Evidence for the Production of Nitric Oxide by Activated Macrophages Treated with the Antitumor Agents Flavone-8-acetic Acid and Xanthenone-4-acetic Acid

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ABSTRACT

Activated peritoneal macrophages, obtained from mice pretreated with Bacillus Calmette-Guérin, after exposure in vitro to flavone-8-acetic acid (FAA; NSC 347512) at a concentration of 890 μM, produce nitrite (3.7 nmol/10⁶ cells), as measured 20 h later by the Griess reaction. Stimulation of nitrite production was inhibited at least 90% by N⁶-monomethylarginine (125 μM), suggesting that nitrite was formed via nitric oxide as a product of arginine metabolism. Stimulation was only partially inhibited by dexamethasone (0.1 μM). The ability of xanthenone-4-acetic acid (XAA) and three of its analogues to stimulate nitrite production was also investigated. 5,6-Dimethyl-XAA stimulated nitrite production (12.6 nmol/10⁶ cells) at an optimal concentration of 80 μM, 8-methyl-XAA was without effect, and XAA and 5-methyl-XAA showed intermediate activity. The optimal in vitro drug concentrations for stimulation by FAA, XAA, and active XAA analogues correlated with the optimal in vivo dose required for the induction of either hemorrhagic necrosis or growth delay of s.c. Colon 38 tumors. These results strongly imply that FAA and active XAA derivatives function as low molecular weight stimulators of nitric oxide formation in macrophages, possibly acting on the same differentiation pathway as do endotoxin and tumor necrosis factor α. We suggest that nitric oxide, which is known to be toxic to tumor cells, contributes to the cytotoxic action of FAA and its analogues.

INTRODUCTION

FAA³ is a new antitumor agent with exceptionally high activity against the Colon 38 tumor in mice (1, 2) and broad spectrum activity against other solid mouse tumors (2–4) and xenografts (4, 5). However, little or no activity has been found against clinical cancer (6). Work on FAA analogues in this laboratory has revealed that XAA (see Fig. 1 for structures) has properties similar to those of FAA against Colon 38 tumors and human tumors xenografts (7). XAA has formed the basis for the synthesis of a large series of analogues which vary in both dose potency and antitumor activity (7, 8). A number of more dose-potent antitumor derivatives of XAA have been synthesized, including 5-MeXAA (8) and 5,6-MeXAA (9). On the other hand 8-MeXAA, despite having very similar physicochemical properties, is inactive against the Colon 38 tumor (7). The availability of closely related compounds with diverse antitumor activity and potency provides a good basis for determining whether in vitro and in vivo effects are related to each other.

While FAA and XAA derivatives cause gross hemorrhagic necrosis of experimental solid tumors within 24 h of administration, tumor cell death is not understood. FAA has a variety of other effects on mice, including the modulation of cytotoxic lymphocyte activity (10–13), induction of cytokines (14–16), and alteration of tumor blood flow (17–19). Observations in this laboratory of the enhancement of in vitro tumoricidal activity of murine peritoneal macrophages by FAA (20, 21) suggest that tumor-associated macrophages may be an important target for the action of FAA. Several products of macrophage metabolism are tumoricidal (22), and recently, L-arginine-dependent pathways have been shown to be induced when endotoxin (23) and tumor necrosis factor α (24) are used as second signals to activate macrophages. Earlier studies showed that macrophage activation for expression of cytotoxicity against tumor cells required several signals including a priming signal and a triggering signal (25–28). The oxidation of a terminal guanidino nitrogen atom of L-arginine, which is induced by these differentiating signals, leads to the synthesis of the cytotoxic product nitric oxide, which subsequently reacts with oxygen and water to produce nitrite and nitrate (29, 30). In vitro, the inhibition of arginine oxidation by the analogue N⁶-MMA suppresses the cytotoxicity of appropriately activated and stimulated macrophages (23).

Because of the similarities in the action of FAA, endotoxin, and tumor necrosis factor α (3, 23, 24, 31, 32) we decided in this study to investigate the ability of FAA to stimulate this L-arginine-dependent cytotoxicity pathway by measuring macrophage-mediated nitrite formation. We have compared the ability of FAA, XAA, and three XAA derivatives to induce macrophages from BCG-treated mice to synthesize nitric oxide in vitro with their ability to affect s.c. Colon 38 tumors in vivo.

MATERIALS AND METHODS

Materials. FAA was obtained from the National Cancer Institute, Bethesda, MD, through the courtesy of Dr. K. Paul. XAA and its derivatives (Fig. 1) were synthesized in this laboratory by Drs. W. A. Denny, G. J. Atwell, and G. W. Rewcastle and were judged pure by thin layer chromatography. Drug solutions were prepared daily by dissolving in a minimal amount of 5% (w/v) sodium bicarbonate in Milli-Q water and diluting to the final concentrations in culture medium. Sodium bicarbonate, at the highest concentration used, was shown not to interfere with the in vitro macrophage culture experiments. Because of their light sensitivity (33), the drug solutions were protected from light in all experiments.

α-Minimal essential culture medium (Gibco, Grand Island, NY) was supplemented with fetal calf serum (10%; Gibco N.Z. Ltd.), 2-mercaptoethanol (50 μM), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). Thioglycollate broth (Becton Dickinson) was prepared as a 10% (w/v) sterile solution in Milli-Q water. Lyophilized BCG organisms (Institute Armand-Frappier, Canada) were prepared as a suspension (4.2 × 10⁹ organisms/ml) in sterile water. Endotoxin (prepared by phenol extraction from Escherichia coli 055:B5; Sigma), N⁶-MMA (Calbiochem), and dexamethasone (David Bull Laboratories, Australia) were dissolved in culture medium.

Macrophage Preparation. C3H/HeN mice were bred in the laboratory animal facility under constant temperature and humidity with sterile...
bedding, water, and food according to institutional ethical guidelines. Mice were killed by cervical dislocation, and PE cells were collected by washing the peritoneum with culture medium. Resident PE cells were collected from mice between 6 and 12 weeks of age. Elicited PE cells were prepared by i.p. injection of 0.2 ml thioglycollate broth per mouse, and were collected 4 to 6 days later. Activated PE cells were prepared by i.p. injection of 10^8 BCG organisms per mouse and were collected 14 to 15 days later.

Macrophage Nitrite Production. Adherent macrophages were prepared by plating 10^5 PE cells/150 μl culture medium in 96-well plates and incubating for 2 h at 37°C in 95% air/5% CO₂. The nonadherent cells and supernatant were then removed and the adherent cells were washed twice with phosphate-buffered saline. The macrophage monolayers were then covered with 200 μl culture medium containing the antitumor agents (concentration range, 3-1450 μM), or endotoxin (20 or 10,000 ng/ml), with or without N³MMA (15-500 μM), or dexamethasone (5-500 nm) and incubated for a further 20 h at the same temperature and atmosphere as above. In all experiments each drug concentration was tested in triplicate or quadruplicate. Control macrophages were prepared and cultured in culture medium without added agents.

Nitrite concentrations in the medium were measured by a microplate assay method, based on the Griess reaction (34). Equal volumes of culture medium supernatant and Griess reagent (0.5% sulfanilamide, 0.05% naphthylenediamine dihydrochloride in 2.5% H₃PO₄) were added to Eppendorf tubes and incubated at 20°C for 10 min. The tubes were then centrifuged for 5 min at 8000 x g, the supernatants were transferred to 96-well microplates and absorbances at 550 nm were determined on a plate reader. The nitrite concentration in cell-free medium alone was determined in each experiment, and this value (0.3-0.4 nmol/well) was subtracted from the values obtained for the wells containing cells.

Macrophage Viability. Macrophage monolayers were prepared by plating 3 x 10^5 cells/450 μl culture medium in 24-well plates, each well containing a glass coverslip. The 2- and 20-h treatment procedures were as described above except that the antitumor agents, endotoxin, N³MMA and control were added in culture medium to a total volume of 500 μl, with each drug concentration tested in duplicate. After culture for 20 h the supernatant medium was removed from each well and 500 μl of 0.1% eosin in phosphate-buffered saline were added. The coverslips with adherent macrophages were then removed from the wells to a counting chamber, and the cells within the grid area of the chamber (range 120-265 cells in all experiments) were assessed for eosin exclusion.

Assay of Tumor Necrosis and Tumor Growth Delay. C57BL/6 x DBA/2 F₁ (hereafter called B6D2F₁) mice (at least 3 mice/dose) with s.c. Colon 38 tumors 5-12 mm in diameter (10-11 days after implantation) were given i.p. injections of drug. Mice were killed by cervical dislocation 24 h later and tumors were removed, sliced in half, and fixed in 10% formalin. Fixed tumors were embedded in paraffin wax and sections through the maximum diameter of the tumor were stained with hematoxylin and eosin by standard methods. Sections were assessed on a grid marked at 0.4-mm intervals by scoring the percentage of intersections which were necrotic (32).

Groups of five B6D2F₁ mice with s.c. tumors (5-10 mm in diameter, randomized with respect to tumor size) were given i.p. injections of the appropriate drug 10 days after implantation. Tumors were measured thereafter three times weekly with digital calipers and tumor volumes were calculated as 0.52a^2b, where a and b were the minor and major axes of the tumor. The growth delay of drug-treated versus control groups was calculated on a logarithmic scale from the time taken to reach 4 times the initial tumor volume.

RESULTS

Stimulation of Macrophage Nitrite Production by FAA and XAA Derivatives. Neither resident nor elicited macrophages produced nitrite when treated with the antitumor agents in the concentration range 0-1450 μM or when treated with endotoxin at 20 or 10,000 ng/ml (35). Activated macrophages were found to produce detectable nitrite, which ranged in individual experiments from 2.5 to 5.6 nmol/10⁶ cells. Nitrite production was significantly enhanced (P < 0.05) following treatment with FAA, XAA, 5-MeXAA, 5,6-MeXAA, or endotoxin (Fig. 2; Table 1). The intracellular accumulation of nitrite was small (average, 4.5%) and the ranking of compounds was the same for all experiments. The greatest effect occurred with 5,6-
MeXAA, which stimulated greater nitrite production than did endotoxin at a concentration (20 ng/ml) which was within the range for optimal activity. In contrast, 8-MeXAA had no significant effect. The optimal concentrations for in vitro nitrite production differed greatly between compounds, ranging from 80 μM for 5,6-MeXAA to 1100 μM for XAA.

Macrophage Viability. Since the stimulation of nitrite production was found to pass through an optimal drug concentration (Fig. 2), viability was tested following 20 h of culture in the presence of the agents. Viability of both resident and elicited macrophages was found to be ≥91% following treatment with drug concentrations up to 1450 μM, 91% with endotoxin (20 ng/ml), and ≥95% with no treatment. Greater than 79% of activated macrophages were viable, as judged by eosin exclusion, following treatment with the optimal in vitro concentrations of FAA, XAA, 5-MeXAA, and 5,6-MeXAA, as compared to 84% with endotoxin (20 ng/ml) and 87% with no treatment. However, significant loss of macrophage viability was observed following exposure to the highest concentrations of 8-MeXAA, 5-MeXAA, and 5,6-MeXAA tested (Table 2).

Relationship between Nitrite Production and Antitumor Effects. Tumor hemorrhagic necrosis and tumor growth delay were measured in mice with s.c. Colon 38 tumors treated with a single drug dose (Table 1). The degree of tumor necrosis in untreated mice ranged from 0 to 40% and varied according to tumor size (32). FAA, XAA, 5-MeXAA, and 5,6-MeXAA derivatives at the optimal dose induced extensive tumor necrosis, whereas 8-MeXAA was within the control range and endotoxin was intermediate. In tumor growth delay experiments, 5,6-MeXAA had the greatest effect, 8-MeXAA and endotoxin had no significant effect, and the other compounds showed intermediate activity. 5,6-MeXAA was more dose potent than 5-MeXAA, which was in turn more potent than XAA or FAA (Table 1). For FAA and the three active analogues, the in vitro concentration for optimal nitrite production was correlated (r = 0.9) with the in vivo dose required for optimal antitumor effect, as measured by either tumor necrosis or tumor growth delay.

Effects of N^G-MMA and Dexamethasone on Nitrite Production. N^G-MMA, an effective inhibitor of nitrite production from endotoxin-stimulated activated macrophages, also inhibited production from activated macrophages stimulated with these antitumor agents (Fig. 3). In the presence of the optimal concentrations of FAA, XAA, 5-MeXAA, 5,6-MeXAA, and endotoxin, N^G-MMA (125 μM) inhibited nitrite production by ≥90%. N^G-MMA (500 μM) had no significant effect on the viability of macrophages exposed to 5,6-MeXAA (1300 μM) or to endotoxin (20 ng/ml). A comparison between 5,6-MeXAA (80 μM) and endotoxin (20 ng/ml) showed that the dose-response curves for the inhibition of nitrite production by N^G-MMA were very similar (Fig. 4).

Dexamethasone, an inhibitor of the FAA-induced in vitro toxicity of resident macrophages (20), inhibited the stimulation of nitrite production by 5,6-MeXAA and endotoxin only partially and showed very similar dose-response relationships for both agents (Fig. 4). In a comparison of FAA, XAA, 5-MeXAA, 5,6-MeXAA, and endotoxin (at optimal concentrations), dexamethasone (100 μM) inhibited stimulation by 49–62% (Fig. 3).

Table 2 Viability of macrophages after 20 h in culture

<table>
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<th>Treatment</th>
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<th>% at optimal</th>
<th>% top conc.</th>
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</table>

* Viable cells (assessed by eosin exclusion) expressed as a percentage of the total number of cells counted. Values are the means of duplicates, each from 1–2 separate experiments.

* Concentration (μM) which gave the greatest nitrite production by macrophages.

* Highest concentration (μM) tested.

* ND, not done.
DISCUSSION

The results demonstrate a new biological action of FAA and XAA derivatives, the stimulation of nitrite production by activated mouse peritoneal macrophages. Production of nitrite is correlated with antitumor activity in the series studied. 8-MeXAA, which is inactive against s.c. Colon 38 tumors, is inactive in the nitrite assay. Of the active compounds, the optimal in vitro concentration (μM) for activity is close to the optimal in vivo dose (μmol/kg) required for antitumor effect (Table 1), with 5,6-MeXAA the most active analogue prepared thus far in our laboratory. The latter compound also shows a greater effect both in vitro and in vivo than does endotoxin (Table 1). Since the viability of activated macrophages (Table 2) was compromised only at the highest in vitro concentrations of three XAA analogues (8-MeXAA, 5-MeXAA 5,6-MeXAA) and was not altered by the addition of N⁶-MMA, the production of nitrite appears to be unrelated to the direct cytotoxicity of these compounds.

The production of nitrite by endotoxin-stimulated activated macrophages has been shown to result from the NADPH-mediated oxidation of one of the terminal guanido nitrogens of arginine (29). Nitric oxide, which is produced as a result of this oxidation, has a life-time of 3–5 sec in oxygen-saturated aqueous solution and combines with oxygen to produce nitrogen dioxide (36). Reaction with water then produces nitrite ions in equal proportions (30). Both nitrate and nitrite, in approximately equal proportions, have been detected in our studies with 5,6-MeXAA. The similarities in the action of endotoxin, FAA and active XAA derivatives reported here, as well as the similarities in their sensitivity to the inhibitors N⁶-MMA and dexamethasone (Figs. 3 and 4), imply that FAA and XAA may stimulate macrophages somewhere on the endotoxin-sensitive, l-arginine-dependent pathway. Because nitrite production is specifically inhibited by N⁶-MMA, the results of Fig. 3 strongly suggest that FAA and active XAA derivatives stimulate the formation of nitric oxide by primed macrophages from BCG-treated mice. The detection of elevated concentrations of nitrate in plasma of mice following treatment with FAA and XAA supports the hypothesis that nitric oxide production occurs in vivo as well as in vitro.

Nitric oxide produced by macrophages in vitro is directly cytotoxic, possibly by forming iron-nitrosyl complexes which inhibit iron-containing enzymes in target cells (37). Nitric oxide production therefore has important implications for the cytotoxicity of FAA and active XAA derivatives. Nitric oxide could act as a naturally produced hypoxia-selective antitumor agent. Since the half-life of nitric oxide is dependent on oxygen tension (36), it should persist in poorly vascularized tissues such as tumor tissue longer than in normal tissues.

If nitric oxide production is stimulated by FAA and related drugs in humans, these results could have specific implications for the treatment of human malignancy: (a) if a sequence of signals is needed for macrophage activation in vivo as is suggested in the present experiments, combined therapy of FAA with a macrophage activating agent such as γ-interferon (24) may be required for optimal treatment; (b) appropriate tumor arginine levels may be essential for the efficient operation of this mechanism, necessitating dietary control as a part of therapy; (c) since hypotension is the dose-limiting toxicity of FAA (6) and since nitric oxide, an endothelium-derived relaxing factor, is known to reduce blood pressure (38), the contribution to hypotension of FAA-induced nitric oxide by macrophages or other cells warrants investigation.

It is likely that other mechanisms of host cell-mediated toxicity as well as nitric oxide production are stimulated by FAA and active XAA derivatives. The stimulation of cytokine production by FAA has been well documented (14–16). FAA stimulates the in vitro tumoricidal activity of resident peritoneal macrophages (20) which do not produce nitrite. Furthermore, this tumoricidal effect is completely inhibited by dexamethasone at concentrations between 50 and 1000 ng (20) whereas nitrite production by activated macrophages is rather insensitive to dexamethasone (Figs. 3 and 4).

In conclusion, while recent investigations suggest that nitric oxide may be an important cytotoxic agent in vitro (23, 24, 29, 35), this study of a series of structurally related antitumor agents whose in vitro stimulation of nitrite production correlates with antitumor activity suggests that nitric oxide makes an important contribution as an endogenous antineoplastic agent in vivo. A better understanding of the response of tumor-associated macrophages is required before the contribution of nitric oxide to the regression of solid tumors can be fully assessed.

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REFERENCES


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4 Unpublished results.


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