing animals with any of the three anti-TGF-β regimens on days 4–8 resulted in increased killing of bone marrow CFU-GM by CDDP (20 mg/kg) in animals bearing either tumor. Administration of the antibodies to TGF-β on days 0–8 produced increased bone marrow CFU-GM killing by CDDP (20 mg/kg) in animals bearing either the EMT-6/Parent or the EMT-6/CDDP tumors ($P < 0.01$) such that, overall, the killing of bone marrow CFU-GM in animals bearing these tumors was equivalent.

Because TGF-β is known to have profound effects on the metabolic status of critical normal tissues such as the liver, the systemic effects produced by the presence of the EMT-6/Parent, EMT-6/CTX or EMT-6/CDDP tumors in the presence or absence of treatment with antibodies to TGF-β were examined. Tumor-bearing animals were either untreated or injected i.p. with anti-TGF-β 2G7 and anti-TGF-β 4A11 on days 0–8 and several metabolic characteristics of the tumors and hosts were determined. Treatment of non-tumor-bearing animals with the anti-TGF-β regimen produced no change in blood ATP or serum glucose levels but did decrease serum lactate levels (Table 1). There was a trend toward increased blood ATP levels in animals bearing the resistant tumors compared with non-tumor-bearing animals and those bearing the EMT-6/Parent tumor. There were decreased glucose serum levels in the tumor-bearing animals compared with the non-tumor-bearing animals. Serum lactate was increased in animals bearing the EMT-6/CTX and EMT-6/CDDP tumors relative to animals bearing the EMT-6/Parent tumor and non-tumor bearing animals.

Table 1 Blood/serum characteristics of animals either treated or untreated with transforming growth factor-β (TGF-β) antibodies. Whole blood ATP content is expressed as mmol ATP/dl; serum lactate is expressed as mg lactate/dl; serum glucose is expressed as mg glucose/dl. (*CTX* cyclophosphamide, *CDDP* cisplatin)

	Untreated			Anti-TGF-β-treated		
Tumor	ATP	Lactate	Glucose	ATP	Lactate	Glucose
Non-tumor-bearing controls	51.3 ± 3.6	37.5 ± 2.0	310 ± 12	53.5 ± 4.2	20.7 ± 0.6	282 ± 4
EMT-6/Parent	56.7 ± 3.5	39.0 ± 2.7	188 ± 9	50.5 ± 3.0	25.5 ± 3.5	198 ± 4
EMT-6/CTX	60.3 ± 3.2	51.1 ± 3.1	206 ± 11	56.0 ± 2.2	37.2 ± 3.8	178 ± 6
EMT-6/CDDP	64.5 ± 3.3	67.9 ± 3.9	218 ± 8	64.9 ± 5.0	40.8 ± 2.9	185 ± 6

Table 2 Tumor characteristics of EMT-6 tumors from animals either treated or untreated with TGF-β antibodies. Tissue ATP is expressed as mmol ATP/mg protein. Tissue lactate and tissue glucose are expressed as mg/kg protein

	Untreated			Anti-TGF-β-treated		
Tumor	ATP	Lactate	Glucose	ATP	Lactate	Glucose
EMT-6/Parent	1.9 ± 0.2	41.2 ± 3.0	95 ± 3	2.1 ± 0.3	31.0 ± 3.0	92 ± 3
EMT-6/CTX	2.2 ± 0.2	31.4 ± 2.1	125 ± 6	2.3 ± 0.3	36.8 ± 3.3	113 ± 9
EMT-6/CDDP	2.4 ± 0.2	32.0 ± 1.8	74 ± 3	1.5 ± 0.1	29.8 ± 2.5	67 ± 5

Table 3 Enzymatic characteristics of EMT-6 tumors from animals either treated or untreated with TGF-β antibodies.Glutathione (*GSH*) is expressed as nmol/mg protein. Glutathione S-transferase activity (*GS-T*), glutathione reductase activity (*GSH red*), glutathione peroxidase activity (*GSH peroxidase*), and cytochrome P450 reductase activity (*P450 red*) are expressed as nmol substrate converted/min per mg protein

				GSH peroxidase		
Tumor	GSH	GS-T	GSH red	H_2O_2	CuOOH	P450 red
Untreated						
EMT-6/Parent	30.0 ± 2.7	194 ± 9	188 ± 6	163 ± 3	285 ± 11	14.1 ± 3.0
EMT-6/CTX	38.8 ± 3.1	291 ± 11	172 ± 4	224 ± 9	271 ± 11	9.2 ± 2.1
EMT-6/CDDP	27.2 ± 1.8	207 ± 8	198 ± 12	306 ± 13	268 ± 8	11.8 ± 2.7
Anti-TGF-β-treated						
EMT-6/Parent	28.7 ± 3.8	149 ± 11	186 ± 7	164 ± 11	286 ± 13	22.0 ± 3.6
EMT-6/CTX	41.4 ± 4.1	180 ± 9	204 ± 12	154 ± 9	255 ± 11	17.4 ± 3.2
EMT-6/CDDP	18.6 ± 3.1	99 ± 7	191 ± 9	178 ± 13	235 ± 10	16.1 ± 2.7

Treatment of animals bearing the EMT-6/CTX and EMT-6/CDDP tumors with the anti-TGF-β regimen decreased the serum lactate levels in these animals to the level of EMT-6/Parent tumor-bearing and non-tumor-bearing animals. When the same parameters were measured in tumor homogenates, no differences were found in ATP or lactate levels, while glucose levels were highest in the EMT-6/CTX tumor and lowest in the EMT-6/CDDP tumor homogenates (Table 2).

Glutathione levels were highest in the EMT-6/CTX tumors and lowest in the EMT-6/CDDP tumors with or without anti-TGF-β treatment (Table 3). Glutathione S-transferase activity was highest in the EMT-6/CTX tumor and similar in the EMT-6/Parent and EMT-6/CDDP tumors. Glutathione reductase levels were similar in all of the tumor homogenates. Glutathione peroxidase activity, as measured by the hydrogen peroxide assay, was increased in the EMT-6/CTX and EMT-6/CDDP tumors compared with the EMT-6/Parent tumor and was decreased in the EMT-6/CTX and EMT-6/CDDP tumors after treatment of the animals with the anti-TGF-β regimen. Glutathione peroxidase activity, as measured by the cupric peroxide assay, was similar in the three tumors in the presence and absence of anti-TGF-β treatment. Cytochrome P450 reductase activity was similar in the three tumors and was increased in each of the three tumors after treatment with the anti-TGF-β regimen.

Livers were collected from the same animals described above. Glutathione levels were similar in the liver homogenates from non-tumor-bearing animals and from animals bearing the EMT-6/Parent, EMT-6/CTX and EMT-6/CDDP tumors (Table 4). Treatment with the anti-TGF-β regimen decreased hepatic glutathione levels to about 60% of normal in both the non-tumor-bearing and the tumor-bearing animals. Glutathione S-transferase activity in the livers of tumor-bearing animals was decreased compared to the non-tumor-bearing control animals. Treatment of the animals with the anti-TGF-β regimen decreased the glutathione S-transferase activity in both the tumor-bearing and non-tumor-bearing animals. Glutathione

Table 4 Enzymatic chracteristics of livers from EMT-6 tumor-bearing animals either treated or untreated with TGF-β antibodies (Abbreviations as in Table 3)

Tumor	GSH	GS-T	GSH red	GSH peroxidase		P450 red
				H_2O_2	CuOOH	
Untreated						
Non-tumor bearing control	84 ± 11	1183 ± 129	129 ± 12	1652 ± 200	2435 ± 289	134 ± 9
EMT-6/Parent	75 ± 10	734 ± 88	99 ± 13	645 ± 127	1657 ± 125	109 ± 16
EMT-6/CTX	68 ± 12	856 ± 95	80 ± 9	1289 ± 190	1818 ± 180	98 ± 12
EMT-6/CDDP	74 ± 17	836 ± 114	105 ± 13	1337 ± 153	1787 ± 180	112 ± 11
Anti-TGF-β treated						
Non-tumor bearing control	52 ± 10	749 ± 59	65 ± 12	647 ± 76	948 ± 120	182 ± 16
EMT-6/Parent	73 ± 11	692 ± 65	71 ± 13	625 ± 68	1001 ± 131	160 ± 12
EMT-6/CTX	41 ± 8	681 ± 70	67 ± 8	726 ± 77	1139 ± 125	179 ± 18
EMT-6/CDDP	45 ± 9	648 ± 67	66 ± 8	537 ± 59	875 ± 93	152 ± 21

reductase activity was highest in the livers of the non-tumor-bearing animals and similar in the livers of the tumor-bearing animals. After treatment with the anti-TGF-β regimen, glutathione reductase activity was decreased to similar levels in the livers of both tumor-bearing and non-tumor-bearing animals. Glutathione reductase activity, as measured by the hydrogen peroxide assay, was highest in the livers of non-tumor-bearing animals, lowest in the livers of animals bearing the EMT-6/Parent tumor, and intermediate in the livers of animals bearing the EMT-6/CTX and EMT-6/CDDP tumors. Treatment with the anti-TGF-β regimen resulted in decreased glutathione peroxidase activity in both the non-tumor-bearing and the tumor-bearing animals. Glutathione peroxidase activity, as determined by the cupric peroxide assay, was highest in the non-tumor-bearing animals and similar in the livers of animals bearing each of the three tumors. After treatment with the anti-TGF-β regimen, this enzyme activity was decreased in both tumor-bearing and non-tumor-bearing animals. There was a trend toward lower cytochrome P450 reductase activity in the livers of the tumor-bearing compared with non-tumor-bearing animals. Interestingly, upon treatment with the anti-TGF-β regimen, there was increased cytochrome P450 reductase activity in livers of both non-tumor-bearing and tumor-bearing-animals.

Discussion

Transforming growth factor-β is a widely occurring cytokine [18]. TGF-β_1 along with other cytokines such as basic fibroblast growth factor, platelet-derived growth factor, tumor necrosis factor, and interleukin-1 are involved in tissue remodeling, that is wound healing, after injury. Excessive or sustained production of TGF-β_1 is a key factor in tissue fibrosis [19–22]. In breast cancer patients, high plasma concentrations of TGF-β_1 measured after induction chemotherapy but prior to high dose combination alkylating agent therapy with autologous bone marrow transplantation have been shown to strongly correlate with the risk of hepatic venoclusive disease and idiopathic interstitial pneumonitis [23]. In another clinical study, persistently elevated plasma TGF-β levels were a strong predictor for developing symptomatic pneumonitis after thoracic radiotherapy [24]. Muir et al. [25] found that high levels of TGF-β expression in prostate carcinoma biopsies from patients correlated with failure of the tumors to respond to hormonal withdrawal. In a cell culture study using human MCF-7 breast carcinoma, it was found that marked alterations in the levels of TGF-β (and TGF-α) may play a role in the molecular events that are involved in the progression of these cells from estrogen-responsive to estrogen-autonomous growth [26]. Welch et al. [27] tested the ability of TGF-β to alter the metastatic potential of a rat mammary carcinoma cell line. Lung colonies were measured 2 weeks after inoculation in syngeneic F344 rats and a bell-shaped dose-response curve with a 2- to 3-fold increase in the number of surface lung metastases was seen. Maximal enhancement occurred at the 50 pg/ml dose level. The effect was specific because addition of neutralizing anti-TGF-β antibody blocked the stimulatory activity at all levels of TGF-β_1 pretreatment, but when antibody was given alone, neutralizing anti-TGF-β antibody had no effect on untreated cells. TGF-β_1 may modulate metastatic potential of mammary tumor cells by controlling their ability to break down and penetrate basement membrane barriers [27]. TGF-β_1 was found to strongly stimulate the ability of human glioma cells to migrate and invade in cell culture [28]. In the clinic, expression of TGF-β_1 correlates with decreased survival, presumably due to its invasion-promoting action [28]. The EMT-6 *in vivo* alkylating agent-resistant tumor lines were developed by treatment of a tumor-bearing animal with a specific alkylating agent, followed by transfer of the tumor cells to a fresh host. Therefore, the changes observed in the host metabolic characteristics amongst the tumors

must be due to factors carried by the tumor cells. The resistant tumors are: (1) more fibrous with collagen bundles visible on electron micrographs of the tumors; (2) more aggressively metastatic than the parent tumor; and (3) much less responsive to the drugs to which resistance was developed than the parent tumor and also somewhat cross-resistant to other cytotoxic therapies [10]. Treatment with antibodies to TGF-β reversed the resistance of these tumors, if the tumors were grown in the presence of antibody treatment. Over a concentration range of 1–1000 pg/ml, addition of TGF-β_1 to the media of EMT-6/Parent, EMT-6/CTX or EMT-6/CDDP cells did not alter the growth (proliferation) of the cells under adherent or non-adherent conditions in culture (data not shown).

TGF-β seems to play an important role in hepatic pathophysiology. It has been reported to be a potent inhibitor of hepatocyte proliferation *in vitro* [29–31]. Moreover, hepatic TGF-β mRNA levels increased after partial hepatectomy in the nonparenchymal liver cells [31]; thus, TGF-β may function *in vivo* as the effector of an inhibitory paracrine mechanism to prevent uncontrolled hepatocyte growth during liver regeneration. TGF-β dramatically decreased accumulation (>95% inhibition) of the negative acute phase protein albumin in both normal human hepatocyte and human hepatoma HepG2 cell line culture media [32]. Several other proteins are also modulated by TGF-β in primary human hepatocytes and HepG2 cells; amongst these is plasminogen activator inhibitor type-1 (PAI-1), a positive acute phase protein. In hepatoma cells, TGF-β decreases apolipoprotein A-1, another negatively regulated protein during acute phase reaction [33]. The fact that TGF-β is able to act on negative (albumin and apolipoprotein A-I) as well as on positive (PAI-1) acute phase proteins, suggests that this cytokine may be an important mediator of the acute phase response.

Kayanoki et al. [34] investigated the effect of TGF-β1 on the expression of the antioxidative enzymes, manganese-superoxide dismutase, zinc-superoxide dismutase, and catalase in cultured hepatocytes of rat. TGF-β1 suppressed expression of all these antioxidative enzymes in time- and cell density-dependent manners. Furthermore, expression of two major classes of the rat glutathione S-transferase subunits 1 and 2 was also reduced by TGF-β1. Treatment of animals bearing the EMT-6/Parent, EMT-6/CTX, and EMT-6/CDDP tumors with antibodies to TGF-β selectively decreased by 30–50% the activity of glutathione S-transferase and glutathione peroxidase (H_2O_2) in homogenates of the resistant tumors (Table 3). The effects on normal liver function were profound, with a 40–60% reduction in the activity of the three sulfhydryl-related enzymes measured. The presence of a tumor in the animals decreased the activity of all the hepatic enzymes measured compared with the activities in normal animals. However, the hepatic activities of glutathione S-transferase and glutathione peroxidase were higher (closer to normal) in the liver homogenates from animals bearing the resistant tumors than in animals bearing the EMT-6/Parent tumor. Treatment of animals bearing the resistant tumors with the anti-TGF-β regimen decreased the activity of glutathione S-transferase, glutathione reductase, and glutathione peroxidase in the livers of these animals by 30–50%. After treatment with the antibodies to TGF-β, the enzymatic activities in the livers of both tumor-bearing and non-tumor-bearing animals were similar. On the other hand, the activity of cytochrome P450 reductase, a flavoprotein which transfers an electron to cytochrome P450, required with molecular oxygen to hydroxylate a substrate, is increased in both the livers and tumors of anti-TGF-β-treated animals. Cytochrome P450 reductase activity is intimately paired with cytochrome P450 activity. Cytochrome P450 is necessary for the conversion of the prodrug CTX to 4-hydroxycyclophosphamide, which is further converted to phosphoramide mustard and other active alkylating species. Overall, therefore, anti-TGF-β treatment decreased the activity of drug-inactivating enzymes and increased the activity of a drug-activating enzyme in the livers of tumor-bearing animals.

Although it cannot be concluded from these studies that increased expression of TGF-β is a direct cause of *in vivo* alkylating agent resistance, it can be concluded that treatment with TGF-β-neutralizing antibodies restored drug sensitivity to the alkylating agent-resistant tumors and that treatment with these antibodies alters both tumor and host metabolic states. Determination of whether these findings reflect a direct or indirect effect of TGF-β requires further investigation.

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