Induction of Intratumoral Tumor Necrosis Factor (TNF) Synthesis and Hemorrhagic Necrosis by 5,6-Dimethylxanthene-4-Acetic Acid (DMXAA) in TNF Knockout Mice

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Abstract

5,6-Dimethylxanthene-4-acetic acid (DMXAA) is a new antitumor drug currently undergoing clinical trial. Administration of DMXAA to mice with tumors leads to cessation of tumor blood flow and the onset of tumor hemorrhagic necrosis, accompanied by the production of the cytokine tumor necrosis factor (TNF). Previous studies have shown that DMXAA induces both tumor and host cells to synthesize TNF and that induced intratumoral TNF production correlates with the antitumor activity of DMXAA. To explore the hypothesis that TNF production by tumor cells contributed to the induction of hemorrhagic necrosis by DMXAA, TNF−/− (C57Bl/6 background) mice were used as recipients for the s.c. implantation of (TNF positive) colon 38 adenocarcinoma. Tumors removed 24 h after treatment with DMXAA (66 or 100 μmol/kg) were found to be hemorrhagic and necrotic. Cells expressing TNF mRNA in tumors removed 2 h after treatment with DMXAA (160 μmol/kg) were found by in situ hybridization to be comparable in frequency and distribution with those in tumors from C57Bl/6 TNF-positive mice. However, the amount of TNF protein extracted from tumors from TNF knockout mice was lower than that from TNF-positive mice. Spleen and liver tissue from TNF knockout mice, in contrast to that from TNF-positive mice, produced no TNF mRNA. TNF protein was undetectable in liver and spleen tissue from TNF knockout mice, but was evident in tissue from TNF-positive mice. These results confirm that DMXAA has the novel ability of inducing tumors to synthesize TNF in situ.

Introduction

Strategies that aim to kill tumors not by direct attack, but by destroying their blood supply have emerged after Folkman’s (1) proposal that the growth of tumors is dependent on a functioning vasculature. DMXAA1, a new antitumor agent synthesized in this laboratory and currently in clinical trials, halts tumor blood flow and causes hemorrhagic necrosis in transplantable murine tumors (2, 3). The inhibition of tumor blood flow and the induction of hemorrhagic necrosis seems to be mediated largely through the production of TNF (4, 5). In contrast to lipopolysaccharide, which induces high levels of tumor necrosis, accompanied by the production of the cytokine tumor necrosis factor (TNF). Previous studies have shown that DMXAA induces both tumor and host cells to synthesize TNF and that induced intratumoral TNF production correlates with the antitumor activity of DMXAA. To explore the hypothesis that TNF production by tumor cells contributed to the induction of hemorrhagic necrosis by DMXAA, TNF−/− (C57Bl/6 background) mice were used as recipients for the s.c. implantation of (TNF positive) colon 38 adenocarcinoma. Tumors removed 24 h after treatment with DMXAA (66 or 100 μmol/kg) were found to be hemorrhagic and necrotic. Cells expressing TNF mRNA in tumors removed 2 h after treatment with DMXAA (160 μmol/kg) were found by in situ hybridization to be comparable in frequency and distribution with those in tumors from C57Bl/6 TNF-positive mice. However, the amount of TNF protein extracted from tumors from TNF knockout mice was lower than that from TNF-positive mice. Spleen and liver tissue from TNF knockout mice, in contrast to that from TNF-positive mice, produced no TNF mRNA. TNF protein was undetectable in liver and spleen tissue from TNF knockout mice, but was evident in tissue from TNF-positive mice. These results confirm that DMXAA has the novel ability of inducing tumors to synthesize TNF in situ.

Materials and Methods

Mice and Tumors. C57Bl/6 TNF−/− mice were generated and bred at the Centenay Institute (Sydney, Australia; Refs. 7–9), and adults were sent to the Auckland Cancer Society Research Center for use in experiments. WT C57Bl/6 mice were purchased from the Department of Laboratory Animal Sciences (Otago Medical School, Dunedin, New Zealand). Colon 38 tumor fragments were implanted s.c. in the left flank of anaesthetized (sodium pentobarbitol, 81 mg/kg) mice. Experiments were carried out when tumors had reached approximately 6–8 mm in diameter. DMXAA, synthesized in this laboratory (10), was dissolved in sterile, endotoxin-free water, and the required dose was injected i.p. in a volume of 0.01 ml/g body weight.

Assessment of Hemorrhagic Necrosis. Tumors were removed 24 h after treatment with DMXAA and fixed in 10% formalin. Fixed tumors were then embedded in paraffin wax, and sections were stained with H&E. The section across the major axis of the tumor was examined on a grid marked at 4-mm intervals and scored for the percentage necrosis by the grid intersection method, as described previously (11).

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 murine TNF. After hybridization, the sections processed for autoradiography, exposed for 14 days, developed, and stained with H&E. Sections of tumors from untreated mice were processed in an identical manner. The antisense riboprobe for murine TNF was transcribed from a cDNA template encoding 700 bp of the murine TNF gene. Probes were radiolabeled using 33P-UTP and a Riboprobe Gemini II labeling kit from Promega.

TNF Determination. Mice were anaesthetized with halothane, and blood was collected from the ocular sinus, coagulated overnight at 4°C, then centrifuged for 30 min at 2000 × g and 4°C. Spleen, liver, and tumor tissues were extracted, weighed, and homogenized in an 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, as described previously (4, 12). Briefly, L929 cells (3 × 104/well) were allowed to adhere overnight to the bottom of flat-bottomed 96-well plates. The cells were then sensitized with actinomycin D (8 μg/ml final concentration) for 1 h before the addition of serial dilutions of the samples to be assayed. Cell killing was assessed after 24 h by a colorimetric assay using...
3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, as described previously (4). One unit of TNF was defined as that required to produce 50% killing of L929 cells. 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 limit of sensitivity was 1 pg/ml.

Results

To determine whether DMXAA was inducing a histological effect on tumors in TNF−/− mice, colon 38 tumors were implanted in C57Bl/6 WT and TNF−/− mice and grown to ~6 mm in diameter. The mice were then treated with a single i.p. dose of DMXAA at a dose of 66 μmol/kg (the optimal antitumor dose) for WT mice and at doses of 66 and 100 μmol/kg for TNF−/− mice. Tumors were removed after 24 h, and tissue was fixed and stained. Tumors from untreated WT (data not shown) and TNF−/− mice (Fig. 1A) showed either little necrosis or central necrosis occupying up to 30% of the section area. Tumors from DMXAA-treated TNF−/− and WT mice demonstrated extensive areas of hemorrhagic necrosis that were identical in appearance (Fig. 1B). The proportions of the tumor section areas that were necrotic (83 ± 12% for 3 TNF−/− mice and 91 ± 6% for 5 WT mice) were not significantly different.

Tumor tissue was excised from WT mice and TNF−/− mice 2 h after treatment with DMXAA at the maximal TNF-inducing dose (160 μmol/kg), sectioned, and subjected to in situ hybridization for TNF mRNA. Both the pattern and extent of cells with TNF mRNA in sections from WT mice and TNF−/− were similar (Fig. 1, C and D). As seen in the liver sections (Fig. 1, E and F), TNF mRNA was detected in normal organs in DMXAA-treated WT mice, but not as expected in TNF−/− mice. TNF mRNA was not found in tumor or normal tissues in untreated WT mice and TNF−/− mice (data not shown).

Functional TNF in serum, liver, spleen, and tumor in DMXAA-treated WT mice and TNF−/− mice was measured using the L929 cytotoxicity assay for biologically active TNF. At 160 μmol/kg, the maximal TNF-inducing dose (4), DMXAA induced greater TNF synthesis in the colon 38 tumor than in the liver, serum, or spleen (6). The TNF activities detected for WT mice in Fig. 2A are consistent with previous observations, and no TNF was detected in untreated WT mice. Induction of TNF in colon 38 tumors from DMXAA-treated TNF−/− mice was observed, but the TNF levels were 200-fold lower than those obtained from tumor tissues in WT mice and have been plotted on a different scale (Fig. 2). No TNF was detected in treated or untreated liver and splenic tissues from TNF−/− mice. A small amount of TNF was detected in the serum of DMXAA-treated TNF−/− mice.

Discussion

The use of TNF−/− mice as hosts for the colon 38 adenocarcinoma clearly demonstrates that tumor cells synthesize both TNF mRNA and TNF protein in response to treatment with DMXAA. Although further work is required to determine the maximum tolerated dose and antitumor activity (including cure rate) of DMXAA in TNF−/− mice, the results corroborate our earlier findings that DMXAA up-regulates

![Fig. 1. A and B, histological appearance (staining with H&E) of sections of colon 38 tumors growing in TNF−/− mice before (A) and 24 h after (B) treatment with DMXAA (100 μmol/kg). Original magnification, ×20. Corresponding sections of colon 38 tumors growing in WT mice and treated with DMXAA (66 μmol/kg) showed the same appearance (data not shown). C and D, localization using in situ hybridization of cells expressing TNF mRNA in colon 38 tumor in WT (C) and TNF−/− (D) mice 2 h after treatment with DMXAA (160 μmol/kg). Untreated colon 38 tumors in WT mice showed no labeling (data not shown). E and F, localization of cells expressing TNF mRNA in liver tissue of WT (E) and TNF−/− (F) mice 2 h after treatment with DMXAA (160 μmol/kg). Original magnification, ×50.](https://cancerres.aacrjournals.org/content/59/23/3304/suppl/DC1/325636D.png)
expression of tumor cell TNF mRNA in human tumor xenografts (6). TNF can induce vascular damage (13), leading to hemorrhagic necrosis (14) in tumors. Although TNF is directly cytotoxic to some types of tumor cells in vitro, colon 38 cells have been reported to be sensitive to TNF in vivo, but insensitive in vitro (15), suggesting that the TNF effects in this tumor are mediated by vascular changes. The induction of tumor hemorrhagic necrosis in TNF−/− mice by DMXAA (Fig. 1) suggests that tumor cell-derived TNF contributes to its antitumor action. Alternatively, other induced proteins may participate in the induction of tumor hemorrhagic necrosis in TNF−/− mice. IFNs and the chemokine IP-10 are induced by DMXAA (16), and both of these have been reported as capable of inducing tumor necrosis (17, 18).

The lower levels of TNF protein in the tumors from TNF−/− mice almost certainly reflect the absence of a contribution from the host-infiltrating leukocytes to TNF protein production in the tumor tissue. However, despite this reduced protein production, in situ hybridization studies show similar amounts and distribution of TNF mRNA-positive cells in tumors from TNF−/− and WT hosts after DMXAA treatment (Fig. 1, C and D). This suggests that in WT hosts, most of the detectable TNF mRNA is from tumor cells, whereas most of the TNF protein is from host cells. Thus, TNF mRNA may be less readily translated in tumor cells as compared with host cells. Studies in human ovarian cancers (19) showed that although TNF mRNA was present in both epithelial tumor cells and infiltrating macrophages, TNF protein was localized primarily to a subpopulation of macrophages within and in close proximity to tumor areas.

No evidence of TNF mRNA-positive cells in colon 38 tumors was found by in situ hybridization in the absence of DMXAA treatment, in agreement with a study of human melanoma and ovarian tumor xenografts in nude mice (6). The distribution of positive cells in the in situ hybridization studies demonstrates that not all tumor cells produce TNF mRNA in response to DMXAA. In the xenograft study, all tumors produced host-derived murine TNF mRNA, but only some produced tumor-derived, human TNF mRNA after DMXAA treatment (6). Again, the distribution of positive cells was not uniform. The results suggest either that the TNF-producing cells are primed to respond by a preexisting stimulus or that a costimulus is required, in addition to DMXAA. Such a stimulus could be provided, for instance, by the microenvironment.

In conclusion, the results of this study on the murine colon 38 tumor, combined with those using tumor xenografts (6), suggest that the biochemical pathway required for TNF production is present in at least some types of cancer cells and can be stimulated by DMXAA. Studies on human ovarian carcinoma, particularly in advanced disease, have shown evidence of TNF production by tumor cells (19–22), suggesting that the response of ovarian cancer to DMXAA would be worthy of further study. Drug-induced intratumoral TNF production by DMXAA represents a new mode of cancer therapy.

References


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