Correlation between in Vivo Induction of Cytokine Gene Expression by Flavone Acetic Acid and Strict Dose Dependency and Therapeutic Efficacy against Murine Renal Cancer

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ABSTRACT

The investigational chemotherapeutic drug flavone acetic acid (FAA) acts as an immunomodulator by augmenting natural killer activity in both humans and rodents after in vivo administration. The accumulated data derived from a series of experiments also demonstrates that FAA synergizes with interleukin 2 (IL-2) for the treatment of murine renal cancer. The immunomodulatory and immunotherapeutic effects of FAA are strictly dose dependent with doses of FAA > 150 mg/kg effectively synergizing with IL-2, and doses < 150 mg/kg exhibiting very little therapeutic effect. The antitumor and immunomodulatory effects of FAA are more pronounced in vivo than in vitro. Collectively, these results suggested that cytokines induced by FAA may contribute to these effects, and that the induction of such cytokines may also be very dose dependent. Studies were therefore initiated to investigate whether the in vivo administration of FAA would alter the expression of cytokine mRNA in leukocytes. Splenic leukocytes or liver nonparenchymal cells from untreated and FAA-treated mice were used as a source of RNA for Northern blot analysis. Interferon α and interferon γ mRNA in the spleen was up-regulated within 1.5 h after FAA administration, with peak induction occurring by about 2 h. An up-regulation of tumor necrosis factor α mRNA was detected in the spleen by 0.5-1 h after treatment with peak induction occurring by 1.5 h. Induction of tumor necrosis factor α mRNA was also detected in hepatic nonparenchymal cells. No up-regulation of splenic mRNA for tumor necrosis factor β, IL-1α or β, or IL-2 was detected after FAA administration. IFN and TNF activities were detectable in the serum by bioassay immediately following the appearance of mRNA in FAA mice. The observed up-regulation by FAA of cytokine mRNA and the corresponding serum protein was strictly dose dependent with substantial induction of both mRNA and proteins occurring only at FAA doses > 150 mg/kg, a dose range also shown to be the minimum required for immunomodulatory and immunotherapeutic effects. In summary, these results demonstrate that FAA acts as a potent inducer of cytokines in vivo, and suggest that the immunomodulatory and immunotherapeutic effects of FAA may be partially mediated by these induced cytokines.

INTRODUCTION

Flavonoids as a class of natural compounds have been reported to cause multiple biological effects (1, 2). These effects include the ability to bind to, and thereby inhibit the activity of, a variety of critical enzymes including lipases, hydrolases, phosphatases, and kinases that are vital to the function of mammalian cells (1, 2). Thus, certain flavones have been selected for evaluation as potential new agents for cancer treatment (3, 4). In addition, some flavonoids have been reported to have activity as BRM. One particular flavone derivative, flavone acetic acid, has been extensively investigated. FAA was used in this investigation because it has already demonstrated both direct antitumor (3) and BRM (5-7) activities. The previously noted BRM effects included augmentation of NK activity and the induction of IFN-α/β activity in the serum (5). Pretreatment of mice with antibodies directed against IFN-α/β partially inhibited the ability of FAA to augment NK activity (5). Further studies showed that FAA and IL-2 synergized in the therapy of established murine renal cancer (6, 8). The combination of IFN-α/β with IL-2 also resulted in somewhat greater in vivo antitumor activity in this model than did either agent alone, although the antitumor effects were appreciably less than FAA plus rIL-2 (8). These results suggested that the antitumor activity of FAA alone and in combination with IL-2, may be at least partially due to induction of cytokines.

Recent investigations have established that cellular and humoral immune responses are under complex regulatory control, often through a cascade of cytokines (9). These factors can have direct effects on tumor cells through their growth regulatory activities, as well as an indirect effect through immunological mechanisms. Thus, the possibility that FAA could regulate the gene expression of other immunologically active cytokines in addition to IFN-α/β was of particular interest in determining a possible relationship between the BRM and direct antitumor activities of FAA.

Therefore, the aim of this investigation was to define, using Northern blot analysis, the ability of FAA to up-regulate in vivo mRNA for IFN-α and other cytokines. A panel of cytokine probes was used for the initial screening and the temporal expression of induced genes was examined. This report demonstrates that administration of FAA up-regulates the expression of mRNA for IFN-α, IFN-γ, and TNF-α in the spleen and TNF-α mRNA is also up-regulated in hepatic leukocytes. The up-regulation of mRNA is rapidly followed by the presence of detectable IFN and TNF activities in the serum.

MATERIALS AND METHODS

Animals. Male BALB/c mice were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility. The animals were used at 7 to 10 weeks of age and were housed under specific pathogen-free conditions.

BRM. FAA was synthesized by Lyonnaise Industrielle Pharmaceutique (LIPHA, Lyon, France) and acquired through the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Frederick, MD). FAA was diluted in Hank’s balanced salt solution for administration to mice. rIL-2 (3 × 10⁶ Biological Response Modifiers Program units/mg protein) was generously supplied by Cetus Corp. (Emeryville, CA).

* The abbreviations used are: BRM, biological response modifier; FAA, flavone acetic acid; IFN, interferon; NK, natural killer; IL-2, interleukin 2; TNF, tumor necrosis factor; NPC, nonparenchymal cell; Renca, murine renal cancer; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; rIL-2, recombinant interleukin 2.
Isolation of Leukocytes. Leukocytes were isolated from the spleen and nonparenchymal cells from the liver as previously described (10) at various times after the i.v. administration of increasing doses of FAA.

Cell Lines. All cell lines were maintained by continuous passage in culture using RPMI-1640 (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT), 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 20 mM L-glutamine. Biological Assays. Mouse L-cells pretreated with actinomycin D were used in a bioassay to detect the presence of biologically active TNF in serum (11, 12). TNF activity is expressed as units/ml where 1 unit results in 50% growth inhibition as assessed by direct Giemsa staining of the remaining cells. This assay does not distinguish between TNF-α and TNF-β. Serum IFN activity was determined with the vesicular stomatitis viral inhibition assay (13), with 1 unit of IFN equal to the amount of IFN in 1 ml of sample that reduces viral lysis by 50% in a bioassay (Clinical Immunology Services, Program Resources, Inc.). It should be noted that this assay does not distinguish between the different IFN types.

Northern Blotting. Total cellular RNA was isolated from cell preparations by using a single step phenol/chloroform extraction procedure of Chomczynski and Sacchi (14). RNA (10 μg) was denatured by heating for 10 min at 50°C with formaldehyde. Separation of mRNA by electrophoresis was performed using a 1% agarose, formaldehyde gel with 50 V of applied current. mRNA was then transferred to a nylon filter membrane (Zetaprobe; Bio-Rad Laboratories, Richmond, CA) by capillary action by using 10x SSC (1x SSC is 0.15M NaCl, 0.015M sodium citrate, ph 7.2) (Quality Biological, Inc., Gaithersburg, MD) as per manufacturer's recommendations. Prehybridization (18 h) and hybridization (48 h) were performed at 42°C in 50% formamide, 5x SSC, 1% sodium dodecyl sulfate, 5% dextran sulfate, 0.05 M sodium phosphate (pH 7.2), 50 μg/ml of denatured salmon sperm DNA (Lofstrand Labs Limited, Gaithersburg, MD), 50 μg/ml of yeast transfer RNA and 1x Denhardt's solution (5'-3', Inc., Paoli, PA). The following DNA probes were used: murine IFN-α cDNA (obtained from Dr. Paula Pitha, Johns Hopkins University, Baltimore, MD, and which hybridizes to all IFN-α subtypes); murine TNF-α (obtained from Dr. Anthony Cerami, Rockefeller University, New York, NY); and murine IFN-γ (obtained from Dr. Ken-ichi Arai, DNAX Corp., Palo Alto, CA). All probes were 32P labeled utilizing dCTP (-3,000 Ci/mmol; Amersham, Arlington Heights, IL) by oligo priming, using a commercially available kit (Pharmacia, Inc., Piscataway, NJ) in a 4-h reaction at room temperature. Labeled probe was separated from free radiolabeled nucleotide by precipitation utilizing NACS (nucleic acid chromatography system) column (Bethesda Research Laboratories, Gaithersburg, MD) as directed by the manufacturer. After hybridization, the filters were washed in 2x SSC and 1% SDS for 20 min at room temperature and then in 0.2x SSC and 0.1% SDS for 20 min at 65°C. The filters were blotted dry and exposed to X-ray film (XOMAT film, Kodak, Rochester, NY) for 24 to 72 h at −70°C. After exposure, all filters were stripped twice by boiling in 0.01% SDS, 0.01x SSC for 20 min, and rehybridized with chicken β-actin cDNA (obtained from Dr. D. W. Cleveland, Princeton University). Lanes within each blot were found to hybridize to this cDNA with approximately equal intensity, indicating that comparable levels of RNA were present in each lane (data not shown).

Experimental Tumor Model. The model used for these studies is the Renca renal adenocarcinoma which originally arose spontaneously (15) in BALB/c mice. The details of tumor progression in this model have been previously described (16). Briefly, after injection of 1 x 106 tumor cells subcutaneously, the solid tumor mass develops rapidly with direct extension to the peritoneal cavity by days 7 to 9 and metastasis to regional lymph nodes and liver shortly thereafter. Surgical resection of the primary tumor-bearing kidney is potentially curative before day 8 but not thereafter when mice succumb to peritoneal carcinomatosis and subsequent metastatic disease. Various doses of FAA were administered by i.v. or i.p. injection, whereas rIL-2 (30,000 units) was delivered by the i.p. route. Routinely, FAA was administered 2 to 4 h after nephrectomy of the primary tumor-bearing kidney on day 11, and rIL-2 was administered once per day for 4 successive days (days 12–14) beginning on the day after nephrectomy and FAA treatment. All long-term survivors were euthanized on day 100 and were determined to be tumor free by both gross and visual examination. Statistical analysis of survival data was performed by χ2 test.

RESULTS

Previous studies demonstrated that the FAA-induced augmentation of splenic NK activity was strictly dose dependent and was related to an induction of IFN-α/β, suggesting the possibility that FAA might also induce the production of other cytokines (5). We have also found that the immunotherapeutic effects of FAA used in conjunction with rIL-2 for treatment of murine renal cancer are similarly dose dependent. The data presented in Fig. 1 represent the accumulated data from four separate experiments. These results clearly demonstrate that doses of FAA ≥ 150 mg/kg effectively synergize with rIL-2 for the treatment of murine renal cancer, such that about 50% of the mice are rendered disease free. The survival incidence obtained at doses of 150–250 mg/kg FAA is significantly greater than that obtained in both the untreated control group (P < 0.01), as well as for all doses of FAA ≤ 125 mg/kg (P < 0.05). These results, along with our previous studies that demonstrated a role for both NK cells and T-cells in the therapeutic effects of FAA plus rIL-2, strongly suggested a strict dose-dependent role for immunoactive cytokines induced by FAA in the immunomodulatory and immunotherapeutic effects of FAA plus rIL-2. Further studies were thus designed to investigate the ability of FAA to induce the production of immunoactive cytokines.

In order to rapidly assess cytokine induction, we studied cytokine gene expression at the level of mRNA. Total cellular RNA was isolated from normal mouse spleen cell suspensions and assayed by Northern blot analysis. Using an IFN-α (type 1) cDNA probe, hybridization was detectable in splenic mRNA from normal mice treated 1.5, 2, and 3 h earlier with FAA (Fig. 2) but not in thymic mRNA in mice treated for 3 h with FAA (data not shown). No increase in IFN-α mRNA expression was detectable at 1 h or in the untreated control mice, and the expression of IFN-α mRNA was lost by 6 h. This induction of IFN-α mRNA, like serum IFN (5), was observed to be strictly dependent on the FAA dose used in combination with rIL-2 for the treatment of Renca. Treatment was initiated 11 days after intrarenal inoculation of Renca. Various doses of FAA were administered 2 h after nephrectomy of the Renca-bearing kidney. On days 12–14 mice received i.p. 30,000 units of rIL-2/day and were then monitored daily for survival until day 100, at which time they were euthanized and determined to be tumor free by both gross and histological analyses. * and **, significantly greater survival (P < 0.01 or P < 0.05, respectively) when compared to all FAA doses ≤125 mg/kg. NS, incidence of survival not significantly increased when compared to untreated Renca-bearing mice.

Fig. 1. Dose-dependent therapeutic efficacy of FAA when used in conjunction with rIL-2 for the treatment of Renca. Treatment was initiated 11 days after intrarenal inoculation of Renca. Various doses of FAA were administered 2 h after nephrectomy of the Renca-bearing kidney. On days 12–14 mice received i.p. 30,000 units of rIL-2/day and were then monitored daily for survival until day 100, at which time they were euthanized and determined to be tumor free by both gross and histological analyses. * and **, significantly greater survival (P < 0.01 or P < 0.05, respectively) when compared to all FAA doses ≤125 mg/kg. NS, incidence of survival not significantly increased when compared to untreated Renca-bearing mice.
cytokine induction by flavone acetic acid

Fig. 2. Hybridization of splenic total cellular RNA to IFN-α cDNA. Top abscissa, time between FAA (200 mg/kg, i.v.) administration and spleen harvest; C, control.

Fig. 3. Hybridization of splenic total cellular RNA to IFNα cDNA. FAA at various doses was administered i.v. 3 h before spleen harvest.

Fig. 4. Hybridization of splenic total cellular RNA to IFNγ cDNA. Top abscissa, time between FAA (200 mg/kg, i.v.) administration and spleen harvest; C, control.

Dose of FAA (mg/kg)

18S

dependent on the dose of FAA administered with induction occurring at doses of 200 and 150 mg/kg, but not at lower doses (Fig. 3). Additional studies investigated the induction of

IFN-γ (type II IFN) in total splenic RNA following FAA treatment. The induction of IFN-γ mRNA was detectable at 1.5 and 2 h as seen in Fig. 4. In contrast, total cellular RNA from total liver and kidney were negative for hybridization with both IFN-α and IFN-γ cDNA (data not shown) in similarly treated groups. As no other tissues were analyzed, we cannot rule out the possibility that other normal tissues produce TNF or IFN-α mRNA.

Because the biological effects of flavones are quite pleiotropic (1) and FAA has been reported to induce hemorrhagic necrosis of colon tumors in BALB/c mice (17), we also performed studies to determine if FAA could induce production of TNF mRNA. Hybridization of a TNF-α cDNA probe to total splenic mRNA from normal mice revealed that mRNA for TNF-α was up-regulated within 0.5 to 1 h following administration of FAA (Fig. 5). This increase in the level of mRNA for TNF-α persisted for 6 h following administration of FAA. No hybridization was observed in total thymus RNA after 3 h of FAA treatment.

Like the FAA-induced increase in IFN mRNA (Figs. 2–4), the induction of TNF-α mRNA was strictly dose dependent with an up-regulation of mRNA detectable only at doses of 150 and 200 mg/kg (Fig. 6). As many drugs are metabolized in the liver, and the liver contains a large population of resident macrophages (Kupffer cells), we postulated that mRNA for TNF-α would be up-regulated in NPC. Subsequent studies confirmed that increased mRNA for TNF-α was detectable in NPC isolated from normal mice treated 3 h earlier with an optimal dose of FAA (200 mg/kg). (Fig. 7). No induction of mRNA for TNF-β, IL-1-α or -β, or IL-2 was detected in the splenic or hepatic leukocytes in similar experiments (results not shown),

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Dose of FAA (mg/kg)

0 200 150 100 50

Table 1 Induction of serum IFN and TNF activities by FAA as function of time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>INF$^a$</th>
<th>TNF$^b$</th>
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<tbody>
<tr>
<td>Control</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>0</td>
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<tr>
<td>FAA</td>
<td>1</td>
<td>&lt;2</td>
<td>1</td>
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<tr>
<td></td>
<td>1.5</td>
<td>1000</td>
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<td></td>
<td>18</td>
<td>80</td>
<td>0</td>
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*a Mice received i.v. either Hanks' balanced salt solution or the indicated dose of FAA and were euthanized at the indicated times after treatment. Serum from 6 or more mice was collected by cardiac puncture and pooled.

*b Interferon activity in the serum was determined by using the vesicular stomatitis viral inhibition assay. One unit equals the amount of interferon in 1 ml of sample that reduces the viral lysis by 50% in the bioassay.

and at this 3-h time point, we did not observe any hybridization to IFN-α or IFN-γ cDNA in the NPC RNA.

Since transcription of mRNA is not always followed by translation of active proteins, further studies were performed to determine whether biologically active proteins were produced in conjunction with induction of cytokine mRNA. The results shown in Table 1 demonstrate that both serum IFN and serum TNF activities are rapidly induced after a single injection of FAA. Peak levels (>4000 units/ml) of IFN activity were de-
monstrable by 2 h postinjection with a rapid decrease to low levels by 18 h. Similarly, peak levels of TNF activity were detected between 1.5 and 3 h, with levels returning to near background by 6 h. Thus, the detection of active serum protein occurs in close proximity to induction of mRNA for both IFN and TNF, 1.5 h (Figs. 2 and 4) and for 0.5 to 1 h (Fig. 5), respectively.

The augmentation of NK activity and the therapeutic synergy with IL-2 by FAA are both sharply dose dependent (6, 8) as is the induction of mRNA for both IFN and TNF (Figs. 3 and 6). Therefore, we also investigated the dose dependency of active cytokine induction by FAA. The results shown in Table 2 demonstrate the strict dose dependency of cytokine induction by FAA. Mice were treated with various doses of FAA for 3 h, which was previously demonstrated to be optimal for induction of both serum IFN and serum TNF activities (Table 1). The results show that maximal induction of both IFN and TNF activities occurred at FAA doses ≥ 150 mg/kg. No induction of TNF activity was detected following treatment of mice with 100 mg/kg, while this dose of FAA induced low levels of IFN activity. Thus, the dose dependency of cytokine induction in the serum also closely parallels the up-regulation of mRNA for IFN-α, IFN-β, and TNF-α.

Overall, these results demonstrate that flavonoid compounds can act as potent inducers of immunoactive cytokines and suggest that induction of cytokines may contribute to the immunomodulatory and immunotherapeutic activities of FAA.

**DISCUSSION**

Recombinant DNA technology has provided the opportunity for molecular characterization of numerous cytokine genes and for gaining insights into the mechanisms by which cytokine genes are induced in response to extracellular stimuli in specific cell types. Many of the cytokine-specific mRNA are controlled primarily at the translational level (18), although mRNA stability is also important. Because FAA has potent antitumor effects against some murine cancers in vivo (3) and demonstrated BRM activity (4), we speculated that the induction of cytokine gene expression could contribute to these activities. Our studies demonstrated that the immunotherapeutic effects of FAA were strictly dose dependent and we hypothesized that a dose-dependent induction of immunoactive cytokines could contribute to this antitumor response. Therefore, we studied the ability of therapeutic and nontherapeutic doses of FAA to induce cytokine gene expression.

Previous studies have demonstrated that FAA can systemically (spleen, liver, and blood) augment NK activity and induce high titers of serum IFN (4, 5, 7, 8, 10). In this study, we demonstrated that the induction of IFN-α and IFN-β mRNA preceded the in vivo induction of the relevant active serum proteins. The expression of mRNA for all three of these cytokines is relatively short lived with peak activity evident at about 2 h. However, the up-regulation of TNF mRNA became detectable as early as 0.5 to 1 h, while mRNA for both types of IFN do not become clearly detectable until about 1.5 h. The production of lymphokine mRNA after FAA treatment appears to be localized to leukocytes, as in preliminary experiments, in situ hybridization of both IFN-α and TNF-α is localized to liver nonparenchymal cells and not in hepatocytes (data not shown). The rapid induction of TNF-α mRNA and the rapid subsequent production of TNF-like activity (Table 1) support a role for TNF-α in the early immunopharmacological effects of FAA. Further, expression of TNF-α mRNA persisted until 6 h after administration of FAA. TNF gene expression has been reported to be controlled by transcriptional and posttranscriptional regulation (19), and our results are consistent with both regulatory mechanisms. FAA-induced transcription of TNF-α resulted in appreciable serum TNF titers followed by a more rapid down-regulation of serum protein than of splenic mRNA. Interestingly, in humans the response to endotoxin was also associated with a brief pulse of circulating TNF detectable in the serum (20).

Some of the reported immunomodulatory effects of FAA may be at least partially attributable to the rapid induction of cytokines. Certainly, induction of TNF-α and IFN-γ, which have been reported to synergize for macrophage activation (21), might result in the in vivo activation of macrophages following treatment with FAA. In fact, Ching and Baguley (22) have reported that peritoneal macrophages isolated from FAA-treated mice (200 mg/kg, i.p.) were activated to kill tumor targets in vitro. Previous studies have already demonstrated that IFN-α/β induced by FAA contributed to the ability of FAA to augment NK activity in vivo (5). NK cells and/or macrophages may be involved in the antitumor effects of FAA used in conjunction with rIL-2, since in vivo treatment with asialo-GM1 serum, which reacts with both cell types (23), partially blocked the antitumor activity of FAA plus IL-2 (6). Thus, cytokines like TNF-α, IFN-α, or IFN-γ could be involved in the antitumor effects of FAA by virtue of their immunoregulatory activity. This hypothesis is supported by the close correlation in active doses of FAA for both induction of cytokines (Table 2) and therapeutic synergy with rIL-2 (6, 8). Studies are in progress to determine whether administration of antibodies with specificity for IFN-α, IFN-γ, or TNF-α can inhibit the therapeutic efficacy of FAA plus rIL-2.

FAA was chosen for preclinical and clinical trials based on its unusual spectrum of activity. It had little activity in vivo against the murine tumors L1210 or P388, but had significant activity against the colon 26 and 38 adenocarcinomas (24). Hemorrhagic necrosis that occurred during the FAA-induced regression of these colon tumors suggested that TNF could be an in vivo mediator of this effect (17). In fact, TNF-α does have antitumor activity against s.c. implants of the Meth A sarcoma (the classical TNF-sensitive tumor) and the colon 26 adenocarcinoma in BALB/c mice when administered i.v. (25). More recently, Bibby et al. (26) presented evidence that FAA changes the blood vasculature of a colon tumor growing s.c. such that
tissue destruction was observed as early as 2 h after i.v. administration of 200 mg/kg FAA. This change in vasculature is similar to in vivo effects seen in tumor tissue following administration of recombinant TNF. The present study is the first to directly demonstrate that TNF can be induced in mice by FAA under similar conditions.

In addition to the immunomodulatory and vasculature-related effects of various FAA-induced cytokines, several of these cytokines, alone and in combination, have been reported to have direct antiproliferative effects on various tumor cells (27-29). Experiments recently performed in our laboratory have demonstrated that IFN-α and IFN-γ have a synergistic antiproliferative effect on the growth of murine renal cancer cells in vitro. Thus, the cytokines induced by FAA may mediate some direct antitumor effects. However, the major effects of FAA are probably mediated via the immune system since the therapeutic efficacy of FAA plus rIL-2 is appreciably reduced by pretreatment of mice with anti-asialo-GM1 serum (8), and FAA plus rIL-2 is ineffective in athymic mice. Furthermore, mice surviving renal cancer after FAA plus rIL-2 therapy are specifically resistant to rechallenge with this tumor.6

Since numerous in vivo and in vitro systems have demonstrated that combinations of cytokines often exhibit enhanced biological effects above those observed with single cytokines (9), it seems likely that BRM which induce multiple pleiotrophic effects and synergize with rIL-2 for treatment of murine renal cancer are specifically induced by FAA. The results also suggest that some of the previously reported biological effects of flavonoid compounds could be cytokine mediated.

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