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Interferon-inducible Protein 10 Induction and Inhibition of Angiogenesis *in Vivo* by the Antitumor Agent 5,6-Dimethylxanthenone-4-acetic Acid (DMXAA)[1]

Zhihui Cao, Bruce C Baguley, and Lai-Ming Ching[2]

Auckland Cancer Society Research Centre, University of Auckland School of Medicine, Auckland, New Zealand 1000

ABSTRACT

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a drug synthesized in this laboratory that halts tumor blood flow and induces tumor hemorrhagic necrosis in transplantable murine tumors, is known to induce the synthesis of antiangiogenic cytokines *in vitro*. We have measured the induction of mRNA for modulators of angiogenesis *in vivo* and investigated whether DMXAA may also have an additional antiangiogenic action through the production of these cytokines. The genes for IFN-α and for interferon-inducible protein 10 (IP-10) were strongly induced in both spleen and Colon 38 tumor tissue after DMXAA treatment, whereas that for IFN-γ was induced in spleen but not in tumor. Expression of mRNA for IFN-β and for the p35 or the p40 subunits of interleukin 12 was not observed in either tissue. Splenic IP-10 mRNA induction was not a result of IFN-γ production induced with DMXAA because spleen tissue from DMXAA-treated mice that lacked functional IFN-γ receptors expressed similar amounts of IP-10 mRNA as those from wild-type mice. A single i.p. injection of DMXAA (20 mg/kg) was sufficient to reduce fibroblast growth factor-induced endothelial cell invasion of Matrigel implants in athymic nude mice by nearly 100%. The inactive analogue 8-methylxanthenone-4-acetic acid did not up-regulate the genes for IP-10 or IFNs and did not inhibit endothelial cell invasion. Antibodies to IP-10 reversed the inhibition of DMXAA of endothelial cell invasion by 58%; antibodies to tumor necrosis factor-α, IFN-γ, and IFN-α reversed inhibition by 7%, 5%, and 0%, respectively. The data support the hypothesis that DMXAA, in addition to antivascular effects mediated by tumor necrosis factor-α, may have an antiangiogenic effect mediated largely by the induction of IP-10.

INTRODUCTION

Because the growth of tumors is critically dependent on a functioning blood supply, strategies that specifically target the vasculature to deprive the tumor of vital nutrients provide an attractive approach to cancer therapy. Advances to date fall into two main categories: prevention of the formation of new blood vessels and destruction of existing tumor vessels. DMXAA[3] (Fig. 1), a new anticancer agent synthesized in this laboratory (1), is currently in Phase I clinical trial and is particularly effective against transplantable murine tumors with an established vasculature (1). DMXAA induces irreversible tumor vascular collapse within min of administration (2, 3), and the ensuing tumor ischemia and hemorrhagic necrosis account for a significant amount of tumor cell death. Although these effects are a prerequisite for DMXAA activity, they appear to be insufficient for complete tumor regression, and other factors may contribute to the overall antitumor action (4).

Several observations raise the question of whether DMXAA possesses antiangiogenic activity. The experimental antitumor activity of DMXAA is enhanced by thalidomide (5), a known inhibitor of angiogenesis (6). Firstly, DMXAA induces IFNs (7, 8), which are inhibitors of angiogenesis (9). Secondly, in primary murine macrophage cultures, DMXAA induces the chemokine IP-10 (7). Although characterized initially for its chemotactic abilities (10, 11), IP-10 is also antiangiogenic, inhibiting bFGF-induced neovascularization *in vitro* and in several rodent models *in vivo* (12, 13). IP-10, which is induced by IFN-γ (11) and IL-12 (14), is thought to mediate the antiangiogenic action of these cytokines (14, 15). Thirdly, DMXAA induces TNF (16), which in addition to its antivascular action (17) alters endothelial cell permeability (18), stimulates angiogenesis at low doses, and inhibits angiogenesis at high doses (19).

In this report, we examine the *in vivo* induction of mRNA for IP-10, IFN-α, IFN-β, IFN-γ, IL-12, and TNF-α in the spleen and tumor of mice after DMXAA administration. We also measure up-regulation of mRNA for MIP-1α. Although not antiangiogenic, MIP-1α shares with IP-10 the chemotactic properties of enhancing lymphocyte migration and diapedesis through the vascular endothelium (20). As a measure of antiangiogenic activity, the ability of DMXAA to inhibit bFGF-induced endothelial cell infiltration into Matrigel plugs *in vivo* has been examined.

MATERIALS AND METHODS

Materials. DMXAA (1) and 8-MeXAA (21) were synthesized in this laboratory and dissolved in 5% sodium bicarbonate. Matrigel, an extract of basement membrane proteins from the Engelbreth-Holm-Swarm murine tumor, was purchased from Becton Dickinson (Bedford, MA). bFGF was purchased from Sigma Chemical Co. (St. Louis, MO).

The anti-IP-10 antibody 5171 was made available through the generosity of Dr. Joshua Farber (Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). It is a rabbit-neutralizing antiserum raised against IP-10 purified from insect cells infected with recombinant baculovirus (22). The other antibodies used were antimouse TNF-α (R&D Systems, Inc.; catalogue number AB-410-NA), antimouse IFN-α (R&D Systems, Inc.; catalogue number AF-485-NA), and antimouse IFN-γ (Serotec Ltd.; catalogue number MCA1431).

Mice. Athymic BALB/c-*nu/nu* mice were from the Animal Laboratories, University of Auckland School of Medicine. Female C57Bl/6 were from the Department of Laboratory Animal Sciences, Otago Medical School, Dunedin, New Zealand. IFN-$\gamma R^{0/0}$ mice and their wild-type counterparts were a generous gift from Dr. James D. Watson (Genesis Research and Development Corporation, Auckland, New Zealand) and were the offspring from the interbreeding of homozygous wild-type or IFN-$\gamma R^{0/0}$ (129/Sv/Ev $\times$ C57Bl/6)F_1 mice (23). All of the mice were maintained under constant temperature and humidity according to institutional ethical guidelines and used between 8–12 weeks of age.

Matrigel Assay for Endothelial Cell Activity. This assay was performed as described by Passaniti *et al.* (24). Matrigel, either alone or mixed with bFGF (final concentration 150 ng/ml) in a total volume of 0.5 ml at 4°C, was injected s.c. into the mid-abdominal region of athymic nude mice. DMXAA (20 mg/kg) was administered as a single i.p. injection (0.01 ml/g body weight) after Matrigel inoculation. Matrigel polymerizes at body temperature to form a solid plug. After 7 days, the Matrigel plug, together with the underlying epidermis and dermis, was removed, fixed in 10% neutral buffered formalin for at least 24 h, dehydrated through ascending concentrations of alcohol, and embedded in paraffin under vacuum. Sections through all three layers were stained with

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[2] To whom requests for reprints should be addressed, at the Auckland Cancer Society Research Centre, University of Auckland School of Medicine, Private Bag 92019, Auckland, New Zealand 1000. Phone (64-9) 3737-999; Fax (64-9) 3737-502; E-mail: l.ching@auckland.ac.nz.

[3] The abbreviations used are: DMXAA, 5,6-dimethylxanthenone-4-acetic acid; IP-10, interferon-inducible protein 10; IL, interleukin; bFGF, basic fibroblast growth factor; TNF, tumor necrosis factor; MIP-1α, macrophage inhibitory protein-1α; 8-MeXAA, 8-methylxanthenone-4-acetic acid; IFN-$\gamma R^{0/0}$, IFN-γ receptor knock-out.

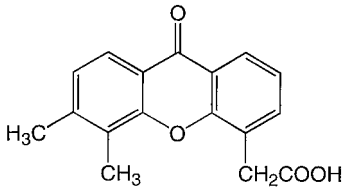

Fig. 1. Chemical structure of DMXAA.

GMasson trichrome. Counterstaining for factor VIII (24) established that >95% of cells stained with Masson trichrome within the Matrigel plug were endothelial cells. The area occupied by infiltrating endothelial cells in histological sections was quantified in four nonoverlapping Matrigel sections/plug (two to four mice/group).

Control rabbit serum was prepared from blood obtained from untreated rabbit and clotted overnight on ice. Undiluted, polyclonal rabbit antimouse IP-10 antibody or normal rabbit serum (50 μl) was mixed with 500 μl of Matrigel plus bFGF/implant and also administered 3 h and 24 h after DMXAA treatment (300 μl/injection i.p.). Neutralizing antibodies to TNF-α were reconstituted at 1 mg/ml, and neutralizing antibodies to IFN-α and IFN-γ were reconstituted at 100 and 200 μg/ml, respectively, according to the manufacturer's instructions. Antibody (50 μl) was added to the Matrigel implant, and each antibody was diluted 3-fold and injected (300 μl) 1 h and 24 h after implantation.

Tumor Implantation. Colon 38 tumor fragments (1 mm^3) were implanted s.c. in the left flank of anesthetized (sodium pentobarbitone; 86 mg/kg) C57Bl/6 and IFN-$\gamma R^{0/0}$ mice. Tumors were used when they had reached approximately 6 mm in diameter, generally 9–10 days after implantation. The implantation rate of Colon 38 tumors was 100% in C57Bl/6 mice and approximately 70% in the IFN-$\gamma R^{0/0}$ mice.

Northern Blot Analysis. Mice were sacrificed by cervical dislocation. Spleens and tumors were removed aseptically, and the tissues were minced using a pair of scalpels. Total cellular RNA was extracted using RNAzol (Life Technologies, Inc.) according to manufacturer's instructions. RNA (10 μg) was denatured and electrophoresed in 1% agarose-formaldehyde gels as described previously (25). RNA was then transferred by capillary action onto nylon membranes (Hybond-N^+; Amersham), which were UV cross-linked (120 mjoule; UV-Stratalinker; Stratagene, San Diego, CA) and baked (80°C for 30 min). Each membrane was prehybridized (2 h; 42°C) in 7 ml of hybridization mix containing 50% formamide, 0.075 M sodium chloride, 0.05 M sodium dihydrogen phosphate, 5 mM EDTA, 0.001% polyvinyl pyrrolidine, 0.001% BSA, 0.001% Ficoll, 0.01 mg/ml herring sperm DNA, and 0.5% SDS. The cDNA to the cytokine gene of interest was labeled with $\alpha[^{32}P]$dCTP (Amersham) using a random priming kit (RTS Radprime DNA labeling system; Life Technologies, Inc.). Excess radioactivity was removed by elution through a G-50 Sephadex column, and labeled probe (10^6 cpm/ml hybridization mix) was then added to the membrane and hybridized for 36 h at 42°C. The blots were washed twice in 2 × SSC with 0.1% SDS for 10 min at 42°C and finally in 0.2 × SSC with 0.1% SDS for 10 min at 65°C. Blots were exposed to X-ray film for 1–3 days at −70°C. After hybridization with one probe, membranes were stripped (two washes in 300 ml of 0.1 × SSC with 1% SDS for 15 min at 80°C) and rehybridized with another probe. Intensity of signals was quantitated by laser densitometric scanning. Loading of lanes was determined from the intensity of bands hybridized with the probe for human β-actin or glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Up-Regulation of Genes for Cytokines with Antiangiogenic Activity in Response to DMXAA in Mice. We investigated the time course of *in vivo* induction of genes for a number of antiangiogenic cytokines in mice that were administered DMXAA. RNA was extracted from spleens 1, 2, 4, 8, 12, and 24 h after DMXAA treatment (22.5 mg/kg) and was subjected to Northern blot analysis. Expression of mRNA for TNF-α, IFN-α, and IFN-γ was maximal at 2 h and then rapidly declined (Fig. 2). IP-10 and MIP-1α mRNA expression peaked at 4 h and was maintained for up to 12 h after DMXAA administration.

We also investigated the induction of the cytokines within Colon 38 tumor tissue after DMXAA treatment and compared it with that in the spleen. Expression of mRNA was followed only up to 5 h after DMXAA administration, because necrosis of the tumor at later times prevented extraction of undegraded RNA. Tumor tissues showed higher expression of mRNA for TNF-α, IP-10, and MIP-1α and lesser amounts of IFN-α than splenic tissue (Fig. 3). Surprisingly, expression of mRNA for IFN-γ was reproducibly not observed in the tumor, although it was induced in the spleen (Fig. 3). Furthermore, upregulation of mRNA for the p35 or p40 subunits of IL-12 and for IFN-β mRNA was not detected in either tissue (Figs. 2 and 3).

Relationship between IP-10 Induction and IFN-γ Production by DMXAA. Other groups have shown that IP-10 is induced by IFN-γ, which in turn is induced by IL-12 (14, 15). The results shown in Figs. 2 and 3 suggested that IP-10 induction in response to DMXAA did not involve IL-12 or IFN-γ. IL-12 mRNA could not be detected (Fig. 2), and IP-10 mRNA expression in the tumor was independent of IFN-γ mRNA induction (Fig. 3). To further clarify whether IFN-γ mediated IP-10 induction by DMXAA, we examined the induction of IP-10 mRNA in mice lacking functional receptors for IFN-γ. DMXAA strongly induced IP-10 mRNA in splenic and tumor tissues of IFN-$\gamma R^{0/0}$ mice to a level that was comparable with that induced in their wild-type counterparts (Fig. 4). Although IFN-γ mRNA was induced in the spleens of both the wild-type and IFN-$\gamma R^{0/0}$ mice, no induction was observed in the Colon 38 tumor tissues implanted in either host (Fig. 4).

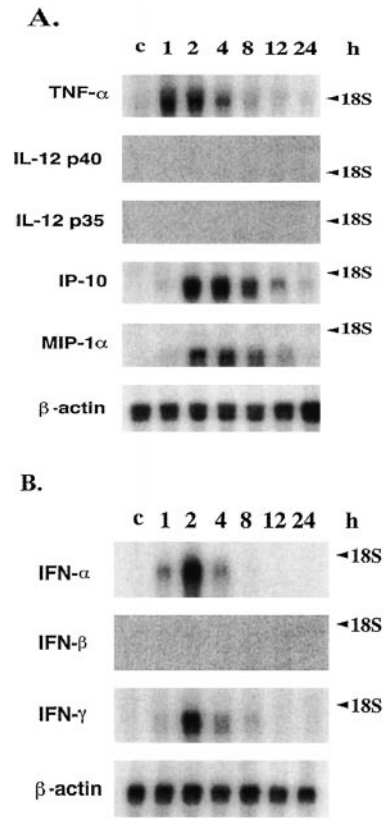

Fig. 2. Induction of cytokine mRNA in murine spleens by DMXAA. Two separate Northern blots (*A* and *B*) of mRNA isolated from spleens of C57Bl/6 mice at indicated times after treatment with DMXAA (22.5 mg/kg) and from untreated control mice (*Lane c*) and probed for the indicated cytokine mRNA. Spleens from three mice were pooled for each time point.

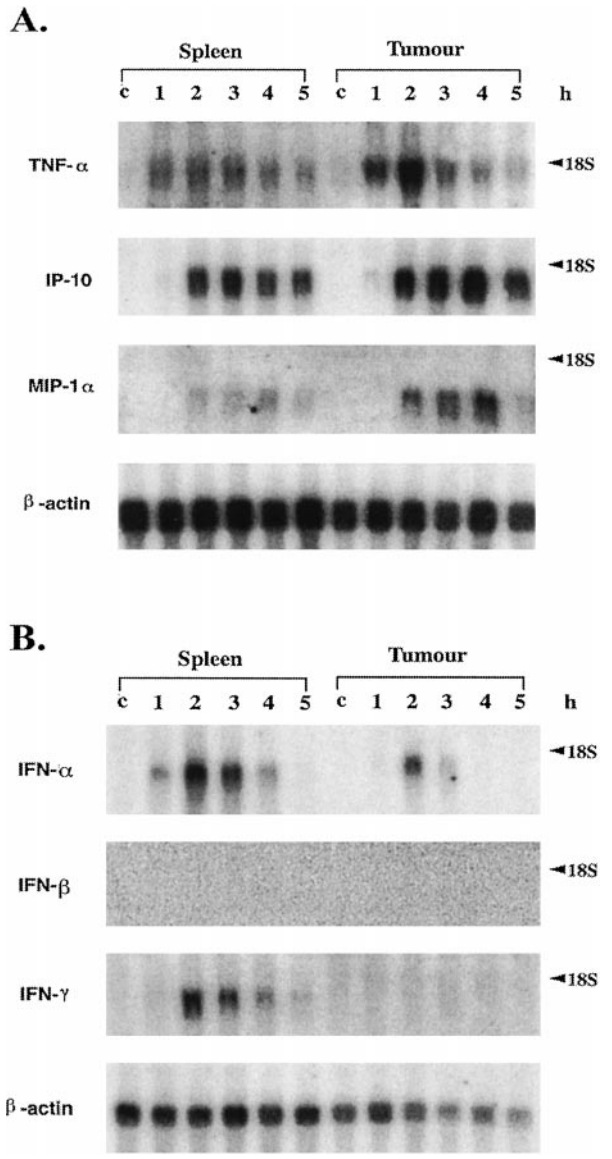

Fig. 3. Comparison of cytokine mRNA induction in Colon 38 tumor tissue and spleen. Two separate Northern blots (*A* and *B*) of mRNA isolated from the Colon 38 and the spleen of C57Bl/6 mice 1–5 h after treatment with DMXAA (22.5 mg/kg) and from untreated mice (*Lane c*) and probed for the indicated cytokine mRNA. Tissues from three mice/group were pooled for mRNA extraction.

Inhibition of bFGF-induced Endothelial Cell Infiltration into Matrigel Plugs after DMXAA Treatment. Athymic nude mice were implanted s.c. with bFGF-impregnated Matrigel plugs and then administered a single dose of DMXAA (20 mg/kg i.p.). Matrigel plugs were removed 7 days after treatment, and sections were cut and examined for endothelial cell infiltration (Fig. 5). The results of multiple sections were analyzed (Fig. 5*E*). Matrigel plugs without bFGF contained few invading endothelial cells compared with plugs of Matrigel plus bFGF. DMXAA treatment reduced the amount of bFGF-induced endothelial cell invasion into the Matrigel by 88% when administered on the same day as the Matrigel implantation. Timing of DMXAA administration after Matrigel implantation was critical, because no inhibition was obtained when DMXAA was given 3 days after Matrigel implantation and only 20% inhibition when given after 24 h.

An analogue of DMXAA, 8-MeXAA, which has no antitumor activity (21), did not inhibit endothelial cell invasion when given at its maximum tolerated dose (220 mg/kg) on the same day as Matrigel implantation (Fig. 5). After administration of 8-MeXAA, no induction of mRNA for IP-10, IFN-α, IFN-γ, and TNF-α was observed in spleens of nude mice used for Matrigel implants (data not shown).

Antibodies to IP-10 Neutralize the Inhibition of DMXAA of Endothelial Cell Invasion. To determine whether cytokines were involved in the inhibition of endothelial cell invasion obtained after

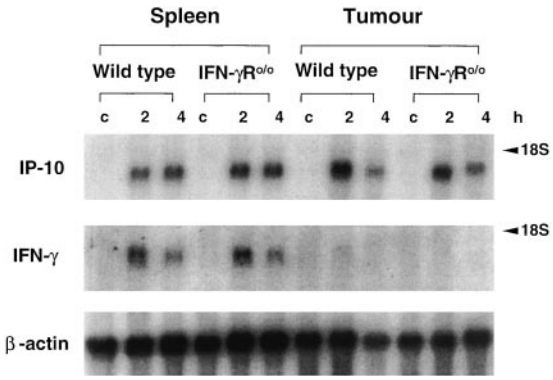

Fig. 4. Induction of mRNA for IP-10 and IFN-γ in spleen and Colon 38 tumor from IFN-$\gamma R^{0/0}$ and wild-type mice (three/group) after DMXAA (22.5 mg/kg) treatment. Northern blots for IP-10 and IFN-γ mRNA from spleen or Colon 38 tumors from IFN-$\gamma R^{0/0}$ and wild-type mice, untreated (*Lane c*), or 2 or 4 h after DMXAA (22.5 mg/kg).

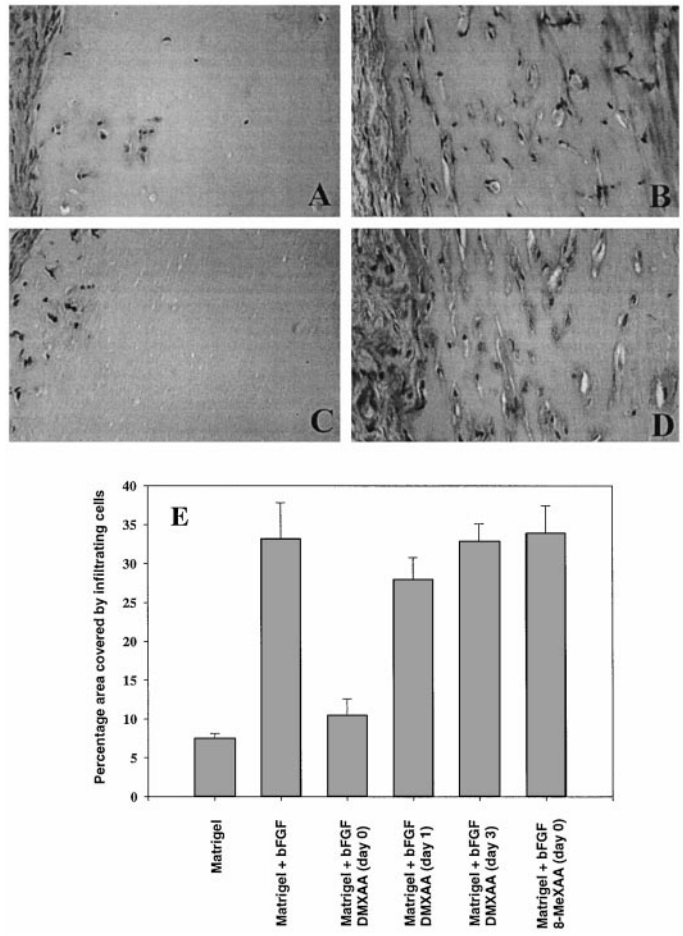

Fig. 5. Inhibition of bFGF-induced neovascularization of Matrigel implanted s.c. in BALB/c nude mice by DMXAA. *A-D*, representative fields (×100 magnification) of endothelial cell invasion after 7 days into (*A*) Matrigel only, (*B*) Matrigel impregnated with bFGF, and (*C*) Matrigel with bFGF from mice that had been treated with a single i.p. injection of DMXAA (20 mg/kg), or (*D*) 8-MeXAA (200 mg/kg) on the day of Matrigel implantation. *E*, quantitation of endothelial cells in Matrigel. Mean ± SE of four sections each from two to four mice/group.

Table 1 *Effect of neutralizing antibodies to IP-10, TNF-α, IFN-α, or IFN-γ on DMXAA-induced inhibition of endothelial cell infiltration of Matrigel plugs in mice*

	% area covered by infiltrating cells (± SE)	% inhibition of neovascularization	% reversal of DMXAA inhibition
Matrigel	8.9 ± 1.2		
Matrigel/bFGF	34.8 ± 5.0		
Matrigel/bFGF DMXAA treatment	12.8 ± 3.1	85	
Matrigel/bFGF/normal rabbit serum DMXAA treatment	13.0 ± 4.1	84	1
Matrigel/bFGF/anti-IP-10 DMXAA treatment	27.8 ± 5.2	27	58
Matrigel/bFGF/anti-TNF-α DMXAA treatment	14.5 ± 4.5	78	7.4
Matrigel/bFGF/anti-IFN-α DMXAA treatment	11.5 ± 6.0	89	0
Matrigel/bFGF/anti-IFN-γ DMXAA treatment	14.1 ± 5.2	80	5

DMXAA administration, normal rabbit serum, anti-IP-10, anti-TNF-α, anti-IFN-γ, or anti-IFN-α antibodies were mixed with the Matrigel implant. Mice were also treated with antibodies 1 or 3 h and 24 h after DMXAA treatment. The number of endothelial cells in the Matrigel was quantitated after 7 days. Addition of anti-IP-10 antibodies reversed DMXAA-induced inhibition of endothelial cells in the Matrigel plug by 58% (Table 1). In contrast, antibodies to IFN-α and normal rabbit serum had no effect, whereas anti-TNF-α and anti-IFN-γ antibodies reversed the inhibition by 7% and 5%, respectively.

DISCUSSION

A growing number of low molecular weight inhibitors of angiogenesis have been identified (26). We have used the Matrigel model for angiogenesis in mice (24) to demonstrate that the antitumor agent DMXAA is also antiangiogenic. In contrast to most agents, which require multiple dosing for an effect, DMXAA caused almost complete inhibition of bFGF-induced neovascularization of Matrigel implanted in athymic mice after a single administration of drug (Fig. 5). The potential of DMXAA to inhibit the development of new tumor blood vessels must therefore be considered in addition to its known immune effects (27, 28) and antivascular effects (2, 3).

DMXAA might exert antiangiogenic activity either directly, *e.g.*, by affecting vascular endothelial cells, or indirectly by inducing angiogenesis-modulating factors. We have extended a previous observation using cultures of primary macrophages (7) by demonstrating that DMXAA up-regulates IP-10 mRNA expression in spleen and tumor tissues (Figs. 2 and 3). At least in spleen tissue, the induction of IP-10 after DMXAA administration was not a consequence of IFN-γ production, because it was induced in the spleens of both the wild-type and IFN-γR$^{0/0}$ mice (Fig. 4). The result is not completely unequivocal in tumor tissue because tumor cells could have IFN-γ receptors and respond to the high levels of circulating IFN-γ present in IFN-γR$^{0/0}$ mice (8). It is possible that DMXAA activates the gene for IP-10 directly, although maximal expression of mRNA for IP-10 occurred later than that of TNF-α or IFN-γ mRNA expression.

Antibodies to IP-10 provided a 58% reversal of the inhibitory effects of DMXAA, suggesting that IP-10 is the main cytokine responsible for endothelial cell invasion. Antibodies to IFN-α had no effect, whereas anti-TNF-α and anti-IFN-γ antibodies reversed the inhibition only marginally. The lack of significant effect with anti-IFN-γ antibodies is consistent with the observation that the induction of IP-10 by DMXAA is independent of IFN-γ production. There is a surprisingly narrow time window for the effect of DMXAA on the Matrigel assay, with administration of DMXAA 3 days after implantation having no effect (Fig. 5). It is likely that the competition between the opposing effects of bFGF and IP-10 determines the outcome of the Matrigel assay, and because both may be relatively short-lived, this competition can be observed only over a short time.

After DMXAA treatment, greater expression of the genes for IP-10 and MIP-1α was seen in tumor tissue than in the spleen (Fig. 3). This behavior is different from that observed in viral infections or after IFN-γ administration, where more IP-10 is produced in the spleen than in the liver (29). The high induction of IP-10 and MIP-1α in tumor tissues raises the question of whether these chemokines contribute to the antitumor response through their chemotactic and antiangiogenic properties. Chemokines produced in an inflammatory response serve to attract leukocytes to the site of inflammation (20), and the production of IP-10 or MIP-1α might mediate an influx of host leukocytes into the tumor. The primary target for both IP-10 and MIP-1α appears to be natural killer cells and activated T lymphocytes (10, 30), and both these cell types have been implicated in the action of DMXAA (28). Inoculation of tumor cells engineered to express high amounts of IP-10 in mice resulted in regression of the tumors, as well as coinoculated tumors that did not produce IP-10 (11). Antitumor activity has been found to depend on the recruitment of T lymphocytes (11), and it has been shown that activated T lymphocytes selectively express the CXCR3 receptor, which is specific for IP-10 and the closely related chemokine Mig (31).

One striking result emerging from these investigations is that, although DMXAA induces mRNA for IFN-γ in spleen, it does not in the Colon 38 tumor (Fig. 3). One possible explanation is that the Th1 subset of T-helper lymphocytes that produce IFN-γ does not infiltrate the Colon 38 tumor. We have yet to examine if expression of mRNA for other cytokines that are produced by the Th1 subset, such as IL-2 and lymphotoxin, is similarly absent in the tumor. Alternatively, Th1 cells could be present, but the production of IFN-γ has been suppressed within the tumor microenvironment. This latter explanation would be compatible with other studies (32) reporting that, although the ability of tumor-associated T cells to produce IL-2 and IFN-γ decreased with tumor progression, transforming growth factor β and IL-6 activity increased. These authors suggested that transforming growth factor β inhibited the production of the T-cell cytokines and that the tumor-bearing state induced an abnormal cytokine network under which T-cell cytokines are negatively regulated. However, we saw no significant difference in IFN-γ mRNA expression between spleens of normal and tumor-bearing mice.

In summary, we have shown here that DMXAA induces the antiangiogenic chemokine IP-10 and that a single administration of DMXAA inhibits bFGF-induced neovascularization of Matrigel. Thus, DMXAA may exert significant antiangiogenic activity through IP-10 production, as well as antivascular action through production of TNF and other cytokines (33, 34). The balance between these two types of antitumor activity may well depend on the schedule of drug administration, with single high doses favoring antivascular effects and repeated low doses favoring antiangiogenesis. Drug combination studies may well facilitate such distinctions, and we are currently studying the effects of administration schedule on the activity of DMXAA in combination with thalidomide (35).

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