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Tumour Necrosis Factor-α Plasma Levels after Flavone Acetic Acid Administration in Man and Mouse

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Flavone acetic acid (FAA) is a synthetic flavonoid with a remarkable spectrum of anticancer activities in mouse tumours, but with no anticancer activity in humans. The mechanism of action of this drug is complex and involves a tumour vasculature action similar to the effects of tumour necrosis factor (TNF). To assess directly the role of TNF in FAA mechanism of action, this cytokine was assayed in both mouse and human plasma after intravenous administration of the drug. In mouse, a species particularly sensitive to FAA antitumour action, FAA plasma concentrations reached 268 μg/ml at 0.5 h and remained high (165 μg/ml) at 6 h following the intravenous administration of an anticancer efficacious dose (540 mg/m²). After FAA administration in mouse, TNF activity (L929 mouse cell bioassay) increased to 300 pg/ml TNF-α-equivalent at 2 h, reached a maximum concentration of 600 pg/ml at 4 h, and declined thereafter to 220 pg/ml at 6 h. TNF activity in mouse plasma was completely abrogated in the presence of mouse TNF-α antibodies. FAA added directly to blank mouse plasma did not show TNF activity. In patients receiving the drug as a 6-h intravenous infusion at doses ranging from 3.6 to 8.1 g/m², FAA plasma levels ranged from 58 to 449 μg/ml at the end of infusion. Human TNF-α levels assayed with an immunoradiometric assay were either not detectable or very low (< 25 pg/ml) before FAA administration. At completion of the FAA infusion, TNF-α remained near background levels in 20 of the 21 courses. A slight increase in plasma TNF-α was observed in 1 patient at the 8.1 g/m² dose level of FAA, from 13 pg/ml before intravenous infusion, to 70 pg/ml at completion of intravenous infusion. Taken together, these data demonstrate a marked interspecies difference with regard to TNF-α secretion after FAA treatment, as this cytokine is produced in mice, whereas it is not significantly secreted in pretreated patients. Although the low TNF-α levels achieved in mice probably do not explain all of FAA antitumour activity in that species, the observed interspecies difference in TNF-α secretion after FAA administration could partly explain the marked difference in FAA antitumour activity observed between mice and humans.
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INTRODUCTION

FLAVONE ACETIC ACID (FAA) is probably one of the most active drugs discovered to date against mouse tumour models [1, 2]. Despite FAA promising preclinical anticancer efficacy, this compound did not show activity in clinical phase I–II studies [3, 4]. The reasons for this marked interspecies difference in FAA antitumour activity are presently unknown.

The mechanism of action of this drug is complex and not completely elucidated at the present time. In mice, FAA antitumour activity was linked to DNA damage [5], decreased tumour ATP levels [6], and tumour blood flow reduction [6–8]. *In vitro*, the drug is poorly cytotoxic at concentrations and exposure times achievable *in vivo* [9, 10]. FAA can be metabolised to cytotoxic species *in vitro* and *in vivo*, suggesting that metabolism is required for the cytotoxic action of this drug [11].

Evidence also exists that FAA may act as an immune modulator. This drug increases natural killer cell activity, induces the production of interferons α/β, and increases the cytotoxicity of mouse peritoneal exudate cells [12, 13]. The cytotoxic action of FAA clearly differs from the majority of other cytotoxic agents because it also induces hyperaemia in tumours, followed by haemorrhagic necrosis [5, 14]. Because of this action on tumour vasculature and the histological appearance of FAA-treated tumours, tumour necrosis factor-like activity was suggested as a possible component of FAA mechanism of action [14]. Tumour necrosis factor-α (TNF-α) is a cytokine produced mainly by activated mononuclear phagocytes. Besides its cytotoxic and cytostatic effects on transformed cells *in vitro* and its antitumour effects *in vivo* [reviewed in 15], TNF expresses multifunctional immunomodulatory activities [16, 17]. This monokine has been shown to play a key role in the cytokine cascade as a modulator of immunocompetent cells [18].

In an attempt to understand better the marked interspecies difference in tumour response to FAA, we assessed directly the possible involvement of TNF in the mechanism of action of this drug *in vivo* in mice and humans. In this paper we report TNF measurement in plasma following FAA administration in both species. Our data demonstrate a clear interspecies difference in terms of TNF-α secretion, that could be partly associated with the lack of clinical response to FAA.

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PATIENTS AND METHODS

Patient plasma samples

Human plasmas used in this study were obtained during a phase I and pharmacokinetic evaluation of FAA at our institution [19]. Patients entered in this study were refractory to conventional therapy, had a good performance status and were meeting standard phase I eligibility criteria. Informed consent was required according to our institution guidelines. FAA was diluted in 500 ml 0.9% sodium chloride solution and administered as a 6-h intravenous infusion every 2–3 days, without alkalinisation or hyperhydration, at doses ranging from 3.6 to 8.1 g/m^2. Before, and at the end of the intravenous infusion, blood was collected into heparinised tubes, centrifuged at 2000 *g* for 10 min, and the plasma was frozen at −20°C until analysis for FAA concentration or TNF-α assay.

Chemicals

FAA sodium salt and the internal standard LM-2320 were kindly provided by Dr Philippe Briet, Lyonnaise Industrielle Pharmaceutique (LIPHA, Lyon, France).

TNF-α and interleukin-2 (IL-2) antibodies

Briefly, plasma samples were diluted 10-fold in microtitre plates and preincubated for 1 h in the presence of specific (anti-TNF) and control (anti-IL-2) antibodies (final volume: 100 μl), and transferred to microtitre plates containing L929 cells. Purified mouse TNF-α and IL-2 antibodies were obtained from Celltech.

Mouse plasma samples

B6D2F1 male mice (C57Bl/6J×DBA/2J; 28 g average) received FAA as an intravenous bolus into a lateral tail vein at the dose of 540 mg/m^2 (180 mg/kg). Three mice per time point were killed by cervical dislocation and their blood was aseptically harvested by cardiac puncture into a heparinised syringe. The pooled blood was centrifuged at 13000 *g* for 5 min at 4°C and the plasma was frozen at −20°C until analysis for FAA concentration or TNF-α activity determination.

FAA HPLC assay in plasma

FAA concentrations in plasma were determined by HPLC. Briefly, to 1 ml of plasma was added 100 μl of HCl (1 N) and 50 μl of the internal standard (LM-2320, 0.5 mg/ml in methanol). After mixing, the plasma was extracted with 5 ml of ethyl acetate, the organic phase was separated by centrifugation (2000 *g*, 10 min), and evaporated under nitrogen. The dry residue was reconstituted with 200 μl of the mobile phase, and 25 μl was injected onto an HPLC system composed of an octadecylsilane column (Nucleosil, 300 × 3.9 mm, 10 μm; SFCC, Neuilly-Plaisance, France) protected by a precolumn, with ultraviolet detection set at 300 nm. The mobile phase was composed of a 10 mmol/l sodium acetate buffer (pH 4) and methanol (40:60, v/v), at a flow rate of 1.3 ml/min.

TNF biological assay in mouse plasma

Biologically active TNF in mouse plasma was determined using mouse L929 cells pretreated with actinomycin D [20, 21]. Duplicate serial dilutions of plasma were incubated with L929 cells for 18–20 h at 37°C. After incubation, the plates were washed and cell lysis was determined by staining cells with 0.5% crystal violet in methanol/water (1/4, v/v). Dye uptake was calculated using an automated microenzyme-linked immunosorbent autoreader (Titertek Multiscan). Standards using human recombinant TNF-α (Knoll, Ludwigshafen, Germany; sp. act. = 107 U/mg protein) were routinely tested for each assay, and activity was reported as TNF-α-equivalent in pg/ml using these calibration curves. When repeated assays of the same plasma sample were performed, interassay variation was less than 15%. A typical calibration curve yielded a 50% growth inhibition concentration of 307 ± 73 pg/ml of TNF-α-equivalent (mean ± S.E.M.). For comparison purposes, one unit was defined as the TNF concentration required to kill 50% of target cells. This assay does not distinguish between TNF-α or TNF-β. To assess whether L929 cell death was actually due to the presence of TNF-α in the plasma samples, antibodies to murine TNF-α were also added in the assay.

TNF-α levels in human plasma

Quantification of immunoreactive TNF-α in human plasma (100 μl) was performed using a sensitive immunoradiometric assay (TNF-α-IRMA, Medgenix Diagnostics, Fleurus, Belgium) according to the kit procedure. Briefly, standards (human recombinant TNF-α) or samples were added to anti-TNF-α-coated tubes in the presence of ^{125}I-labelled anti-TNF-α monoclonal antibody directed against a different epitope. After a 18-h incubation (room temperature), tubes were washed with 20% Tween 20 and the remaining radioactivity (reflecting TNF-α concentrations) was measured on a gamma counter (Kontron). The TNF-α-IRMA does not cross-react with TNF-β, interleukins 1 and 2, and interferons α, β and γ. The sensitivity of this assay was 5 pg/ml.

Effect of FAA on TNF-α cytotoxicity in vitro

To assess the influence of FAA on the *in vitro* TNF-α-induced cytotoxicity, L929 cells were exposed for 24 h at 5 and 50 ng/ml of TNF-α in the absence or presence of FAA at pharmacologically relevant concentrations (5, 25, 50, 100 and 500 μg/ml). After incubation, the plates were washed and cell lysis was determined by staining cells with 0.5% crystal violet in methanol/water (1/4) as described above.

RESULTS

FAA levels and TNF activity in mouse plasma after FAA administration

FAA plasma concentrations achieved after an intravenous bolus administration in mouse of an antitumour efficacious dose of 540 mg/m^2 are depicted in Fig. 1(a). FAA plasma concentration at 0.5 h was 268 μg/ml, and the latest time point sampled at 6 h displayed a concentration of 165 μg/ml.

TNF activity was then determined using the mouse L929 bioassay in the same plasma samples used in the above pharmacokinetic study. There was no detectable TNF activity in blank mouse plasma, or in plasma spiked *in vitro* with FAA concentrations up to 1000 μg/ml (data not shown). Immediately after FAA administration, when drug concentrations were highest (at 0.5 and 1 h), TNF activity was not detectable [Fig. 1(b)]. However, 2 h after drug administration, TNF activity increased to 300 pg/ml TNF-α-equivalent, peaked at 4 h at 600 pg/ml, and declined thereafter to 220 pg/ml TNF-α-equivalent at 6 h [Fig. 1(b)].

The addition of an antibody to mouse TNF-α completely abrogated the cytotoxicity of mouse plasma samples against L929 cells [Fig. 1(b)]. This indicated that TNF activity detected in mouse plasma was mainly due to TNF-α and not to TNF-β. As a control for unspecific protein binding, an antibody to interleukin-2 added to mouse plasmas did not appreciably alter the TNF cytotoxicity profile [Fig. 1(b)].

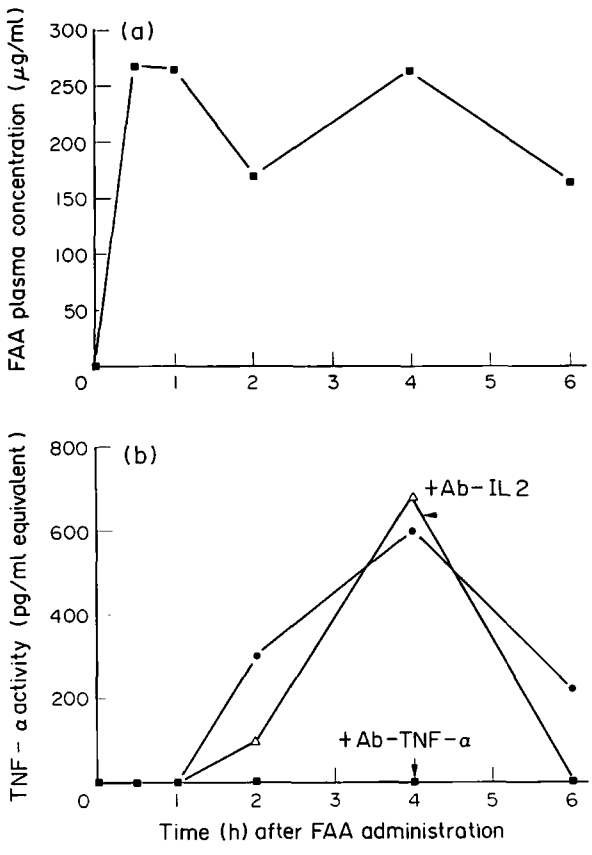

Fig. 1. FAA plasma concentrations and TNF-α activity in pooled mouse plasma (three mice) before and after FAA administration (540 mg/m², intravenous bolus). (a) FAA plasma concentrations (solid squares). (b) TNF activity in plasma alone (solid circles); addition of interleukin-2 antibodies (+ Ab-IL2) (open triangles); and addition of mouse TNF-α antibodies (+ Ab-TNF-α) (solid rectangles).

FAA and TNF-α levels in human plasma after FAA administration

In patients, FAA was administered as a 6-h intravenous infusion at doses ranging from 3.6 to 8.1 g/m², every 2–3 days. Some patients received as many as 18 courses of the drug. FAA plasma concentrations achieved at completion of the intravenous infusion ranged from 58 to 449 μg/ml, which were comparable to the concentrations achieved above in mice (Table 1).

Human TNF-α plasma levels, determined using a sensitive immunoradiometric assay, were either not detectable or low (< 25 pg/ml) before FAA administration (Table 1). At completion of the 6-h intravenous infusion of FAA, TNF-α levels did not increase compared to preinfusion levels, and remained near background levels in 20 of the 21 courses. In only one patient (no. 12) a slight increase in TNF-α level was noted over pretreatment levels (13 pg/ml), with a maximum value of 70 pg/ml at completion of the intravenous infusion (Table 1).

Influence of FAA on TNF-α-induced L929 lysis

FAA has been shown to exhibit *in vitro* cytotoxicity against a number of tumour cell lines, suggesting that FAA could act as a mediator in killing tumour cells. Experiments were therefore performed to test the effect of FAA alone or in the presence of TNF-α on L929 cell lysis. Results depicted in Fig. 2 indicate that FAA, at pharmacologically achievable concentrations (5–500 μg/ml), had no effect by itself on cell viability. Furthermore, when added to TNF-α-treated L929 cells, FAA did not interfere with the TNF-α-induced cell lysis.

DISCUSSION

Despite its promising preclinical activity in mouse tumour models [1, 2], FAA did not demonstrate clinical antitumour activity in phase I–II studies [3, 4]. The reasons for this marked interspecies difference in FAA antitumour activity, as well as its precise mechanism of antitumour action in mice, are presently unknown. In an attempt to understand better the marked interspecies difference in tumour response to this drug, we evaluated the production of TNF after FAA administration in both mouse and man.

In terms of pharmacokinetics, the *in vivo* FAA concentrations achieved with the administration of maximum tolerated doses were comparable in mouse and man. The levels achieved in mice after an intravenous administration of an efficacious dose (540 mg/m²) were well within the previously reported therapeutic range for this species [22, 23]. FAA levels achieved in humans were similar to mouse levels, and were also within previously published concentrations [3, 4, 19]. Consequently, FAA plasma levels alone cannot explain the marked interspecies difference in antitumour activity. In addition, although FAA protein binding is higher in man than in mouse, it alone does not explain the marked difference in anticancer activity between the two species [23–25].

FAA mechanism of action is rather complex and not completely elucidated at the present time. In mice, the antitumour action of this drug clearly differs from most other classical cytotoxic agents because FAA causes a decrease in tumour blood flow [6–8], provokes a decrease in tumour ATP levels [6], induces DNA damage [5], and induces tumour haemorrhagic necrosis [5, 14]. There is also evidence that FAA may act as an immune modulator in mice, because it increases natural killer cell activity, induces the production of interferons α and β, and increases the cytotoxicity of mouse peritoneal exudate cells [12, 13]. TNF-like activity was also indirectly suggested to be part of FAA mechanism of action because of its action on vasculature and histological appearance of tumours from FAA-treated mice [12, 14].

To assess more directly the role of TNF-α in FAA mechanism of antitumour action, this cytokine was assayed in mouse and human plasmas after FAA administration. TNF was indeed present in mouse plasma after an intravenous administration of FAA, but at low levels. Contrary to what was noted in mice, TNF-α was not produced in patients even at high FAA doses, with the exception of one patient in which a marginal increase of the cytokine was observed. These data demonstrate a marked interspecies difference in terms of TNF production that could be partly responsible for the marked interspecies difference in tumour response to FAA.

Although TNF is produced in mice after FAA administration, the contribution of this cytokine to direct tumour cytotoxicity is questionable because of the low levels achieved, since the peak concentration achieved of 600 pg/ml is equal to about 2 U, which is considered a low TNF activity. For example, the levels needed to achieve cytotoxicity *in vitro* are in the range of 300 pg/ml for an 18-h continuous exposure, whereas TNF levels superior to 300 pg/ml are achieved for only 4 h in mice. TNF concentrations needed to exert significant cytotoxicity *in vitro* are 30–50-fold higher [26, 27]. *In vivo*, TNF plasma concentrations reaching 10 000 U/ml are achieved only after the administration of high doses (100 000 U/mouse) [28]. Although a direct contri-

*Table 1. FAA concentrations and TNF-α levels in human plasma after intravenous administration of FAA**

Patient no.	FAA dose (g/m^2)	Course no.	Before infusion FAA (μg/ml)	Before infusion TNF-α (pg/ml)	After infusion FAA (μg/ml)	After infusion TNF-α (pg/ml)
1†	3.6	1	—	12	144	14
		5	—	25	126	nd
2†	3.6	1	—	—	58	—
		2	—	—	69	3
3‡	6.4	1	—	3	349	4
		10	0.1	3	139	—
4‡	6.4	1	—	3	260	10
		10	12	2	88	3
5†	8.1	2	—	6	170	5
		11	4	3	165	8
6†	8.1	1	—	3	449	7
		10	8	17	434	—
7†	8.1	1	—	4	152	nd
		18	—	3	222	3
8‡	8.1	1	—	5	352	8
		7	—	10	135	3
9‡	8.1	1	—	10	185	—
10‡	8.1	1	—	12	378	12
11‡	8.1	1	—	4	305	—
		4	0.1	2	242	—
12‡	8.1	1	—	13	181 (end)	70
					101 (4 h)	35
					26 (12 h)	18

* FAA was administered as a 6-h intravenous infusion at the indicated dose. FAA concentrations were determined by HPLC, and TNF-α levels were determined using an immunoradiometric assay.
† FAA administration every 3 days.
‡ FAA administration every 2 days.
—, Undetectable level. nd, Not determined.

bution of TNF-α to cytotoxicity is unlikely to occur *in vivo* because of the low levels achieved, we cannot rule out an indirect contribution of this cytokine to FAA antitumour activity in mice, since a synergy between FAA and TNF could be synergistic *in vivo*, as recently shown for FAA in mice [29] and for other chemotherapeutic agents [26, 27].

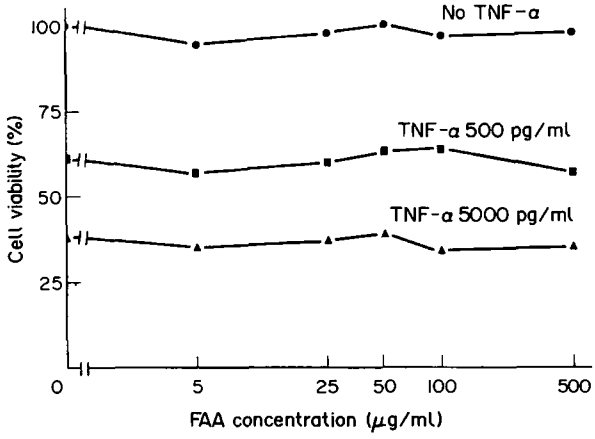

Fig. 2. Effect of FAA on TNF-α-induced cytotoxicity *in vitro* against mouse L929 cells. Cells were exposed for 24 h without TNF-α, or at 500 or 5000 pg/ml of TNF-α, with increasing concentrations of FAA, and cell viability was determined.

Since the present work was undertaken, Mace *et al.* [30] have also reported the production of TNF-α after FAA treatment in mice. However, their maximum TNF levels are in the range of 66 U in BALB/c mice, whereas in our B6D2F1 mice, the maximum TNF levels reached a maximum of only 2 U (600 pg/ml). This apparent discrepancy may be due to the different strain of mice used. Despite the low TNF-α levels observed in our B6D2F1 mice, it should be noted that FAA is none the less a very active drug in this animal model [1, 2], thus questioning the direct contribution of TNF to the anticancer activity observed in this species.

The complete reversal of mouse plasma cytotoxicity by antibodies specific to TNF-α demonstrated that TNF-β is not secreted after FAA administration. These data are in agreement with similar results showing that FAA does induce TNF-α mRNA, whereas it does not induce TNF-β mRNA in mice [30].

FAA metabolism may also be needed for full FAA cytotoxic activity. *In vitro*, FAA is poorly cytotoxic at concentrations and exposure times achievable *in vivo* [9–11]. However, FAA can be metabolised to cytotoxic species *in vitro* and *in vivo*, suggesting that metabolism is required for the cytotoxic action of this drug [11]. Reactive and cytotoxic species are indeed formed by *in vitro* metabolism of FAA, in conditions where TNF is absent [11, 31]. The causes of the vascular collapse and hyperhaemia observed in tumours of FAA-treated mice could also be due to reactive metabolite(s) that could be formed *in vivo* in mice. It is also possible that the vascular effects of TNF could contribute

to FAA metabolism within the tumour, by entrapment of the drug within the target tissue.

One major difference that has to be taken into consideration when comparing human and mouse responses to FAA is the clinical status of patients entered in clinical phase I studies, which are often heavily pretreated and probably immunosuppressed prior to FAA administration. This difference in host status prior to FAA administration could be partly responsible for the marked interspecies discrepancy observed between mouse and man in the anticancer activity of FAA. The immune status of the host is probably important in tumour response to FAA because mouse colon tumours responsive to the drug in their normal host become less responsive in immune-deprived animals [32].

In conclusion, FAA was shown to induce the secretion of TNF-α in mice, but did not elicit the production of this cytokine in humans at maximum tolerated doses, with the exception of 1 patient in which a marginal increase of the cytokine was observed. These data therefore demonstrate a major interspecies difference in terms of TNF production that could be partly responsible for the marked interspecies difference in tumour response to FAA.

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