Stimulation of Tumors to Synthesize Tumor Necrosis Factor-α in Situ Using 5,6-Dimethylxanthenone-4-acetic Acid: A Novel Approach to Cancer Therapy

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ABSTRACT

The selective induction of tumor vascular collapse represents an exciting approach to cancer treatment. However, clinical evaluation of tumor necrosis factor-α (TNF), an agent that accomplishes this goal, has been limited by systemic toxicity, and clinical approaches using bacterial components to induce TNF production have also been disappointing. Our laboratory has developed synthetic low molecular weight inducers of TNF, including 5,6-dimethylxanthenone-4-acetic acid (DMXAA), as an alternative strategy. DMXAA induces rapid vascular collapse in transplantable murine tumors and induces TNF synthesis in vitro in both murine and human systems. We show here that the extent of DMXAA-induced TNF synthesis is greater in tumors than that in the spleen, liver, or serum. As shown by in situ hybridization studies of the murine Colon 38 tumor, DMXAA induced tumor as well as host cells to express TNF mRNA. The distribution of cells containing TNF mRNA in tumor tissues after DMXAA administration contrasted significantly with that obtained after lipopolysaccharide (LPS) treatment, although splenic and hepatic tissues showed a similar distribution of TNF mRNA-positive cells. In the Colon 38 tumor, the action of LPS was limited to host cells in the periphery of the vessels. DMXAA treatment induced 7-fold higher peak TNF levels in tumor than in serum. In contrast, LPS treatment induced 9-fold higher TNF levels in serum than in tumor. DMXAA induced 35-fold higher TNF activity in the Colon 38 tissue than did LPS. One ovarian, one squamous, and three melanoma human tumor xenografts implanted in athymic nude mice expressed TNF mRNA of human and murine origin in response to DMXAA, confirming that DMXAA can activate both host and tumor cells. The use of low molecular weight agents to induce TNF synthesis in situ in the tumor represents a novel approach to TNF-mediated therapy of cancers.

INTRODUCTION

Since its discovery over 20 years ago, TNF has offered the promise of a new approach to cancer therapy (1). This M, 17,000 protein, produced by macrophages and lymphocytes in response to bacterial infections, induces hemorrhagic necrosis and, in some cases, regressions of transplanted murine tumors (2, 3). Although TNF may have a direct cytotoxic action against tumor cells in culture (4, 5), the induction of hemorrhagic necrosis is thought to be mediated primarily by its action on the tumor vasculature (5). Tumor vascular hemorrhage is observed 1–2 h after TNF administration, vascular congestion at 4–6 h, and blockage of blood flow at 24 h (6). Treatment of vascular endothelial cells in culture with TNF promotes production of procoagulant factors (7, 8) and disruption of cytoskeletal connections, leading to induction of vascular leakage involving redistribution and phosphorylation of platelet/endothelial cell adhesion molecule 1 (9).

Although inhibition of tumor blood flow and the ensuing ischemia accounts for a significant proportion of early tumor cell death (3, 10), the hemorrhagic necrosis preceding tumor regression also serves to create conditions conducive to the entry and functioning of immune effector cells (3, 11). Among its many activities, TNF stimulates leukocyte adhesion to endothelial cells through the regulation of expression of cell adhesion molecules, intercellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1, CD44, and α2 integrin (12–15). Although endothelial cells express both TNF-R55 and TNF-R75 receptors, the regulation of expression of the adhesion molecules appears to be controlled exclusively by the TNF-R55 (12). Despite its spectacular activity against murine tumors, TNF has been disappointing in clinical trials because of severe side effects that limit the administered dose to subtherapeutic levels (16). Several strategies have been developed to deliver high doses of TNF selectively to tumor tissue, including genetic insertion of the TNF gene into TILs (17), ILP (18), and radiation-targeted TNF gene therapy (19, 20). Studies using genetically engineered TILs were curtailed when it was found that TILs did not traffic specifically to the tumor site (21). Excellent results have been obtained with ILP and high-dose TNF therapy (18), but application is limited to the treatment of melanomas and sarcomas, in which it is possible to confine the administration to a limb. Nevertheless, ILP has demonstrated the efficacy of TNF as an anticancer agent if high doses can be delivered selectively to the tumor. TNF gene therapy targeted by radiation is still at an experimental stage.

An alternative approach, which followed the success of Coley’s toxins last century (22), is to stimulate endogenous TNF production. Preparations of bacterial components such as LPS (23) and ImuVert (24) have been used in trials with little success. In this laboratory, we investigated the use of synthetic, small molecule cytokine inducers as an alternative to bacterial macromolecules to stimulate TNF in situ. DMXAA (Fig. 1), a prototype of this approach to cancer therapy, induced cure rates of 60–80% against the Colon 38 tumor (25–27) and is currently in Phase I clinical trial.

In murine systems, DMXAA exhibited a similar profile of immunomodulation, cytokine induction, and antitumor activity to that of its predecessor, FAA (28), except that DMXAA was 12-fold more potent (25). The antitumor activity and tumor blood flow effects of FAA were inhabitable by antibodies to TNF, consistent with an important role for TNF in its antitumor action (29, 30). Although both agents up-regulated TNF mRNA in a murine cell line, only DMXAA induced TNF mRNA in a human cell line (31). Thus, DMXAA was not only more potent but could also overcome the species preference exhibited by FAA, providing great potential as a clinical candidate. DMXAA caused irreversible cessation of tumor blood flow within 4 h of administration, with little effect on perfusion of normal tissues (32, 33). Hemorrhagic necrosis, discernible within 2–4 hours of DMXAA administration, had, by 24 h, pervaded the entire tumor, providing a histological appearance similar to that of tumors treated with TNF (34).

Serum TNF activity was elevated after administration of DMXAA in a manner that correlated with the response of Colon 38 tumors (35). However, LPS, a more potent inducer of serum TNF than DMXAA,
did not induce cures or growth delays against this tumor (36). In this report, we have investigated the cause of this apparent anomaly. We have compared the ability of DMXAA and LPS to induce TNF in Colon 38 tumor and normal tissues. We have also examined eight human tumor xenografts (one ovarian carcinoma, one squamous cell carcinoma, and six melanomas), excised from athymic nude mice that had been treated with DMXAA, for expression of human and murine TNF mRNA. These studies support the feasibility of using low molecular weight molecules to induce therapeutic levels of TNF selectively within the tumor tissue as a novel approach to cancer therapy.

MATERIALS AND METHODS

Mice and Tumors. C57Bl/6 × DBA/2 mice, between 8 and 12 weeks of age, were anesthetized (sodium pentobarbital, 90 mg/kg) and Colon 38 tumor fragments were implanted s.c. in the left flank. Mice were treated when tumors were ~6 mm in diameter (9–10 days after implantation). Human tumor xenografts were obtained by s.c. inoculation of 106 cells from a human tumor cell line into athymic C57Bl/6 nu/nu mice. The human tumor lines used were developed in this laboratory (37–39).

Mice were treated with drug by i.p. injection. DMXAA, synthesized in this laboratory (25), was dissolved in 5% sodium bicarbonate and injected at 50 mg/kg in a volume of 0.01 ml/g body weight. LPS (Escherichia coli serotype 055:B5; Sigma Chemical Co.) was dissolved in water and injected at 175 μg/ml per mg in 0.1 ml/mouse. The doses were used that induced maximal serum TNF activity in previous studies (35, 36).

In Situ Hybridization. Cryosections (15 μM) were fixed with paraformaldehyde, deproteinized using proteinase K, equilibrated in triethanolamine to block positive charges, and dehydrated with ascending concentrations of ethanol. Sections were hybridized with 32P-labeled antisense riboprobe (2 × 106 cpm/ml) for human or murine TNF. After hybridization, the sections were processed for autoradiography, exposed for 2–3 weeks, developed and stained with H&E. Sections of tissues from untreated mice were processed in an identical manner. The antisense murine TNF riboprobe was transcribed from a cDNA template encoding 820 bp of the human TNF gene. Probes were radiolabeled using [33P]UTP and a Riboprobe Gemini II labeling kit from Promega. The antisense human TNF riboprobe was found not to bind murine TNF RNA samples, and the antisense murine riboprobe did not bind human TNF RNA samples.

Northern Analysis. Organs were excised from sacrificed mice and minced using scalpels, and total cellular RNA was extracted using Trizol (Life Technologies, Inc.) according to manufacturer’s instructions. RNA samples (10 μg) were fractionated by electrophoresis on a formaldehyde-denaturing 1% agarose gel and transferred overnight to a nylon membrane (Hybond N+; Amersham). The membrane was hybridized with an 1100 bp TNF cDNA probe that had been labeled with [α-32P]dCTP (Amersham) using a random priming kit (RTS Radprime DNA labeling system; Life Technologies, Inc.), and blots were exposed to X-ray film for 1–3 days at −70°C. Membranes were then stripped and rehybridized with a 32P-labeled cDNA probe for human β-actin to determine loading of the lanes. Signal intensity was quantitated by laser densitometric scanning.

TNF Determination. Blood or organs were combined from three mice per group. To measure serum TNF concentrations, mice were anesthetized with halothane, and blood was collected from the ocular sinus, coagulated overnight at 4°C, and centrifuged for 30 min at 2000 × g at 4°C. To measure extractable TNF in spleen, liver, and tumor, tissues were extracted, weighed, and homogenized in α-modified MEM (2 ml) using a tissue homogenizer. The homogenates were centrifuged at 2,000 × g for 30 min at 4°C, and the supernatant was removed and centrifuged at 14,000 × g for 30 min at 4°C. TNF activity was assayed using the standard L929 cytotoxicity assay (35, 40). L929 cells (3 × 104 per well) were allowed to adhere overnight to the bottom of flat-bottomed 96-well plates. The cells were sensitized with actinomycin D (8 μg/ml final concentration) for 1 h before addition of serial dilutions of the samples. Cell killing was assessed after 24 h using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (35), and one unit of TNF was defined as that required to produce 50% killing. A purified, recombinant murine TNF standard was titrated alongside, and the amount of standard that gave 1 unit of activity in each assay was calculated. The sample TNF values were expressed as ng/g of tissue or ng/ml of serum.

RESULTS

Localization of Cells Expressing TNF mRNA in Liver, Spleen, and Tumor in response to DMXAA and LPS. To locate cells expressing TNF mRNA following treatment with DMXAA or LPS, liver, spleen, and Colon 38 tissues were processed for in situ hybridization with an antisense TNF riboprobe. Sections of tissues were examined 1–4 h posttreatment. Sections from the 2-h time point after DMXAA (Fig. 2, A2–D2), and the 1-h time point after LPS (Fig. 2, A3–D3) showed the greatest number of labeled cells (Fig. 2). The responses to LPS and DMXAA in the spleen and liver were similar in terms of both the numbers of positive cells and their distribution and location. In the spleen, TNF mRNA positive cells were located in the central regions of the lymphatic nodules (Fig. 2, A2 and A3), whereas, in the liver, cells with TNF mRNA were scattered individually throughout the parenchyma (Fig. 2, B2 and B3). Striking differences were observed between the LPS- and the DMXAA-treated Colon 38 sections. Tumor sections presented two patterns of histology depending on whether or not the region contained areas of spontaneous necrosis. In regions of predominantly viable tumor tissue, many of TNF mRNA positive cells from DMXAA-treated sections were associated with the host stromal elements (Fig. 2C2). In regions of extensive spontaneous necrosis, a ring of Colon 38 cells, in typical acinar formation, surrounded each vessel (Fig. 2D1). These rings of viable tumor tissue in the DMXAA-treated sections were very heavily labeled (Fig. 2D2), suggesting that Colon 38 cells were contributing to the overall intratumoral TNF response. The number of cells expressing TNF mRNA in the tumor after treatment with LPS, especially in the regions with spontaneous necrosis, was much lower than that from DMXAA-treated animals. The labeled cells in LPS-treated sections were confined to the periphery or the lumen of the vessels only (Fig. 2D3), whereas, in the DMXAA-treated sections, the entire thickness of the ring of viable tissue contained cells with TNF mRNA (Fig. 2D2). Labeled cells were not observed in sections from untreated animals (Fig. 2, A1–D1).

DMXAA Induces Greater TNF mRNA Transcription in the Tumor than Liver and Spleen, as Compared to LPS. The relative amounts of TNF mRNA following DMXAA administration were quantitated in liver, spleen, and tumor using Northern blot analysis and correlated with the in situ hybridization results. TNF mRNA was induced to a higher extent in tumor tissue than in spleen and liver, with peak expression 2 h following DMXAA administration (Fig. 3). Whereas LPS and DMXAA at their respective maximal TNF-inducing dose induced comparable amounts of TNF mRNA in the liver and spleen, DMXAA induced higher amounts of TNF mRNA in the Colon 38 tumor than LPS (Fig. 4).

Comparison of TNF Activity in Serum and Tumor following Treatment with DMXAA or LPS. Northern analysis (Fig. 4) showed DMXAA to induce greater amounts of TNF mRNA because
of stimulation of a greater number of cells in the tumor than LPS (Fig. 2, D2 and D3). Because biological and antitumor effects are mediated by the mature protein, we determined the relationship of intratumoral and circulating TNF activity after treatment with DMXAA and LPS at their respective maximal TNF-inducing dose (35, 36). The differences between LPS- and DMXAA-induced TNF activity in the spleen, liver, serum, and tumor were striking (Fig. 5). LPS-induced concentrations of TNF (273 ng/ml) in the serum 1 h after treatment that were 9-fold higher than the 2-h peak activity obtained in the tumor (20.3 ng/g).

DMXAA, on the other hand, induced low concentrations of TNF in the serum (100 ng/ml), maximal after 2 h and consistent with the slower kinetics of TNF mRNA expression observed with the in situ hybridization studies and the Northern analyses. Intratumoral TNF activity increased with time after DMXAA treatment and, at 4 h (716 ng/g), was 7-fold higher than the peak serum concentration. In a separate experiment, TNF activity after DMXAA administration was found to accumulate progressively in the tumor over 6 h. Measurements beyond this time could not be carried out due to the progressive
necrosis and disintegration of the tumor. Intratumoral TNF activity thus appeared to be independent of TNF in the circulation, indicating that the TNF in the tumor has been synthesized \textit{in situ}. At their respective maxima, LPS induced 3-fold higher levels of TNF activity in the serum than did DMXAA, whereas DMXAA induced 35-fold higher TNF in the tumor than did LPS. The area under the TNF activity-time curve was calculated as an estimate of the total exposure of cells in the various organs to TNF over the first 4 h (Table 1). Highest exposure was obtained in the tumor following DMXAA treatment. The intratumoral exposure was 7.6-fold higher than that in the serum, 1.5-fold higher than the liver, and 38-fold higher than the spleen (Table 1). With LPS, highest exposure occurred in serum and liver, with the tumor receiving the lowest. Thus, DMXAA delivered a 27-fold greater TNF exposure to the tumor than LPS and, at the same time, a 2.4-fold lower exposure in serum.

\textbf{Induction of Murine and Human TNF mRNA in Human Tumor Xenografts by DMXAA.} The \textit{in situ} hybridization studies using the Colon 38 tumor suggested that DMXAA might stimulate tumor cells as well as infiltrate host leukocytes to synthesize TNF. We,

![Fig. 3. Comparison of relative amounts of TNF mRNA induced in spleen, liver, and Colon 38 tumor with DMXAA. Mice with Colon 38 tumors were treated with DMXAA (50 mg/kg), and at indicated times, tissues from three mice per group were removed and combined, and total RNA was extracted and processed for Northern blot analysis. A, Northern blot probed for TNF mRNA; B, same membrane blotted with \(\beta\)-actin to show loading of individual lanes; C, relative intensity of TNF mRNA bands after correcting for loading. Controls (Lanes C) were untreated.](image)

![Fig. 4. Comparison of TNF mRNA up-regulation in spleen, liver, and tumor by DMXAA or LPS. Mice with Colon 38 tumors were administered DMXAA (50 mg/kg) or LPS (175 \(\mu\)g/mouse), and spleen (A), liver (B), and tumor (C) from three mice per group were excised at the indicated times, and total RNA was extracted and processed using Northern blotting for TNF mRNA. Loading of lanes was assessed from the binding of the \(\beta\)-actin probe. Relative intensity of the TNF mRNA bands was assessed by scanning densitometry and corrected for loading as determined from the actin signals.](image)

![Fig. 5. TNF activity in spleen, liver, serum, and tumor after treatment with DMXAA or LPS. Tumor-bearing mice were treated with DMXAA (50 mg/kg; A) or LPS (175 \(\mu\)g/mouse; B), and blood and tissues collected at the indicated times. Tissues were homogenized and the supernatant after centrifugation was assayed along with the serum for TNF activity using the L929 bioassay. TNF activity was expressed as ng per ml of serum or per g of tissue.](image)
In situ

**DISCUSSION**

6

B (all melanomas) expressed murine but not human TNF mRNA (Fig. 6). Of the eight xenografts examined, three

antisense murine TNF riboprobes, which, in a separate experiment, were probed with 820-bp antisense human and 700-bp human tumor xenografts, excised from athymic lines used for the xenografts have been found not to produce TNF. Moreover, some of the human tumor cell lines used for the xenografts demonstrated a response only in the host cell component, raising the question of whether tumor TNF expression controls responsiveness to DMXAA. Xenografts could provide a valuable tool for characterizing tumor phenotypes that influence sensitivity of individual tumors to DMXAA therapy.

In general, xenografts have lower responses to DMXAA than do murine tumors implanted in euthymic mice, and this has been attributed to a lack of functional T-cell immunity (51, 52). However, the number of cells expressing TNF mRNA in human xenografts is lower than that in murine tumors, and three of the six melanoma xenografts demonstrated a response in tumors after DMXAA treatment, as determined by in situ hybridization.

Therefore, examined human tumor xenografts, excised from athymic nude mice hosts after DMXAA treatment, for expression of both host (murine) and tumor (human) TNF mRNA. Sections of each xenograft were probed with 820-bp antisense human and 700-bp antisense murine TNF riboprobes, which, in a separate experiment, were demonstrated to be non-cross-reacting. Three xenografts (one ovarian carcinoma and two melanomas) expressed both murine and human TNF mRNA (Fig. 6A). Of the eight xenografts examined, three (all melanomas) expressed murine but not human TNF mRNA (Fig. 6B; Table 2). The responses were DMXAA-dependent because positive cells were not seen with either probe in untreated tumors.

**IN SITU INDUCTION OF TNF IN TUMORS**

**REFERENCES**

Table 1. TNF exposure after treatment with DMXAA or LPS

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Area under the concentration-time curve (ng · h · g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMXAA</td>
<td>Spleen 788, 155, 1176</td>
</tr>
<tr>
<td>LPS</td>
<td>Liver 423, 376, 43</td>
</tr>
</tbody>
</table>

Table 2. Expression of murine and human TNF mRNA in tumor xenografts after DMXAA treatment, as determined by in situ hybridization

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Human TNF mRNA</th>
<th>Murine TNF mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZM1 Melanoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NZM2 Melanoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NZM4 Melanoma</td>
<td>+</td>
<td>-</td>
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<tr>
<td>NZM6 Melanoma</td>
<td>+</td>
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<tr>
<td>NZM7 Melanoma</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NZM10 Melanoma</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NZOV2 Ovary</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NZSQ1 Squamous</td>
<td>+</td>
<td>+</td>
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</table>

Therefore, examined human tumor xenografts, excised from athymic nude mice hosts after DMXAA treatment, for expression of both host (murine) and tumor (human) TNF mRNA. Sections of each xenograft were probed with 820-bp antisense human and 700-bp antisense murine TNF riboprobes, which, in a separate experiment, were demonstrated to be non-cross-reacting. Three xenografts (one ovarian carcinoma and two melanomas) expressed both murine and human TNF mRNA (Fig. 6A). Of the eight xenografts examined, three (all melanomas) expressed murine but not human TNF mRNA (Fig. 6B; Table 2). The responses were DMXAA-dependent because positive cells were not seen with either probe in untreated tumors.

**DISCUSSION**

The data presented here point to the feasibility of using synthetic, small molecules to induce *in situ* TNF synthesis in tumors as a novel approach to TNF therapy of cancers. Peak TNF activity in Colon 38 tumors after DMXAA treatment was 7-fold greater than in serum. Moreover, intratumoral TNF activity was maintained for 5 h, whereas activity in spleen, liver, and serum was maximal at 2 h and sharply declined thereafter.

DMXAA induced 27-fold higher intratumoral TNF levels than did LPS (Table 1), providing an explanation for its higher antitumor activity. Because the molecular weight of DMXAA is small compared to LPS, its greater activity could be due to its better diffusion through tumor tissue. Alternatively, DMXAA could activate a wider spectrum of cell types, inducing tumor cells as well as cells of the host immune system to produce TNF. Evidence that DMXAA treatment activates tumor cells themselves comes from *in situ* hybridization studies on human tumor xenografts implanted in nude mice, where part of the response is of human origin (Fig. 6A). In contrast, the response to LPS appears to reside within cells of the immune system, which express cell surface receptors that specifically recognize bacterial components. The presence of receptors such as CD-14 (41) and p73 (42), which interact with endotoxin, confers on cells of the lymphoreticular system an extremely efficient system for transducing activation signals from the cell surface to the nucleus (43). TNF gene transcription occurred faster with LPS than DMXAA. Despite the slower kinetics, perhaps one of the greatest advantages of DMXAA is that it acts intracellularly and is not restricted to cells expressing the appropriate surface receptors like LPS.

Not all of the tumor cells in a responding tumor are activated by DMXAA to synthesize TNF. Moreover, some of the human tumor cell lines used for the xenografts have been found not to produce TNF in response to DMXAA *in vitro*. This suggests that other factors are important in the action of DMXAA. In the Colon 38 tumor, positive cells are seen near regions with spontaneous necrosis (Fig. 2B), suggesting that hypoxia or other microenvironmental stress might act to prime cells to respond to DMXAA. Hypoxia up-regulates a number of factors, including vascular endothelial growth factor and tissue factor (44–46). Nitric oxide, which regulates angiogenesis (47), is induced by DMXAA (48) and is costimulated by hypoxia (49). Both hypoxia (44) and TNF (50) are angiogenic, suggesting common pathways. We are investigating the properties of the tumor microenviron-ment that lead to selective DMXAA-induced TNF production.

In general, xenografts have lower responses to DMXAA than do murine tumors implanted in euthymic mice, and this has been attributed to a lack of functional T-cell immunity (51, 52). However, the number of cells expressing TNF mRNA in human xenografts is lower than that in murine tumors, and three of the six melanoma xenografts demonstrated a response only in the host cell component, raising the question of whether tumor TNF expression controls responsiveness to DMXAA. Xenografts could provide a valuable tool for characterizing tumor phenotypes that influence sensitivity of individual tumors to DMXAA therapy.

In summary, the results demonstrate that the action of DMXAA is mediated through TNF synthesized in situ within the tumor rather than through circulating TNF (Fig. 5). Transcription of TNF mRNA is maintained much longer in tumor tissue than in plasma (Fig. 3). Although externally administered TNF has a half-life in the blood of less than 30 min (6), intratumoral TNF induction by DMXAA results in a much longer exposure to TNF, possibly because the induced tumor vascular collapse can trap the secreted TNF. The use of small molecules that induce tumors, through cytokine synthesis, to mediate their self-destruction represents an attractive new approach to cancer therapy.

**REFERENCES**


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