Activation of Tumor-Associated Macrophages by the Vascular Disrupting Agent 5,6-Dimethylxanthenone-4-Acetic Acid Induces an Effective CD8+ T-Cell–Mediated Antitumor Immune Response in Murine Models of Lung Cancer and Mesothelioma

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Abstract

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a small molecule in the flavanoid class that has antitumor activity thought to be due to ability to induce high local levels of tumor necrosis factor (TNF)-α that disrupt established blood vessels within tumors. The drug has completed phase 1 testing in humans and is currently in phase 2 trials in combination with chemotherapy. Although characterized as a “vascular disrupting agent,” there are some studies suggesting that DMXAA also has effects on the immune system that are important for its efficacy. The goal of this study was to carefully define the immune effects of DMXAA in a series of murine lung cancer and mesothelioma cell lines with varying immunologic characteristics. We show that DMXAA efficiently activated tumor-associated macrophages to release a variety of immunostimulatory cytokines and chemokines, including TNF-α; IFN-inducible protein-10; interleukin-6; macrophage inflammatory protein-2; monocyte chemotactic protein-1; and regulated on activation, normal T-cell expressed, and secreted. DMXAA treatment was highly effective in both small and large flank tumors. Animals cured of tumors by DMXAA generated a systemic memory response and were resistant to tumor cell rechallenge. DMXAA treatment led to initial tumor infiltration with macrophages that was followed by an influx of CD8+ T cells. These CD8+ T cells were required for antitumor efficacy because tumor inhibitory activity was lost in nude mice, mice depleted of CD8+ T cells, and perforin knockout mice, but not in CD4+ T-cell–depleted mice. These data show that activation of tumor-associated macrophages by DMXAA is an efficient way to generate a CD8+ T-cell–dependent antitumor immune response even in animals with relatively nonimmunogenic tumors. Given these properties, DMXAA might also be useful in boosting other forms of immunotherapy. (Cancer Res 2005; 65(24): 11752-61)

Introduction

Most clinically effective anticancer therapies are based on their ability to directly kill dividing tumor cells. However, therapies are now being developed that target other cells within the tumor. Possible strategies for antitumor therapy include activating the tumor-associated macrophages (1–3) or targeting the tumor vasculature (4).

Compared with the normal tissue beds, the vasculature in tumors exhibits an increased rate of proliferation, structural differences, and expression of unique genes and gene products (5). Tumor endothelium has been successfully targeted by drugs that inhibit angiogenesis. The efficacy of an antivascular endothelial growth factor (VEGF) antibody to inhibit tumor angiogenesis has recently been shown in colon and lung cancer (6, 7). Drugs classified as “vascular disrupting agents” have also been developed that can specifically affect established tumor vessels (8). Vascular disrupting agents are distinct from antiangiogenic agents in that they target existing tumor vessels rather than prevent growth of new vessels.

One class of small molecule vascular disrupting agents under study are tubulin-depolymerizing agents, such as the Combretastatins (natural products from the African willow tree; ref. 9). Another group of drugs being investigated for their antivascular properties is the flavone acetic acid (FAA) derivatives (10). Flavanoids have a unique mechanism of action and are believed to exert their effects primarily by inducing localized release of TNF-α and other cytokines within tumor tissue. A more potent analogue of FAA, called 5,6-dimethylxanthenone-4-acetic acid (DMXAA), has been extensively studied (10, 11). Treatment of tumor-bearing mice with DMXAA results in a rapid reduction in tumor blood flow followed by tumor necrosis with only a rim of viable tumor tissue remaining by 24 hours. Despite these viable cancer cells, smaller tumors can be eradicated, although in larger tumors, cures are usually not achieved, perhaps because cells in this rim are capable of rapid proliferation. DMXAA is in early clinical trials and seems to be well tolerated, with some patients experiencing disease stability (12, 13), a reduction of tumor blood flow (12), and localized increases in TNF-α activity (10).

Although flavanoids are classified as vascular disrupting agents, studies of mice treated with flavone acetic acid suggested that effects on the immune system were also important. This seems reasonable given their ability to both induce tumor necrosis and to stimulate an array of cytokines and chemokines. FAA was shown to activate natural killer cells, although this did not seem to mediate hemorrhagic necrosis (14, 15).

However, the role of the acquired immune system in the effects of flavanoids remains uncertain. A central role for T cells...
was suggested by Pratesi et al. (16) and Bibby et al. (17) who found that FAA lost much of its therapeutic effect when mouse tumor cells (CT26 or MAC26 colon cancer cells) were grown in nude or thymectomized mice versus syngeneic hosts despite the fact that hemorrhagic necrosis was still seen. This same group also saw no effect of DMXAA on MAC15A tumors grown in nude mice with good efficacy in syngeneic mice (18). In marked contrast, both DMXAA and FAA had good antitumor activity against mouse colon 38 tumor explants that were grown in nude mice, or thymectomized mice, although the percentage of cures were decreased compared with that in euthymic mice (19). Moreover, immunosuppression with high-dose cyclosporine only altered the time that that colon 38 tumor explants took to completely regress but did not reduce the number of complete regressions obtained with DMXAA (20). The specific role of T cells is also unclear. In the studies of Pratesi et al. (16), depletion of CD8+ T cells had no effect on efficacy, but antitumor activity was lost after depletion of CD4+ T cells. In contrast, studies examining the effects of FAA combined with interleukin (IL)-2 in the murine renal cell carcinoma (RENCA) model by Franco et al. (21) showed that depletion of CD4+ T cells had a partial effect on efficacy, whereas depletion of CD8+ T cells completely abrogated antitumor effects.

Additionally, although both tumor graft tissue and host stromal tissue have been implicated as producers of cytokines in response to DMXAA, a clear cellular source has not been identified (22, 23). Thus, the role of the immune system in mediating the effects of DMXAA is not well defined. However, clarification of this question is important for a number of reasons. First, a more complete understanding of the mechanisms by which DMXAA exerts its effects could help target particular tumors that might be more amenable to therapy or in designing improved dosing schedules and treatment regimens. Second, if immune effects are induced, DMXAA could potentially be combined with a variety of other immune modulating therapies that could synergize with its activities.

The goal of this study was to identify the key effector cells and carefully define the role of immune system in mediating the effects of DMXAA in a series of murine thoracic tumors with varying immunologic characteristics. We show that DMXAA efficiently activates tumor-associated macrophages to release a variety of immunostimulatory cytokines and chemokines. DMXAA treatment was highly effective in both small and large flank tumors, leading to initial tumor infiltration with macrophages followed by CD8+ T cells. These CD8+ T cells were required for antitumor efficacy because tumor inhibitory activity was lost in nude mice, mice depleted of CD8+ T cells, and perforin knockout mice but not in CD4+ T-cell-depleted mice. The ability of DMXAA to activate tumor macrophages and create a tumor microenvironment that is conducive to antitumor immune responses suggests that it could also be a useful adjunct to immunotherapy.

### Materials and Methods

#### Cell lines.

AB12 (a murine mesothelioma cell line; ref. 24) and L1C2 (a murine bronchoalveolar carcinoma cell line; ref. 25) are syngeneic to BALB/c mice. Lewis lung carcinoma (LLC) cells (syngeneic to C57/B6 mice) and the 2H-11 and H5V mouse endothelial cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). AB12, L1C2, LLC, 2H-11, and H5V cells were cultured and maintained in high-glucose DMEM (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

The TC-1 cell line, derived from transformed primary lung epithelial cells of C57/B6 mice, was obtained from ATCC. TC-1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mmol/L glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

All cell lines were regularly tested and maintained negative for Mycoplasma spp.

#### 5,6-Dimethylxanthenone-4-acetic acid.

The sodium salt of DMXAA was synthesized at the Auckland Cancer Society Research Center (26). DMXAA was formulated in normal saline and administered by i.p. injections at a dose of 18 mg/kg in 200 μL saline.

#### In vitro cell proliferation assay.

To evaluate the direct antiproliferative effects of DMXAA, cells were seeded in 96-well plates at a density of 3 x 10^3 cells per well. Twenty-four hours later, medium containing various concentrations of DMXAA was added. Survival of treated cells was compared with untreated cells 24 hours after addition of DMXAA using the MTS assay, a colorimetric test for the quantification of cell viability and proliferation (MTS Cell Proliferation Assay, Promega Corp., Madison, WI).

#### Table 1. DMXAA up-regulates mRNA for cytokines and chemokines in the tumors

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Experiment 1 (fold change ± SE)</th>
<th>Experiment 2 (fold change ± SE)</th>
<th>Average fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-10</td>
<td>90.1 ± 6.7</td>
<td>55.9 ± 2.0</td>
<td>73.0</td>
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<tr>
<td>RANTES</td>
<td>8.2 ± 0.4</td>
<td>32.9 ± 4.0</td>
<td>20.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>15.4 ± 1.4</td>
<td>10.7 ± 1.5</td>
<td>13.0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>18.4 ± 2.8</td>
<td>5.7 ± 0.6</td>
<td>12.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>12.8 ± 1.0</td>
<td>7.1 ± 0.4</td>
<td>9.95</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5.6 ± 0.2</td>
<td>8.0 ± 0.7</td>
<td>6.8</td>
</tr>
<tr>
<td>MCP-1</td>
<td>6.8 ± 0.6</td>
<td>6.0 ± 1.4</td>
<td>6.4</td>
</tr>
<tr>
<td>INOS</td>
<td>1.7 ± 0.2</td>
<td>7.1 ± 1.5</td>
<td>4.4</td>
</tr>
<tr>
<td>MIG</td>
<td>4.2 ± 0.3</td>
<td>4.3 ± 0.3</td>
<td>4.2</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>2.3 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

NOTE: Flank L1C2 tumors were established by s.c. injection in mice. The mice were treated with DMXAA i.p. when the tumor size was ~ 200 mm^3. Two hours later, tumors from control and DMXAA-treated mice were harvested and RNA was isolated. Semi-quantitative analysis of gene expression was done using real-time RT-PCR. cDNA concentrations from each pool were normalized using β-tubulin as a control gene. Relative level of expression of each of the selected genes (fold change in DMXAA-treated versus control) was determined. Each sample was run in triplicate.
Mice. Pathogen-free female BALB/c, BALB/c nude, and C57/B6 mice (6-8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Perforin knockout mice on a C57/B6 background were obtained from Taconic Laboratories (Germantown, NY). Animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA). The animal use committees of the Wistar Institute and University of Pennsylvania approved all protocols in compliance with the care and use of animals.

Animal tumor models. To establish tumors, a single cell suspension of 10^6 tumor cells in 100 μL serum-free medium was injected s.c. into mice flanks. Tumors were measured twice weekly and volumes were estimated using the formula \( \frac{\pi}{6} \times \text{largest diameter} \times (\text{perpendicular diameter})^2 \). Treatment was administered when tumors were \( f_{200} \) mm^3 in size and mice followed for tumor growth. Mice were sacrificed when the tumors became \( >10\% \) body weight or the mice showed signs of distress.

Real-time reverse transcription-PCR in tumors. To evaluate the effect of DMXAA on expression level of selected cytokine and chemokine mRNAs in tumors, L1C2 tumors from control and DMXAA-treated mice (n = 6) were harvested 2, 4, 8, and 24 hours after i.p. injection of DMXAA, homogenized, and mediator levels were measured using a Lumines buffer assay. Columns, fold change (DMXAA-treated versus control); bars, SE. * , \( P < 0.05 \), significantly different than controls. C, to identify the source of cytokines, tumors were harvested from L1C2-bearing (i) or TC-1-bearing (ii) mice (n = 6) and digested. Macrophages were isolated using CD11b magnetic beads and equal numbers were incubated with DMEM (Control) or DMXAA (10 μg/mL) for 5 hours. TNF-α was measured in the supernatant by ELISA. Columns, average of two measurements done on cells aggregated from six mice each.

Figure 1. DMXAA up-regulates production of proinflammatory cytokines and chemokines in tumors. BALB/c mice with established L1C2 tumors were treated i.p. with either saline (Control) or 18 mg/kg DMXAA. A, tumors were harvested from control and DMXAA-treated mice (n = 3) and chopped into small pieces. Supernatants were collected 5 hours later and TNF-α levels were ascertained by ELISA and standardized to total protein levels. Columns, mean TNF-α (pg/mL/mg protein); bars, SE. B, to assess cytokine/chemokine levels within tumors, tumors (n = 6) were harvested 6 hours after i.p. injection of DMXAA, homogenized, and mediator levels were measured using a Luminex bead assay. Columns, fold change (DMXAA-treated versus control); bars, SE. * , \( P < 0.05 \), significantly different than controls. C, to identify the source of cytokines, tumors were harvested from L1C2-bearing (i) or TC-1-bearing (ii) mice (n = 6) and digested. Macrophages were isolated using CD11b magnetic beads and equal numbers were incubated with DMEM (Control) or DMXAA (10 μg/mL) for 5 hours. TNF-α was measured in the supernatant by ELISA. Columns, average of two measurements done on cells aggregated from six mice each.

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Animal tumor models. To establish tumors, a single cell suspension of 10⁶ tumor cells in 100 μL serum-free medium was injected s.c. into mice flanks. Tumors were measured twice weekly and volumes were estimated using the formula \( 3.14 \times \text{largest diameter} \times (\text{perpendicular diameter})^2 \) / 6. Treatment was administered when tumors were \( \approx 200 \text{ mm}^3 \) in size and mice followed for tumor growth. Mice were sacrificed when the tumors became \( >10\% \) body weight or the mice showed signs of distress.

Real-time reverse transcription-PCR in tumors. To evaluate the effect of DMXAA on expression level of selected cytokine and chemokine mRNAs in tumors, L1C2 tumors from control and DMXAA-treated mice were harvested 2, 4, 8, and 24 hours after DMXAA treatment and homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA). Three micrograms of RNA from each tumor were reverse-transcribed using 0.5 μg oligo(dT) (Promega), 10 mmol/L deoxyribose triphosphates (Clontech, Palo Alto, CA), and 1 unit of PowerScript Reverse Transcriptase in 5× First-Strand Buffer and 100 mmol/L DTT (Clontech) for 80 minutes at 42°C. Primers were designed using the protocol available in Sambrook and Russell (27). Primer sequences can be obtained from the authors upon request. Semiquantitative analysis of gene expression was done using a Cepheid Smart Cycler (Sunnyvale, CA) following the protocol of the manufacturer for SYBR green kit supplied by Roche. cDNA concentrations from each pool were normalized using \( \beta\)-actin as a control gene. Relative level of expression of each of the selected genes (fold change in DMXAA-treated versus control) was determined. Each sample was run in triplicate. The 2-hour time point was found to show maximal responses and the experiment was repeated at this time point (data presented for this time point only).

Protein studies for cytokine and chemokine levels. The amount of TNF-α secreted by tumors activated by DMXAA was quantified using an ELISA kit to detect murine TNF-α according to the instructions of the manufacturer (BD OptEIA ELISA set, BD Biosciences PharMingen, San Diego, CA). Briefly, mice bearing L1C2 flank tumors were treated with DMXAA and tumors were harvested 2 and 21 hours later. These tumors were chopped into small pieces and immersed in serum-free DMEM containing 50 mg/L polymyxin B (Sigma-Aldrich, St. Louis, MO) in 48-well plates for a further 3 hours. The supernatant was then collected and tumors were weighed. Polymyxin was added because previous studies have shown that endotoxin contamination can stimulate DMXAA-mediated cytokine secretion (28). Note that there was no DMXAA in culture medium and the tumors were treated with DMXAA only in vivo. Preliminary studies showed
that the 5 hours (2 hours in vivo + 3 hours in culture medium) time point showed higher levels of TNF-α. Therefore, in replicate experiments, only this time point was used. The supernatants were spun at 14,000 rpm for 10 minutes and then aliquoted at −80°C until use.

To compare changes in cytokine and chemokine mRNA expression with protein levels within tumors, mice bearing L1C2 flank tumors were treated with DMXAA. After harvesting at 6, 24, and 48 hours, the tumors were sonicated for 30 seconds in 1 mL of complete buffer (50 mL PBS containing one tablet of antiprotease cocktail, Roche, Indianapolis, IN). Tissues were then spun at 3,000 rpm for 10 minutes and filtered through a 1.2 μm syringe filter unit. Total protein in each sample was determined. Mouse cytokine expression was measured using a multiplex Luminex bead assay system as previously described (29). Samples were incubated for 2 hours at room temperature with a mixture of anti-KC/CXCL1; IL-6; monocyt chemotactic protein-1 (MCP-1)/CCL2; ITAC/CXCL11; regulated on activation, normal T-cell expressed, and secreted (RANTES)/CCL5, macrophage inflammatory protein-2 (MIP-2)/CCL2/3; and IFN-inducible protein-10 (IP-10)/CXCL10 beads in a 96-well plate. A mixture of the same panel of biotinylated antibodies was added to each well and the plate was incubated at room temperature for 1.5 hours. Streptavidin-phycoerythrin was added to the wells and the plate was incubated for 30 minutes. Next, 0.2% paraformaldehyde was added to the wells and the plate was read on a Luminex 100 IS instrument. The concentration of cytokines was determined from a standard curve assayed at the same time with known amounts of recombinant proteins. Data from the 6-hour time point showed maximal values and is presented here.

Isolation of macrophages from tumors. L1C2 and TC-1 flank tumors were harvested from BALB/c and C57/B6 mice respectively, chopped, and digested with 1 mg/mL DNase I and 2 mg/mL collagenase type IV (Sigma, St. Louis, MO) at 37°C for 1 hour. Six to eight tumors were pooled together to obtain the required yield of cells. The resulting mixture was filtered and washed thrice in medium containing 50 mg/l polymyxin B. Polymyxin B has no effect on the ability of DMXAA to induce TNF-α; however, because mouse macrophages are very sensitive to low amounts of lipopolysaccharide contamination, it was routinely included in experimental medium. Cells were sorted for macrophages using CD11b magnetic beads (Miltenyi Biotec, Inc., Auburn, CA) and a relatively pure population (~95%) of macrophages was obtained. Both CD11b-positive and CD11b-negative cells were collected and plated in 12-well plates at concentration of 2.5 × 10^6 cells per well in 500 μL of control DMEM or medium containing DMXAA (10 μg/mL). Cells were incubated for 5 hours at 37°C, supernatants were collected and frozen at −80°C until use. The TNF-α level in the supernatants were measured by ELISA.

Immunohistochemical studies. Animals bearing flank tumors were treated with i.p. injections of DMXAA. Three mice were euthanized at different time points, tumors were harvested and immediately placed in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA) to be stored at −80°C. Five-micrometer sections were cut. Monoclonal antibodies against leukocytes (anti-CD45), macrophages (anti-CD11b), and CD8+ cells (anti-CD8) were obtained from BD Biosciences and immunohistochemical staining was done according to established protocols. Tumor cell infiltrate was quantified by counting the number of positively staining cells in multiple fields (>5) on multiple slides per high-powered (×40) field.

In vivo depletion of CD4+ and CD8+ T cells. To deplete CD4+ or CD8+ T cells, mice were injected i.p. with monoclonal antibodies purified from the anti-CD4 hybridoma GK1.5 or the anti-CD8 hybridoma 53-6.7, respectively (both obtained from ATCC). Mice were injected i.p. with 300 μg of purified antibody in 200 μL of PBS. Antibody was administered 3 days and 1 day before tumor cell injection. Thereafter, a maintenance dose of 300 μg was delivered every 6th day to ensure persistent depletion of targeted

Figure 2. DMXAA has significant antitumor effects in murine mesothelioma and non–small cell lung cancer tumors grown in syngeneic mice. Immunocompetent mice (five mice per group) with established LLC, L1C2, TC-1, and AB12 tumors were treated with DMXAA (18 mg/kg i.p.) when they were either relatively small (120-300 mm³; A and B) or larger (400-600 mm³; C and D). Tumor growth curves (A and C) or measured tumor volumes at 22 to 32 days after implantation (B and D) of both the small and the large LLC, L1C2, TC-1, and AB12 tumors were significantly smaller in the DMXAA-treated mice than in the control mice. Points and columns, mean volume (mm³); bars, SE. *, P < 0.05. Arrow, time of treatment with DMXAA.
lymphocyte population. For some experiments, the schedule for antibody administration varied with experimental goals; such variation has been described in Results. CD4+ and CD8+ lymphocyte depletion was confirmed by flow cytometry of splenic cell suspension (data not shown).

Statistical analysis. Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student’s t test. Multiple comparisons were made using ANOVA with appropriate post hoc testing. Differences were considered significant when \( P < 0.05 \).

Figure 3. DMXAA causes extensive tumor necrosis and a biphasic cellular influx into the tumor. A, wild-type mice. Mice bearing either LLC (A-B and E-P) or TC-1 (C-D) tumors were treated with DMXAA and tumors were harvested at 24 hours, 48 hours, and 7 days after treatment for histology and immunohistochemical studies. By H&E staining, both the LLC (A and B) and TC-1 (C and D) tumors showed presence of a significant areas of necrosis with peripheral sparing of tumor tissue at 24 hours after treatment (arrowheads). Immunohistochemical studies done in LLC tumors over a time course of 24 hours to 7 days showed a heavy infiltration of white cells (stained with anti-CD45 antibody) into tumor (E-H). Most of these cells were macrophages (stained with anti-CD11b; I-L). CD8+ T cells were scant in the tumors initially (M and N) but appear at the periphery of the tumor at day 3 (O, arrows) and infiltrate into the tumor by day 7 (P). B, CD8+ T-cell-depleted mice. LLC flank tumors were established in mice depleted of CD8+ T cells. These mice were treated with DMXAA and tumors were harvested 48 hours after treatment. H&E and immunohistochemical staining was done. Compared with controls (A), the DMXAA-treated tumors (B) showed the presence of large area of necrosis, which was sharply demarcated from a rim of viable tissue (arrowheads). CD11b immunostaining revealed a significant macrophage influx into the DMXAA-treated tumors (D) compared with the controls (C).
Results

Mature tumor and tumor endothelial cell lines are relatively resistant to 5,6-dimethylxanthenone-4-acetic acid in vitro. Three murine lung cancer cell lines and one murine mesothelioma cell line were exposed to various concentrations of DMXAA to determine direct antiproliferative effects. The concentrations of DMXAA, which inhibited the proliferation of tumor cells by 50% compared with the control cells (IC50) for L1C2, LLC, TC-1, and AB12 cell lines were 310, 285, 252, and 516 μg/mL, respectively. DMXAA may cause tumor vascular disruption by directly acting on tumor endothelium (30), so we also checked the direct antiproliferative effect of DMXAA on the H5V and 2H11 tumor endothelial cell lines in vitro. The IC50 of DMXAA for 2H11 cells was 665 μg/mL and was >1,000 μg/mL for H5V cells. Because administration of maximal dose of DMXAA to mice corresponds to a maximal free drug concentration of 9 μg/mL in the plasma (31), it is unlikely that direct tumor or tumor endothelial cell killing plays a significant role in DMXAA activity in vivo.

5,6-Dimethylxanthenone-4-acetic acid markedly up-regulates mRNA levels of proinflammatory and chemotactant cytokines/chemokines in tumors. To confirm and extend previous work showing up-regulation of mRNA for various cytokines in tumors in response to DMXAA (32), we did real-time reverse transcription-PCR (RT-PCR) studies on control and DMXAA-treated L1C2 tumors 2 hours after DMXAA administration using primers for several cytokins and chemokines. The mRNAs for IP-10, RANTES, IL-6, IFN-γ, TNF-α, MIP-1α, MCP-1, iNOS, Mig, and intercellular adhesion molecule (ICAM) were up-regulated between 73-fold and 2-fold (Table 1).

5,6-Dimethylxanthenone-4-acetic acid up-regulates production chemokines and cytokines in tumors. To ascertain if mRNA up-regulation correlated with protein up-regulation, we evaluated the levels of some of the proteins secreted by tumors after treatment with DMXAA. We did ELISA to quantitate TNF-α secreted by tumors. L1C2 tumors treated with DMXAA showed TNF-α levels of 283 ng/g total protein compared with only 5 ng/g in the control tumors (56-fold increase; Fig. 1A). We also tested for up-regulation of other proteins in tumor homogenates using a Luminox bead assay and found that IP-10 (CXCL10), IL-6, KC (CCL2), MIP-2 (CCL2/3), MCP-1 (CCL2), and RANTES (CCL5) were significantly up-regulated (12 to 2 fold) in DMXAA-treated tumors (Fig. 1B).

Macrophages are the major source of tumor necrosis factor-α in the tumors. To test the hypothesis that the major source of TNF-α in the tumors were macrophages, we isolated CD11b-positive and CD11b-negative cells from L1C2 and TC-1 flank tumors. Both the CD11b-positive and CD11b-negative cells were exposed to either DMEM or DMEM containing 10 μg/mL DMXAA. A concentration previously shown to be optimal for in vitro studies with DMXAA (33). Supernatants were collected 5 hours later and TNF-α levels were determined by ELISA. The supernatants from CD11b-positive cells (macrophages) extracted from both the tumor cell types exposed to DMXAA showed very high levels of TNF-α (1,565 pg/mL from L1C2 tumors and 1,125 pg/mL from TC-1 tumors; Fig. 1C). The CD11b-negative cells secreted minimal amounts of TNF-α in the supernatant. These data show that the main cellular source of TNF-α is the tumor-associated macrophages and that DMXAA possesses the ability to trigger these macrophages to secrete TNF-α.

5,6-Dimethylxanthenone-4-acetic acid shows significant antitumor effects in AB12, L1C2, LLC, and TC-1 tumors grown in syngeneic mice. DMXAA has been shown to be highly effective in some cancer models. We evaluated the efficacy of DMXAA in our syngeneic lung cancer (LLC, TC-1, and L1C2) and mesothelioma (AB12) models. In small pilot experiments, a dose of 18 mg/kg was found to be optimal for our experiments (data not shown). Mice bearing flank tumors were treated with DMXAA when the average tumor size was between 120 and 300 mm3. DMXAA was very effective in causing reduction in tumor size in all four cell lines. Cure rates of 100% (AB12), 80% (LLC), 100% (TC1), and 60% (L1C2) were obtained (Fig. 2A and B).

To ascertain the dependency of tumor size on effectiveness of treatment, we treated large tumors (between 440 and 600 mm3) with DMXAA. We found that even in large tumors, DMXAA was effective in restricting tumor growth. The average tumor size in the treated animals compared with controls was 49% (TC1), 36% (L1C2), and 38% (LLC; Fig. 2C and D).

Grossly, DMXAA-treated tumors had a deep blue, congested appearance noticeable at 4 hours. Over the next 2 days, these tumors developed frank ulcers. In smaller tumors, this ulcer was later covered with a scab, which eventually fell off leaving behind intact skin with no residual tumor. In larger tumors, there was a macroscopically visible rim of viable tumor tissue surrounding the

Table 2. Quantification of tumor cell infiltration after DMXAA treatment

<table>
<thead>
<tr>
<th>Cells per high power (×40) field (mean ± SE)</th>
<th>Control</th>
<th>24 h post DMXAA</th>
<th>72 h post DMXAA</th>
<th>7 d post DMXAA</th>
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<tr>
<td>CD11b+ cells in wild-type mice</td>
<td>12.8 ± 5</td>
<td>129 ± 8*</td>
<td>102 ± 11</td>
<td>159 ± 14*</td>
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<tr>
<td>CD11b+ cells in CD8-depleted mice</td>
<td>19 ± 3</td>
<td>175 ± 7*</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>CD8+ cells in wild-type mice</td>
<td>14 ± 8</td>
<td>ND</td>
<td>ND</td>
<td>74 ± 5*</td>
</tr>
</tbody>
</table>

Note: Mice bearing LLC flank tumors were treated with DMXAA. Tumors were harvested and flash frozen at 24 hours, 72 hours, and 7 days after treatment. Frozen sections were cut and stained with antibodies against macrophages (CD11b) or T-cells (CD8). The number of positively staining cells per ×40 high-power field (mean ± SE) are shown. CD8 cells were not quantified in the 24- and 72-hour samples because the T-cell distribution was very inhomogeneous with cells only at the periphery of the tumors (see Fig. 3). CD11b cells were also measured in tumor-bearing mice depleted of CD8 T cells. CD8 depletion had no effect on macrophage accumulation. Abbreviation: ND, not determined. *P < 0.05 compared with control.
ulcer, which subsequently became thicker, and tumors began to regrow after a brief posttreatment growth arrest.

5,6-Dimethylxanthenone-4-acetic acid causes a biphasic influx of leukocytes into tumors. To assess the tumors microscopically, we did staining on tumor sections. Flank LLC tumor-bearing mice were treated with DMXAA and tumors harvested at 24, 48, and 72 hours and 7 days after treatment. H&E staining showed the presence of a large area of central necrosis with a diffuse infiltration of white cells into the tumor. A peripheral rim of intact tumor tissue was observed (Fig. 3A, A-D). Antibodies against CD45 (pan-WBC marker) showed that white cells infiltrated into the tumors at 24 hours and were present at 7 days after treatment (Fig. 3A, E-H). CD11b staining revealed that most of the infiltrating white cells were macrophages. Macrophage infiltration was significantly \( P < 0.05 \) increased in these tumors 24 hours after DMXAA administration and remained elevated up to 7 days after treatment (Fig. 3A, I-L). Table 2 quantifies this infiltration by demonstrating the number of CD11b cells per high power field \((\times 40)\). There were very few CD8\(^+\) T cells at 24 and 48 hours after DMXAA treatment. The CD8\(^+\) cells began to appear, mostly at the periphery of the tumors, at 72 hours and were seen infiltrating into the tumors in significant numbers at 7 days posttreatment \(( P < 0.05 \) compared with baseline; Fig. 3A, M-P; Table 2). Tumors with the most prominent CD8\(^+\) T-cell infiltrates were smaller than the tumors without these infiltrates. These data show a shift, with time, in the spectrum of cell types involved in the antitumor effects: macrophages predominating the early posttreatment phase and CD8\(^+\) T cells being important in the later stages.

Cured animals are resistant to tumor growth when rechallenged with same tumor type. Influx of CD8\(^+\) T cells into the tumors after DMXAA treatment suggested a role of the adaptive immune system in the process of tumor eradication. We, therefore, looked for generation of a memory response. Animals that were injected with flank tumors and were cured by DMXAA treatment were rechallenged with \( 1 \times 10^6 \) cells of same tumor cell type injected into the opposite flank. Naïve animals were injected at the same time and tumor growth compared between the two groups. The growth of tumors in the rechallenged animals was significantly inhibited when compared with the naïve controls. Whereas tumors were detected in all of the control animals, 0/5 AB12, 1/4 L1C2, and 0/8 LLC tumors grew in the rechallenged mice on day 14 after rechallenge. These data indicate generation of a systemic immune response that develops after treatment with DMXAA resulting in the rejection of rechallenge with tumor cells.

Anti-tumor efficacy of 5,6-dimethylxanthenone-4-acetic acid is lost in immunodeficient mice. Prevention of tumor growth in rechallenged mice suggested that systemic immunity was generated in response to DMXAA treatment. Therefore, we studied the effects of DMXAA on tumors grown in mice lacking functional T-cells. L1C2, LLC, and TC1 flank tumors were grown in athymic nude mice and DMXAA was administered when the mean tumor volume was 270 to 386 mm\(^3\). The efficacy of DMXAA was almost completely lost in these mice in all the three cell lines. As shown in Fig. 4A, B, and C, the average tumor volume in the treated group compared with control was 100\%, 87\%, and 91\% in L1C2, LLC, and TC1 tumor-bearing mice, respectively. These data show a crucial role for T-cell-mediated immunity in mediating antitumor effects of DMXAA.

CD8\(^+\) T lymphocytes mediate the antitumor effects of 5,6-dimethylxanthenone-4-acetic acid. To further identify the T-cell subset involved in mediating effects of DMXAA, we used antibodies to specifically deplete either CD8\(^+\) or CD4\(^+\) T lymphocytes. Flank tumors were studied in two cell lines, L1C2 (Fig. 4D) and LLC (Fig. 4E). Mice were treated with DMXAA when the mean tumor volume in the nondepleted mice was between 260 and 306 mm\(^3\). In both cell lines, the antitumor effect of DMXAA was completely lost when the CD8\(^+\) T cells were depleted but was retained when the CD4\(^+\) T lymphocytes were depleted. These results show that CD8\(^+\) T lymphocytes are essential for the therapeutic effects of DMXAA.
Depletion of CD8+ T cells does not inhibit necrosis and macrophage influx into tumors. Possible explanations for loss of effect of DMXAA in CD8-depleted mice would be the absence of necrosis or limited migration of macrophages into tumor. To examine these hypotheses, flank LLC tumors were grown in mice in which CD8+ T cells had been depleted. These mice were treated with DMXAA and tumors were harvested 48 hours later for histologic studies. H&E staining revealed the presence of significant amounts of necrosis in treated mice (Fig. 3B, A and E). Immunohistochemical staining using the CD11b antibody showed increased infiltration of macrophages into tumors (Fig. 3B, C and D) similar in magnitude as seen in wild-type mice (Table 2). These data show that tumor necrosis and influx of macrophages can occur in the absence of circulating CD8+ T cells but are not sufficient to significantly inhibit tumor growth.

CD8+ T lymphocytes are essential after administration of 5,6-dimethylxanthenone-4-acetic acid to produce tumor cures. To confirm that CD8+ T cells were involved in the antitumor response after the administration of DMXAA, we started CD8+ T cell depletion at the same time as DMXAA treatment. As shown in Fig. 4F, treatment with DMXAA in these animals results in an initial decrease in tumor size but tumors began to grow again and thereafter grew at the same rate as the control tumors. These data show that CD8+ T cells are not required in the early posttreatment phase but suggest a role of CD8+ cells in the late posttreatment phase.

Figure 4. A, antitumor effect of DMXAA is lost in immunodeficient mice. Treatment of immunocompetent mice with DMXAA with L1C2 flank tumors led to marked growth inhibition. *, P < 0.001. B, in contrast, treatment of BALB/c nude mice bearing established L1C2 tumors with DMXAA had virtually no effect on tumor growth. **, P > 0.9. C, similarly, treatment of BALB/c nude mice with established LLC or TC-1 tumors (n = 5) with DMXAA had no significant antitumor effects compared with control tumors when measured at 21 or 22 days after tumor inoculation. #, P > 0.1; ##, P > 0.4. D and E, L1C2 (D) and LLC (E) tumors were grown in the immunocompetent mice and mice that were depleted of either CD8+ or CD4+ T cells using monoclonal antibodies for the duration of the experiment (shadowed column). These mice were then divided into control (n = 5) or the treatment (DMXAA 18 mg/kg i.p.; n = 5 or 6) groups. Mean tumor volume in the DMXAA treatment group in the intact and CD4+ T-cell-depleted mice was significantly smaller than the control group for both the L1C2 (D) and LLC (E) tumors. *, P < 0.01; #, P < 0.001. The treatment groups in the CD8+ T-cell-depleted mice were not significantly smaller than their respective controls. **, P = 0.46; ##, P = 0.84. F, L1C2 tumors were established in immunocompetent mice. Mice were treated with DMXAA on day 12. At the same time, CD8+ T-cell depletion was commenced in selected mice (n = 5) and maintained through the further course of the experiment (shadowed column). Although the tumors in the CD8+ T-cell-depleted mice were significantly smaller than the control group, there was only an initial brief growth delay in response to treatment after which the tumors began to regrow at almost the same rate as the controls. **, P < 0.05. G, LLC tumors were established in perforin knockout (K.O.) mice and received either no treatment (n = 5) or treatment with 18 mg/kg DMXAA i.p. (n = 5). Although the tumors in the treatment group were smaller than the control, DMXAA did not produce tumor cures and after an initial delay growth began to regrow at almost the same rate as the controls. **, P < 0.05. Points and columns, mean volume; bars, SE. Arrow, time of treatment with DMXAA.

Discussion

DMXAA has completed phase 1 clinical trials (12, 13) and a phase 2 trial has recently been initiated in combination with chemotherapy for patients with non–small cell lung cancer. Because the mechanisms of action of DMXAA are still unclear, we studied the effects of DMXAA in murine models of thoracic cancers.

Although DMXAA has been classified as a “vascular disruptive agent” causing TNF-α secretion and hemorrhagic necrosis in tumors, our data confirm other studies showing that the necrosis caused by DMXAA is not enough to produce tumor cures. In the tumor models that we studied, DMXAA does not produce its antitumor effects due to necrosis or by its direct antiproliferative actions but by generation of a potent antitumor immune response involving CD8+ T cells. This conclusion is supported by several pieces of data. First, treatment with DMXAA results in a delayed, but impressive intratumoral infiltration of CD8+ T cells (Fig. 3; Table 2). Second, animals “cured” of their tumors after DMXAA treatment show immunity to tumor cell rechallenge. Third, antitumor efficacy of DMXAA is lost in nude and CD8+ T-cell-depleted mice despite the presence of hemorrhagic necrosis (Fig. 3B; ref. 4).
We specifically wanted to study the effects of DMXAA on tumors with different levels of immunogenicity as defined by the inability of injected tumor cells to grow after previous vaccination of mice with irradiated cells. Using these variables, LLC cells were nonimmunogenic, TC-1 cells were intermediate immunogenic, and AB-12 and L1C2 cells were highly immunogenic (data not shown). Interestingly, even in the nonimmunogenic LLC tumors, the effect of DMXAA was completely dependent on CD8+ T cells.

Our data showing lack of DMXAA effect in nude mice are similar to the studies of Pratesi et al. (16), Bibby et al. (17), and Law et al. (18); however, these investigators found that CD4+ T cells were required for FAA effects, not CD8+ T cells. Our data are consistent with the observations of Franco et al. (21) who showed an important CD8+ T-cell component to antitumor efficacy of FAA (in combination with IL-2) in a RENCA murine carcinoma model. Our findings differ from those that showed retention of activity of DMXAA against colon 38 tumors in nude mice (19). The reason for these differences is not known for certain but may relate to the specific tumor types studied. The mouse colon 38 tumor explant system may behave differently from the lung cancer and mesothelioma cell lines studied here.

Our data support a model in which both the innate and adaptive immune systems are required for optimal antitumor effects, with an early effector phase driven by macrophages and a late effector phase driven by CD8+ T cells. Systemic administration of DMXAA rapidly activates CD11b+ tumor-associated macrophages (within 2 hours) causing up-regulation of mRNA for a variety of cytokines and chemokines, including IP10, RANTES, IL-6, IFN-γ, TNF-α, MIP-1α, MCP-1, MIG (Table 1), as well as inducing the secretion of many of these proteins (documented for TNF-α, IP-10, IL-6, KC, MIP-2, MCP-1, and RANTES; Fig. 1). In in vitro studies, DMXAA did not induce tumor cells to chemotactic macrophages (data not shown). Based on previous studies (34), although not directly evaluated in this study, macrophage cytotoxicity for tumor cells is also likely enhanced. Histologic studies at early time points (24-48 hours) show the presence of large areas of necrosis within the treated tumors, with a peripheral rim of viable tumor tissue (Fig. 3). The tumors were found to be heavily infiltrated with leukocytes, the majority of which were macrophages. CD8+ T cells were rare in the tumors immediately after treatment but appeared in tumors ~3 days posttreatment and infiltrated into tumors in significant numbers 1 week after DMXAA administration (Table 2). We postulate that this initial combination of vascular activation, tumor necrosis, and the presence of inflammatory cytokines and chemotactants generates an immunostimulatory microenvironment that attracts an early monocyte (and possibly dendritic cell) infiltration into the tumor (Fig. 3), which, in turn, supports effective tumor antigen presentation and consequent generation of CD8+ CTLs. These CTLs subsequently infiltrate the tumor between days 3 and 7 post-DMXAA treatment (Fig. 3), causing tumor destruction. This effect can be blocked in the absence of perforin, suggesting involvement of direct cytolytic pathways in eradication of tumors by CD8+ T cells (Fig. 4G).

Our model explains the presence of necrosis, but lack of tumor cures, in nude mice and the mice depleted of CD8+ T cells. Although we have focused on the role of CD8+ T cells in the effector phase of DMXAA treatment, they may also play a role in “priming” the tumor-associated macrophages. This idea is based on the observation that when CD8+ T cells were depleted just before treatment (compared with depleting CD8+ cells before injecting tumor cells), there was an initial (3-4 day) delay in tumor growth (Fig. 4F) followed by a rapid regrowth of the tumor. This initial effect was not seen in nude mice or in mice depleted of CD8+ T cells at the time of tumor cell injection (Fig. 4). We speculate that this initial effect may have been due to innate immune responses induced by the tumor-associated macrophages that have been primed by CD8+ T cells. This idea is supported by the presence of a similar partial antitumor effect seen in the perforin knockout mice, suggesting that the effects of CD8+ T cells in the pretreatment phase are perforin-independent and may be mediated by cytokine secretion. Experiments to examine the role of CD8+ T cells in priming phase are ongoing and will be reported separately. Interestingly, the CD8+ T cell effect does not require CD4+ T cell help as complete tumor cures can be achieved even in the complete absence of CD4+ T cells.

Although the role of CD8+ T cells was crucial for producing tumor cures, the fact that DMXAA primarily acts on tumor-associated macrophages highlights the potential key role of this cell type in initiating and maintaining adaptive antitumor immune responses. Two distinct phenotypes have been described for tumor macrophages (35). M1 macrophages promote TNF-α, nitric oxide, and antiangiogenic chemokines (like Mig and IP10) and inhibit tumor growth. M2 macrophages produce cytokines like IL-10, transforming growth factor-β, and VEGF that suppress T-cell activity and promote angiogenesis. Unfortunately, in most established mouse and human tumors, abundant numbers of the M2-pheno type macrophages are usually present (34). This immunosuppressive/angiogenic environment in the tumor is one of the major hurdles in developing efficacious immune therapies, which, if overcome, could potentially augment exogenous immune therapy. A recent study demonstrating synergistic effects with combination of the chemokine CCL-16 with an IL-10 receptor antibody and CpG supports this hypothesis and underscores the role of tumor-associated macrophage activation to overcome immune tolerance (3). DMXAA seems a highly effective way to convert M2 macrophages into cells with an M1 phenotype, as well as recruiting new macrophages that are immunostimulatory. Thus, properly stimulated macrophages generate an innate antitumor response that inhibits, rather than promotes, tumor growth.

In addition to initiating effective antitumor immune responses, DMXAA may be a potential agent that can be combined with other forms of immunotherapy to generate synergistic effects. Treatment with DMXAA can markedly alter tumor microenvironment by switching macrophages into an activated phenotype and result in secretion of chemotactic cytokines that can traffic immunocytes to tumor. Additionally, an inflammatory milieu within the tumor may allow for more effective activation and function of these trafficked cells. Further, the necrosis induced within the tumors, in addition to reducing tumor burden, could also release tumor-specific antigens that could be presented by the antigen-presenting cells (dendritic cells and macrophages) to T cells, resulting in better activation of the adaptive immune system. Previous studies have shown that the combination of FAA with IL-2 enhanced the efficacy of FAA (36, 37). The antitumor efficacy of DMXAA was enhanced by the intratumoral injection of a plasmid encoding the costimulatory molecule B7.1 (CD80) followed by i.p. administration of DMXAA (38). ICAM-1...
immunogene therapy was similarly enhanced with DMXAA that was dependent on CD8+ and natural killer cells (39). We also have preliminary data to support the value of combining DMXAA with an antitumor vaccine.4

In summary, we have shown a tumor-associated macrophage activator, DMXAA, that can induce strong CD8+ T-cell-mediated antitumor immune responses in both immunogenic and non-immunogenic tumors and have implicated a role for both innate and adaptive immunity in the mechanism of the antitumor action of DMXAA. Initially, DMXAA activates tumor-associated macrophages to produce inflammatory cytokines and chemokines. These mediators lead to tumor necrosis and to the generation of CD8+ T cells that are critical mediators of the ultimate antitumor effects of DMXAA. Our data provides strong rationale for further studies involving the use of DMXAA to induce antitumor immune responses and the combination of DMXAA with other forms of immunotherapy.

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References


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