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Serotonin involvement in the antitumour and host effects of flavone-8-acetic acid and 5,6-dimethylxanthenone-4-acetic acid

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Abstract. The relationship of serotonin (5-HT) receptors to the action of the experimental antitumour drugs flavone-8-acetic acid (FAA) and 5,6-dimethylxanthenone-4-acetic acid (5,6-MeXAA) was studied. Both FAA and 5,6-MeXAA are known to induce the synthesis of tumour necrosis factor-α (TNF) and to stimulate nitric oxide synthesis in vivo, as measured by elevation of plasma nitrate. Serotonin potentiated the effect of a subtherapeutic dose of 5,6-MeXAA (20 mg/kg) as measured both by plasma nitrate increase and by growth delay of s.c. implanted colon 38 tumours. On the other hand, administration of the serotonin 5-hydroxytryptamine-2 (5-HT_2) antagonist cyproheptadine (20 mg/kg) inhibited both the plasma nitrate response and, to a lesser extent, the induction of tumour haemorrhagic necrosis by 5,6-MeXAA, FAA and TNF. Reduction of circulating plasma serotonin by pre-treatment with *p*-chlorophenylalanine and reserpine reduced the plasma nitrate response, but not the tumour necrosis response, to 5,6-MeXAA (30 mg/kg). It is suggested that serotonin is necessary for the induction of nitric oxide synthases and acts, either directly or indirectly, in concert with TNF. Serotonin agonists may have utility in increasing nitric oxide synthesis in response to TNF or to agents that induce TNF as part of their antitumour action.

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Introduction

Serotonin, a vasoactive amine, has been reported to induce a rapid decrease in tumour blood flow [5–7, 13, 18]. Over a longer time scale, serotonin also induces, in some tumours, tumour haemorrhagic necrosis similar in appearance to that induced by tumour necrosis factor-α (TNF) [5, 16] and growth delays [13, 16]. TNF, a cytokine with a variety of potentially cytotoxic effects, induces tumour haemorrhagic necrosis [4], reduces tumour blood flow [19] and induces nitric oxide synthesis [26]. Serotonin can be distinguished from TNF in that it does not induce nitric oxide synthesis [2].

The serotonin 5-hydroxytryptamine-2 (5-HT_2) antagonist cyproheptadine has two unexpected effects when given together with TNF. It reduces the degree of induced tumour haemorrhagic necrosis [2, 16] as well as the induction of increased plasma nitrate [3]. Since plasma nitrate is derived from oxidation of nitric oxide in the presence of erythrocytes, the latter result implies that cyproheptadine inhibits nitric oxide synthesis. These observations suggest a relationship between the functions of TNF and serotonin.

The experimental antitumour drug flavone-8-acetic acid (FAA) induces tumour haemorrhagic necrosis that is histologically similar to that induced by TNF [1, 23]. 5,6-Dimethylxanthenone-4-acetic acid (5,6-MeXAA), a potent FAA analogue developed in our laboratory and scheduled for clinical trial, has the same effect [1, 22]. Both FAA [15] and 5,6-MeXAA (L.-M. Ching, personal communication) have been found to be small-molecule inducers of TNF, and both agents induce nitric oxide synthesis in vitro [24] and in vivo [25]. In this report we address the question as to whether serotonin receptors are involved in the action of either FAA or 5,6-MeXAA.

Materials and methods

Materials and mice. FAA was supplied by the National Cancer Institute (USA). 5,6-MeXAA was synthesized in our laboratory by Drs. W. A. Denny, G. J. Atwell and G.W. Rewcastle, and was pure as determined by

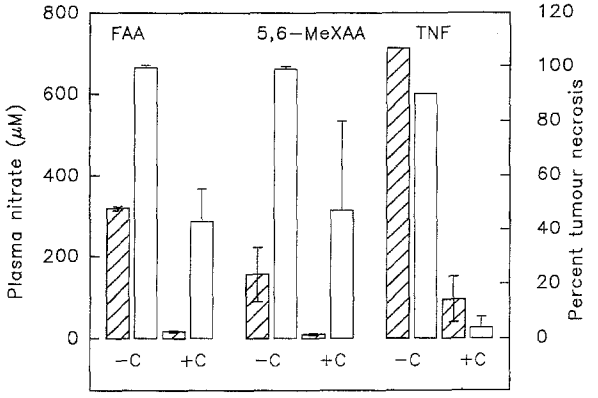

Fig. 1. Plasma nitrate concentrations (*hatched bars*) and percentage of tumour necrosis (*open bars*) determined in colon 38 tumour-bearing mice 12 h after i.p. injection of FAA (330 mg/kg), 5,6-MeXAA (30 mg/kg) or rh-TNF (0.8 mg/kg), either without (*-C*) or with (*+C*) co-administration of cyproheptadine (20 mg/kg)

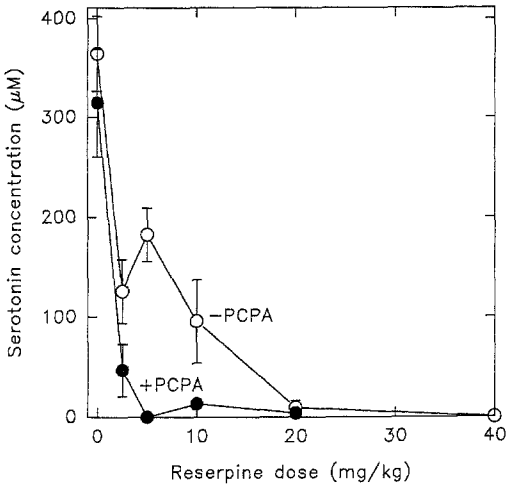

Fig. 2. Plasma serotonin concentrations measured in non-tumour-bearing mice 24 h after injection of reserpine at the doses indicated, either without (○) or with (●) co-administration of PCPA (200 mg/kg)

thin-layer chromatography. Cyproheptadine and ketanserin were obtained from Sigma Chemical Co. (USA), and phentolamine and cimetidine were supplied by Auckland Hospital. Recombinant human TNF (rh-TNF) was kindly provided by Prof. J. D. Watson, Department of Molecular Medicine, University of Auckland. Mice (Jackson Laboratory, Bar Harbor, Me., USA) were bred in the laboratory and housed according to institutional ethical guidelines [25].

Tumours. Colon 38 tumours were grown s.c. in $C_{57}BL/6 \times DBA/2$ F_1 hybrid mice following the implantation of 1-mm^3 fragments using anaesthesia (pentobarbitone, 90 mg/kg). For tumour histology studies, tumours were grown to a diameter of 5–10 mm before treatment of the mice with single i.p. doses of the drugs under test. After 12 h, mice were anaesthetized with ether and blood was removed and placed in heparinized tubes, which were centrifuged (1300 rpm, 5 min) to provide plasma samples. Tumours were removed and fixed, embedded, sectioned, stained and then evaluated using a grid-marking system as previously described [1]. The percentage of necrosis was determined as the number of grid intersections showing necrotic tumour divided by the total number of intersections counted. For growth delay experiments, tumours were grown to a diameter of 5–6 mm (8 or 9 days after implantation) before treatment of the mice with single i.p. doses of the drugs under test. Tumour diameters were measured with calipers and volumes, calculated as $0.52 \times a^2b$, where *a* and *b* represent the minor and major tumour axes. Tumour growth delays were measured at a time when mean logarithmic tumour volumes had increased 4-fold from their initial volume.

Nitrate determination. Plasma nitrate/nitrite concentrations were determined as previously described [25] by precipitation of plasma proteins with zinc sulphate, reduction with cadmium powder and colorimetric assay of the resulting nitrite with the Griess reagent. Plasma serotonin concentrations were determined by high-performance liquid chromatography [21]. Plasma proteins were precipitated by the addition of 1.2 vol. of 0.1 *M* perchloric acid containing 0.23 m*M* ascorbic acid (prepared fresh). After centrifugation (1300 rpm, 2 min), 100 μl of the supernatant was chromatographed on a C-18 Bondclone reverse-phase column using a Waters pump and a Princeton Applied Research (model 400) electrochemical detector. The mobile phase was 0.1 *M* sodium acetate buffer (pH 4.7) containing 10% methanol, the flow rate was 2 ml/min and the detector was set to a potential of 700 mV.

Results

Effect of cyproheptadine on the induction of tumour necrosis and nitric oxide

Plasma from untreated colon 38-bearing mice contained 18 ± 2 μ*M* (mean ± SEM) nitrate. The mean degree of spontaneous haemorrhagic necrosis detected in tumours removed from these mice was 15%. FAA (330 mg/kg), 5,6-MeXAA (30 mg/kg) and rh-TNF (0.8 mg/kg) induced plasma nitrate concentrations significantly above the control values, and all three agents induced haemorrhagic necrosis of treated tumours (Fig. 1). Co-administration of cyproheptadine substantially reduced both the degree of haemorrhagic necrosis and the elevation of plasma nitrate levels induced by each agent (Fig. 1). Ketanserin (20 mg/kg), another 5-HT_2 antagonist, reduced tumour necrosis in response to FAA and 5,6-MeXAA and also inhibited nitrate production in response to 5,6-MeXAA by 76%. Phentolamine (100 mg/kg), an α-adrenergic antagonist, and cimetidine (100 mg/kg), a 5-HT_3 antagonist, had no significant effect on FAA-induced tumour necrosis. Preliminary experiments using 5,6-MeXAA indicated that cyproheptadine did not antagonize the effect of 5,6-MeXAA on tumour growth (results not shown). However, cyproheptadine also increased the toxicity of 5,6-MeXAA, making such experiments difficult to assess.

Effect of serotonin depletion in plasma on nitric oxide induction

Cyproheptadine is not completely specific for 5-HT_2 receptors, and the requirement of high doses for the inhibition of host and tumour effects raised the question as to whether a non-specific action was involved. To determine whether depletion of serotonin inhibited drug action, mice were treated with a combination of *p*-chlorophenylalanine (PCPA, 200 mg/kg) to inhibit serotonin synthesis and reserpine (20 mg/kg) to inhibit serotonin storage in platelets [10]. Preliminary experiments were carried out to determine the optimal doses of each of these agents (Fig. 2) as well as the time course of plasma serotonin depletion. Tumour-bearing mice were treated with 5,6-MeXAA (30 mg/kg) 12 h after injection of reserpine with or without

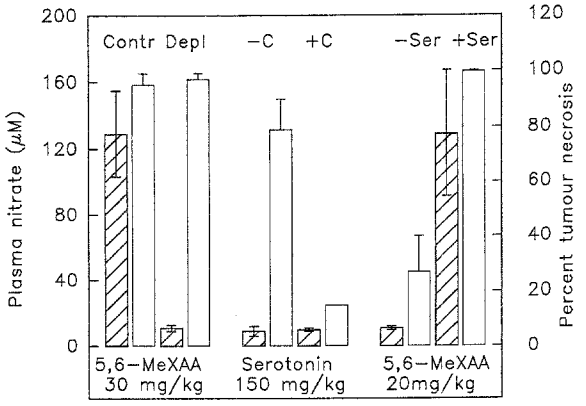

Fig. 3. Plasma nitrate concentrations (*hatched bars*) and percentage of tumour necrosis (*open bars*) determined in colon 38 tumour-bearing mice 12 h after i.p. injection of agents. *The left-hand set of bars* indicates the response to 5,6-MeXAA (30 mg/kg) in control mice (*Contr*) or mice depleted of serotonin (*Depl*) by two successive courses of PCPA (200 mg/kg) and reserpine (40 mg/kg). *The central set of bars* indicates the response to serotonin (150 mg/kg) either without (*-C*) or with (*+C*) co-administration of cyproheptadine (20 mg/kg). *The right-hand set of bars* indicates the response to 5,6-MeXAA (20 mg/kg) alone (*-Ser*) or in combination with serotonin (*+Ser*, 150 mg/kg)

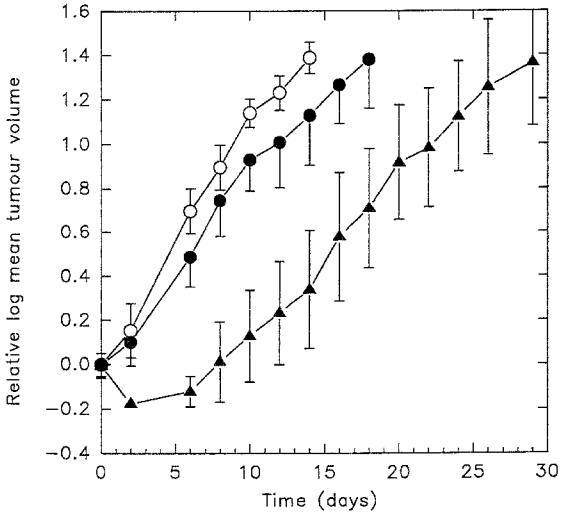

Fig. 4. Growth of s.c. colon 38 tumours in untreated BDF_1 mice (○) or following treatment with 5,6-MeXAA (20 mg/kg) either alone (●) or together with serotonin (150 mg/kg, ▲). *Points* represent mean log tumour volumes relative to the initial group tumour volume (approx. 100 mm^3). *Vertical bars* represent the standard error of logarithmic tumour volumes. In a separate experiment, administration of serotonin alone at this dose caused a growth delay of less than 2 days

PCPA, and tumour necrosis and plasma nitrate levels were measured 12 h later. Values for both plasma nitrate and tumour necrosis were in the normal range. Serotonin was undetectable in mice not treated with 5,6-MeXAA but was detectable in mice treated with drug, suggesting that it was still available and was in some way released in response to 5,6-MeXAA. Two courses of PCPA and reserpine (24 h apart) were then given, 5,6-MeXAA was injected 12 h after the second course, and tumour necrosis and plasma nitrate levels were measured 12 h later. In this case, plasma serotonin was undetectable in both untreated and 5,6-MeXAA-treated groups, and the increase in plasma nitrate was strongly inhibited (Fig. 3). However, pre-treatment did not prevent 5,6-MeXAA-induced tumour necrosis.

Augmentation of the effects of 5,6-MeXAA by serotonin

When given alone, serotonin (150 mg/kg), although inducing tumour necrosis at 12 h, induced less than a 2-day delay in the growth of colon 38 tumours (results not shown) and failed to increase plasma nitrate concentrations above the control levels (Fig. 3). Cyproheptadine, as expected, inhibited this effect (Fig. 3). However, co-administration of serotonin (150 mg/kg) significantly augmented the stimulation of plasma nitrate following administration of a sub-optimal dose (20 mg/kg) of 5,6-MeXAA (Fig. 3). Serotonin also augmented the antitumour effect of 5,6-MeXAA as measured by tumour growth delay (Fig. 4). On the other hand, serotonin did not increase the toxicity of the maximal tolerated dose of 5,6-MeXAA, which was 30 mg/kg in these mice, and did not strongly augment the effect of 5,6-MeXAA when given at the maximal dose.

Discussion

The induction of both plasma nitrate elevation and tumour haemorrhagic necrosis by FAA, 5,6-MeXAA and TNF was significantly inhibited by the serotonin 5-HT_2 antagonist cyproheptadine. The degree of inhibition of tumour necrosis was variable and incomplete, but it is possible that cyproheptadine, an aromatic cation, is not efficiently taken up by tumour tissue. Depletion of plasma serotonin levels by pre-treatment with reserpine and PCPA (Fig. 2) strongly inhibited the ability of 5,6-MeXAA to elevate plasma nitrate concentrations (Fig. 3). Serotonin depletion did not prevent the development of tumour necrosis in response to 5,6-MeXAA (Fig. 3), but it is possible that serotonin is not fully depleted from the tumour on the PCPA/reserpine protocol and that serotonin-dependent pathways can thus operate within the tumour microenvironment. The reason why an increased proportion of apoptotic tumour cells was observed in 5,6-MeXAA-treated tumours from serotonin-depleted animals is under further investigation.

The similarity of the effects of cyproheptadine and the structurally unrelated antagonist ketanserin, as well as the results of serotonin depletion, argue in favour of the specific involvement of serotonin (5-HT_2) receptors rather than of non-specific inhibitor effects. Serotonin cannot be a direct mediator of the effects of TNF because when given alone, it fails to cause an elevation of plasma nitrate [3]. Furthermore, while TNF is known to cause accumulation of platelets in the liver, it does not induce the release of serotonin from platelets [10].

Since the antagonist cyproheptadine inhibits the action of TNF (Fig. 1), the serotonin effect appears to act downstream from TNF production. An attractive hypothesis to explain the results is that serotonin is required, either directly or indirectly, for the in vivo induction of nitric oxide synthase in response to TNF. Cyproheptadine does

not prevent the induction of nitric oxide by activated macrophages in vitro (results not shown), which argues against a direct effect on macrophages. 5-HT_2 receptors are present on many types of cells and serotonin is known to stimulate the release of chemotactic cytokines and other factors [8, 12]. These products may be required in conjunction with TNF for the induction of nitric oxide synthases. It should be noted that systemic nitric oxide does not originate from tumour tissue alone [25] and that liver nitric oxide synthase may be a major source [11, 27]. If the main effect of serotonin is to facilitate the induction of nitric oxide synthases, the results obtained with cyproheptadine imply that nitric oxide production within the tumour contributes, along with blood flow changes [28], to the induction of haemorrhagic tumour necrosis.

When given alone, serotonin induces a reduction in tumour blood flow [5, 6, 13], but this effect is of short duration and studies in athymic mice have shown that it can be separated from the antitumour effect of serotonin [13]. The observation that the administration of serotonin alone induces tumour necrosis over a period of 12–24 h could be explained if it is conjectured that serotonin acts in concert with TNF produced within the tumour microenvironment to release nitric oxide locally. By inhibiting tumour cell respiration [9], nitric oxide could induce tumour necrosis, thus complementing the effects of decreased tumour blood flow. In response to a suboptimal dose of 5,6-MeXAA in colon 38-bearing animals, serotonin stimulates the production of nitric oxide (Fig. 3) and increases the tumour growth delay (Fig. 4), consistent with synergy between serotonin and local TNF production.

In conclusion, the observations reported herein demonstrate a role for serotonin in both host and tumour responses either to TNF or to drugs that induce TNF as part of their action. Although clinical trials of TNF have been disappointing, recent trials using isolated limb perfusion [14] and i.p. treatment of tumour ascites [20] have demonstrated its potential as an antitumour agent. Serotonin potentiates the antitumour effect of a suboptimal dose of the drug 5,6-MeXAA but does not increase toxicity, suggesting that serotonin (or a non-emetic serotonin 5-HT_2 agonist) might be considered in the context of clinical studies of 5,6-MeXAA. Measurement of circulating serotonin concentrations could be an important part of clinical trials in view of the observation that platelets in some cancer patients are depleted of serotonin [17]. Further work is required to elucidate the nature of the interaction between serotonin and TNF.

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