Flavone Acetic Acid Increases the Antitumor Effect of Hyperthermia in Mice

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ABSTRACT

The combined effects of flavone acetic acid (FAA), a synthetic flavonoid, and hyperthermia on B16 melanoma cells were investigated. In vitro, FAA alone at concentrations below 100 μg/ml was not cytotoxic with a 60-min exposure at 37°C. Hyperthermia at 43°C for 60 min enhanced the cytotoxicity of FAA only at concentrations over 100 μg/ml. Inhibition of the growth of B16 melanoma solid tumor by FAA and/or hyperthermia was examined in vivo. FAA (100–200 mg/kg) inhibited tumor growth in a dose-dependent manner. The combined treatment of FAA (200 mg/kg) and hyperthermia (43°C, 15 min) significantly inhibited tumor growth compared to a treatment of FAA or hyperthermia alone. The maximum antitumor effect of FAA combined with hyperthermia was obtained when FAA was administered 2 or 4 h before heat. The significantly increased cytotoxicity of FAA combined with hyperthermia seems to relate to specific decreases in tumor blood flow, a reduction in tumor pH, and an increased tumor temperature, without altering pH in the normal tissues. This combined treatment of FAA and hyperthermia warrants further study for treating subjects with solid tumors.

INTRODUCTION

FAA, a synthetic flavonoid synthesized by Lyonnaise Industrielle Pharmaceutique (LIPHA, Lyon, France) (1), was found in the National Cancer Institute Screening Program to have antitumor activity (2). FAA has definite antitumor activity in various experimental models in vitro and the mode of action of this drug is unique. FAA is more cytotoxic in vivo than in vitro (3, 4), and has greater antitumor activity on solid tumors than on ascites or hematological tumors (2, 5, 6). FAA also shows greater antitumor activity on established than on newly implanted tumors (3, 6), and induces hemorrhagic necrosis within the tumor (7, 8). The effect of this compound on tumor growth may be indirect and may involve interaction with the host. Chabot and Rutkowski (9) suggested that FAA needed metabolic activation by the host, and cytotoxic FAA metabolites were investigated. Rubin et al. (10) discussed alteration of platelet function by FAA. The immunomodulation effects of FAA were evident in laboratory animals (11, 12) and in humans (13). Reduction in blood flow to the tumor was seen in various experimental tumor systems (14–17). Zwi et al. (18) did experiments using avascualar and vascularized multicellular spheroids and noted that the establishment of a tumor vasculature was important and essential for the antitumor effects of FAA. In the present studies, we examined FAA-mediated reduction of tumor blood flow leading to acidic states of tumor cells. As acidity increases the thermosensitivity of tumor cells (19–21), FAA may enhance the cytotoxicity of hyperthermia. We investigated the mechanism of FAA-enhanced thermal effects on tumors, since little is known of the combined effects of FAA and hyperthermia.

MATERIALS AND METHODS

Drug. FAA was supplied by Dr. R. A. Newman, the University of Texas, M. D. Anderson Cancer Center, Houston, TX. FAA, in freeze dried storage, was dissolved in physiological saline just prior to use. For in vivo experiments, FAA was administered by i.p. injection at a final concentration of 10 mg/ml.

Mice. C57BL/6NCrj male mice (6–8 weeks old) were obtained from Charles River Japan (Tokyo, Japan), and were housed under conditions of constant temperature and humidity. Food and water were provided ad libitum.

Tumors. For in vitro experiments, B16 melanoma cells (obtained from Dr. S. Taniguchi, Medical Institute of Bioregulation of Kyushu University, Fukuoka, Japan) were cultured in monolayers on plastic dishes, using Eagle's minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY), l-glutamine (292 mg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (40 μg/ml). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

For in vivo experiments, we used B16 melanoma solid tumors, grown and maintained by biweekly s.c. inoculations in C57BL mice. Tumors were removed from carrier mice by mechanical disaggregation and then minced with scissors. Tumor fragments (1 mm³) were implanted with a trocar into the s.c. tissue of the foot. Mice with tumor exceeding 6–8 mm in diameter were used for all experiments. The mice weighed between 18 and 23 g at the time of treatment.

Colony Formation Assay. Three × 10⁵ B16 melanoma cells were plated in 60-mm dishes in the absence of the drug and were incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. Cells were then exposed to various concentrations of FAA (0.1–1000 μg/ml) at 37°C for 60 min. In the combined treatment group (FAA and hyperthermia), cells were incubated at 43°C for 30 or 60 min, simultaneously with FAA exposure. After FAA treatment, the cells were washed 3 times with phosphate-buffered saline and incubated in fresh medium. Six days later the dishes were scanned by inverted microscopy and colonies of more than 50 cells were counted. The control dishes contained 140–160 colonies, and the plating efficiency was about 50%. Effects of treatment were evaluated by the rate of inhibition of colony formation. This assay was done in triplicate.

Treatment of B16 Melanoma Solid Tumor. (a) To investigate the effects of FAA alone, drug (100, 150, or 200 mg/kg) was administered i.p. to 8 tumor-bearing mice in each group. (b) To investigate the combined effects of FAA and hyperthermia, FAA (200 mg/kg) was administered alone or in combination with hyperthermia. FAA was administered just prior to hyperthermia, which was induced by immersing the foot of the mouse into a circulating waterbath (22) at 43°C for 15 min. Each group included 8 mice. (c) To investigate the most effective timing for the combination of FAA and hyperthermia, FAA (200 mg/kg) was administered at −8, −6, −4, −2, 0, +2, +4, +6, +8 h, using 0 h as the time of the start of hyperthermia. Each group included 5 mice.

In all the experiments, there were no differences among the mean tumor volumes in each group on day 0 (100–120 mm³). FAA was administered as a single dose on day 0. Size of the tumor was measured by digital calipers (DP-1 HS, Mitutoyo Co., Tokyo, Japan), in two dimensions every 2 days, and body weight was recorded. Tumor volume was estimated from the measurement of two perpendicular diameter: length and width, by the formula (23)

Tumor volume = length × (width)² + 2
Relative tumor volume was expressed as a ratio of tumor volume on each day to initial tumor volume at the time of treatment. Tumor growth time was calculated as the time required to reach a tumor volume 3 times greater than the initial tumor volume. Tumor growth delay was calculated by subtracting the tumor growth time of control tumors from that of treated tumors.

Measurement of Tissue pH. The pH of tissues of mice, treated with FAA alone, was measured with a tissue pH electrode (IW202, Iwaki Co., Tokyo, Japan), and pH meter (Beckman Instruments, Inc., Fullerton, CA). The electrode used was a combined glass/reference electrode, the conical tip of which had a maximum diameter of 1 mm; it was calibrated in standard solutions at pH 4.01 and 6.86. To introduce the fragile glass electrode into the tissue, a small incision was made on the skin and tumor surface. The pH of muscle of the femur and the tumor was measured from 1 h before to 8 h after the administration of 200 mg/kg FAA, in 4 tumor-bearing mice.

Measurement of Tumor Temperature. The tumor temperature was measured in mice treated with hyperthermia, given alone or in combination with FAA. FAA, 200 mg/kg, was given 2 h before the initiation of hyperthermia, and a needle probe (Type NST, Shibaura Electronics Co., Tokyo, Japan) attached to a thermometer (MGA III-219, Nihon Kohden Co., Tokyo, Japan) was inserted immediately before hyperthermia. The temperature was recorded, in 4 tumor-bearing mice in each group, every 2.5 min during the heating phase and every 1 min during the cooling phase.

Statistical Analysis. Differences in data were analyzed by Student’s t test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect on Colony Formation. The combined effects of FAA and hyperthermia, expressed as the surviving fraction, are shown in Fig. 1. FAA alone was not cytotoxic when the concentration was below 100 μg/ml, and the suppression of colony formation by FAA over 100 μg/ml was minimal. Heat at 43°C alone, when the concentration of FAA was 0, suppressed clonogenicity, but hyperthermia for 30 min did not affect the cytotoxicity of FAA. Hyperthermia for 60 min enhanced the cytotoxicity of FAA only when the concentration of FAA exceeded 100 μg/ml.

Effect on B16 Melanoma Solid Tumor. (a) When the effect of a single administration of FAA was evaluated, FAA (100–200 mg/kg) inhibited tumor growth in a dose-dependent manner (Fig. 2) and induced a delay in tumor growth by 1.7 days with a dose of 100 mg/kg, 2.4 days with 150 mg/kg, and 3.2 days with 200 mg/kg. The mice tolerated these doses of FAA well and there was no obvious toxicity. (b) The combined effects of FAA (200 mg/kg) and hyperthermia, given concomitantly, were also observed. While tumor growth was inhibited by either FAA or hyperthermia alone, the inhibition was greatest when FAA and hyperthermia were combined (Fig. 3). The combination treatment significantly delayed tumor growth time (P < 0.01) compared with findings in other treatment groups (Table 1). Tumor growth delay was 1.9 days by hyperthermia, 3.3 days by FAA, and 7.4 days by the combined modality. Mean body weight on day 10 in the combination group decreased slightly,
FAA INCREASES HYPERTHERMIC EFFECT

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor growth time (day)*</th>
<th>Body wt change (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.4 ± 0.9</td>
<td>98.6</td>
</tr>
<tr>
<td>Hyperthermia</td>
<td>5.3 ± 0.9</td>
<td>99.0</td>
</tr>
<tr>
<td>FAA</td>
<td>6.7 ± 1.6</td>
<td>99.5</td>
</tr>
<tr>
<td>FAA + hyperthermia</td>
<td>10.8 ± 1.9</td>
<td>95.2</td>
</tr>
</tbody>
</table>

* Mean ± SD of tumor growth time required to reach 3 times the initial tumor volume in each group.
‡ The mean body weights on day 10, expressed as a percentage of that on day 0 in each group.
§ Different from control group (P < 0.01).
¶ Different from all other groups (P < 0.01).

DISCUSSION

In the B16 melanoma cells we used, we found that FAA was cytotoxic with a 60-min exposure at 37°C but only at concentrations over 100 μg/ml, and that when combined with hyperthermia at 43°C for 30 min there were no effects on cytotoxicity of FAA in vitro. In vivo studies on mice showed that the concentration of FAA achieved in tumors is lower than needed for direct cytotoxicity in vitro (24). However, we found that FAA plus hyperthermia for 15 min inhibited the growth of B16 melanoma solid tumors in vivo. The mechanism of the combined effects we observed is probably not a direct interaction of FAA and hyperthermia; rather, FAA may reduce tumor blood flow, thereby increasing the effect of tumor cell killing by hyperthermia.

A reduction in tumor blood flow would support the unique features of actions of FAA. Some investigators noted a reduction in tumor blood flow and stated that it was a main determinant in the antitumor activity of FAA (15–17). The mechanism of this reduction of tumor blood flow may be related to a passive steal phenomenon with increased perfusion of normal tissues (15), or to vascular occlusion following intravascular coagulation (25), or to vascular failure by induction of tumor

but with no statistical significance. (c) Differences in the combined effects relating to the timing of administration of FAA and hyperthermia were also investigated. Relative tumor volumes on day 10 of each group treated with FAA and hyperthermia at various times are shown in Fig. 4. In groups treated with FAA before or concomitantly with hyperthermia, there was a more prominent inhibition in growth of the tumor, compared to findings of groups in which FAA was administered after hyperthermia. The antitumor effect was most remarkable when FAA was administered 2 or 4 h before hyperthermia, with a significant difference between these groups and the groups treated with FAA after hyperthermia (P < 0.01).

Change in Tissue pH. Change in tissue pH was noted when FAA (200 mg/kg) was administered to tumor-bearing mice (Fig. 5). The mean initial pH of the muscle before FAA treatment was 7.27 and that of the tumor was 7.07. pH of the muscle remained unchanged in the presence of FAA; however, pH of the tumor decreased immediately after FAA administration and remained at about 6.8 for 4 h before gradually returning to base-line values. FAA selectively reduced pH of the tumor (P < 0.05), but not that of the normal tissue.

Change in Tumor Temperature. Change in temperature of the B16 melanoma solid tumor is shown in Fig. 6. During the heating phase, the temperature of tumor in the FAA group was significantly higher than that in the control group (P < 0.05). During the cooling phase, there was no statistical difference. FAA increased the heating efficiency of hyperthermia.

FAA increases the cytotoxic effect of hyperthermia in B16 melanoma cells, with the most significant effect observed when FAA was administered 2 or 4 h before hyperthermia.
necrosis factor (26). However, vasoactive agents inducing passive steal phenomenon (e.g., misonidazole) or drugs inducing tumor blood flow failure (e.g., hydralazine) do not have the antitumor activity seen with FAA, in a single use. Thus, we suggest that various actions of FAA are involved in the antitumor effects of FAA.

Although solid tumors contain hypoxic fractions, the pH of which is relatively low due to an elevated level of lactic acid production and its subsequent inadequate removal (27), reduction of tumor blood flow makes the tumor more hypoxic and acidic. Previous studies showed that hypoxia and especially acidity increased the cytotoxicity of hyperthermia. Overgaard (19) and Overgaard and Bichel (20) reported that lysosomal activity is accelerated with low pH and this lysosomal intensity and a heat-induced cell membrane damage are important events for the increased sensitivity to heat. Edwards et al. (28) showed that FAA caused a reduction in tumor blood flow and increased hypoxia of the tumor. We found that FAA selectively reduced the pH of tumor, but not the muscle pH, probably as a result of reduced tumor blood flow. FAA seems to increase the thermosensitivity of B16 melanoma solid tumor by reducing tumor blood flow with a resultant tumor acidity. Experiments on the timing of FAA administration in relation to hyperthermia showed that the combined effects in inhibiting tumor growth were evident when hyperthermia was given after FAA administration and were most prominent when FAA was administered 2 or 4 h prior to hyperthermia. These results are consistent with the findings that FAA reduced the B16 melanoma tumor pH for 4 h. We assume that the pH, decreased by FAA, plays an essential role in the interaction of FAA and hyperthermia, and the timing of treatments is important to get remarkable antitumor effects. Although the data in Fig. 3 showed that the combined effects of FAA and hyperthermia which were given concomitantly were considered to be additive, the effects were increased further by giving FAA before hyperthermia. FAA should be able to act synergistically with hyperthermia by establishment of the best timing of treatment.

In addition to a reduction in tumor pH, pretreatment with FAA rapidly elevated the temperature in the tumor during heating, perhaps as a result of a decreased cooling effect by the circulation, secondary to the FAA-induced reduction of tumor blood flow.

In conclusion, FAA enhances the antitumor effects of hyperthermia on solid tumors in vivo by increasing thermosensitivity of the tumor following a selective decrease in the tumor pH, and by increasing the heating efficiency of hyperthermia following a reduction of blood flow to the tumor. The combined treatment of FAA and hyperthermia warrants further study for possible treatment of subjects with solid tumors.

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