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Potentiation of the cytotoxicity of carboquone by flavone acetic acid combined with hyperthermia

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Flavone acetic acid (FAA) has shown the effectiveness of vasoactive drugs in the selective reduction of tumor blood flow. A FAA-mediated decrease in tumor blood flow may produce sufficient hypoxic conditions within the tumor. Carboquone (CQ), a naturally occurring prototype bioreductive alkylating agent like mitomycin C (MMC), has been shown to be selectively more cytotoxic toward hypoxic tumor cells. We have reported enhancement of the combined antitumor effects of MMC plus FAA and hyperthermia (HT). In this study, we examined the combined effects of FAA, CQ and HT. *In vitro*, although HT (43°C, 60 min) reduced the colonogenicity to 0.58 in CQ (0.01 μg/ml) alone, the combined cytotoxicity of CQ and HT was not enhanced with exposure to FAA at a concentration of 100 μg/ml. *In vivo*, the tumor growth time, calculated as the time required to reach twice the initial tumor volume, for CQ (2 mg/kg) alone, FAA (150 mg/kg) alone, CQ+FAA, CQ+HT (43°C, 15 min), FAA+HT and FAA+CQ+HT was 6.1, 5.1, 7.1, 8.0, 7.6 and 13.4 days, respectively. A significant enhancement of antitumor effects by trimodality therapy with CQ, FAA and HT was observed, when compared to the treatment with CQ and FAA ($p < 0.05$). The possible mechanisms of an increased antitumor response achieved with the combination of CQ, FAA and HT may be explained in the following way: the FAA-mediated decrease in tumor blood flow produced sufficient hypoxic conditions within the tumor, and these resulted in a significant increase of the antitumor effects of CQ and HT.

Key words: Flavone acetic acid, carboquone, hyperthermia, tumor blood flow.

Introduction

Several vasoactive drugs that can selectively reduce the tumor blood flow have been reported to enhance the antitumor effects of hypoxic-targeted anticancer drugs and hyperthermia (HT) against a variety of tumors.[1,2] Reduction of tumor blood flow may be potentially useful for enhancing the antitumor effects of such anticancer drugs and HT.

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Flavone acetic acid (FAA) has been an effective anticancer drug in rodent models and has also shown the same effectiveness as vasoactive drugs in the selective reduction of tumor blood flow. The phase I clinical trial of FAA was done safely,[3,4] and was found to induce serum interferon and natural killer cell activity.[5] FAA differs from most cancer chemotherapeutic agents in that it has no antimetabolic properties and that it induces greater antitumor effects in solid tumors than in cells *in vitro* or in disseminated lymphomas or leukemia.[6] Although the mode of action of FAA is still unclear, tumor vascular disruption by administration of FAA has been shown to be largely attributable to its cytotoxic action toward solid tumors[7] and an FAA-mediated decrease in tumor blood flow was supposed to produce sufficient hypoxic conditions within the tumor.

Carboquone (CQ), a naturally occurring prototype bioreductive alkylating agent like mitomycin C (MMC), has been used to treat cancer patients in Japan,[8] and has better or equal therapeutic potential against human gastric and lung carcinoma, when compared to MMC.[9,10] The cytotoxicity of CQ was increased in combination with HT and was shown to be selectively more cytotoxic toward hypoxic tumor cells.[11,12]

HT has been used to treat various kinds of malignancy, locally[13] and systematically[14] in clinical situations, and would appear to be effective in the treatment of solid tumors, especially when used in combination with other treatment modalities, such as radiation[15] and/or chemotherapy,[16,17] including CQ[18] and FAA.[19] The antitumor effects of HT are greatly enhanced by hypoxic conditions.[20]

We have previously reported the enhancement of the combined antitumor effects of MMC plus FAA and HT.[21] In the present study, since CQ and HT are effective in hypoxic cell killing, we investigated whether the antitumor effects of CQ could be enhanced against B16 melanoma cells in combination with HT in an FAA-induced hypoxic state; here we

discuss the potential mechanism of the enhanced cytotoxicity of this trimodality treatment.

Materials and methods

Drugs

CQ was obtained from Sankyo Pharmaceutical (Tokyo, Japan). FAA was kindly supplied by Professor RA Newman (Department of Clinical Investigation, MD Anderson Cancer Center, Houston, TX). CQ was dissolved in Hank's solution and FAA was dissolved in physiological saline immediately prior to use at the appropriate concentration.

Mice

C57BL/6NCrj male mice (5 weeks old) were obtained from Charles River Japan (Tokyo, Japan), and were housed under constant temperature and humidity conditions. The mice were housed six per cage in a controlled environment with a 12 h light/dark cycle, and fed a diet of standard laboratory chow and allowed access to water. An environmental adaptation period of 1 week was allowed prior to the use of these animals for experimentation.

Tumor

B16 melanoma cells (obtained from Dr S Taniguchi, Medical Institute of Bioregulation of Kyushu University, Fukuoka, Japan) were cultured in monolayers on 60 mm plastic dishes (Corning 25010; Iwaki Glass, Tokyo, Japan) using Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell survival assay

Cell survival assay was carried out using the colony formation method. Three hundred cells were plated in 60 mm dishes in the absence of the drug and were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were exposed to various concentrations of drugs for 60 min. In combination with HT, cells were incubated at 43°C for 60 min, simultaneously. After treatment, cells were washed 3 times with phosphate-buffered saline and incubated in a fresh medium for 7 days. Colonies of each group were stained with Giemsa solution and counted. A colony composed of more than 50 cells was considered as one colony. The effectiveness of treatment was evaluated by the rate of inhibition of colony formation. All experiments were independently done at least 3 times.

Treatment of B16 melanoma solid tumor

B16 melanoma cells were transplanted by s.c. injection of 5×10^5 tumor cells in a volume of 0.05 ml of Hank's solution on the lower part of the thigh. Tumors grew to 7 mm in diameter within 7–9 days after tumor innoculation. All experiments were performed with tumors of this size. Each group included six or seven tumor-bearing mice. In combination experiments, drugs were administered just prior to HT, which was induced by immersing the tumor-bearing lower part of the mouse thigh into a circulating water bath (Model T-10; Thermonics, Tokyo, Japan) at 43°C for 15 min.

The mean tumor volume of each group at the time of treatment on day 0 was similar. Control groups of mice received saline instead of drugs. Tumor size was measured with a digital caliper (DP-1 HS; Mitutoyo, Tokyo, Japan) every second day following treatment. Tumor volume was determined from measurements of two perpendicular diameters using the following formula:[22] tumor volume=$1/2 \times$ length $\times$ $(\text{width})^2$.

Relative tumor volume, tumor growth time (TGT) and tumor growth delay (TGD) were used to evaluate the anti-tumor effect. Relative tumor volume was expressed as the ratio of tumor volume at each treatment day to the initial tumor volume on day 0. TGT was calculated as the time required to reach twice the initial tumor volume. TGD was calculated by subtracting the TGT of the control tumor from that of treated tumors. Body weight was measured and macroscopic toxicity such as diarrhea or nasal and urogenital bleeding was carefully examined.

Statistical analysis

Statistical differences in data were analyzed by Student's *t*-test, where p values less than 0.05 were considered to be significant.

Results

Effect on colony formation

The survival fractions of exponentially growing B16 cells exposed to CQ and FAA at 37 or 43°C are shown

in Figure 1(A and B, respectively). HT (43°C, 60 min) reduced the colongenicity to 0.58 in CQ (0.01 μg/ml) alone. The combined cytotoxicity of CQ and HT was not enhanced with exposure to FAA at a concentration of 100 μg/ml.

Effect of B16 melanoma solid tumors

Tumor growth curves after treatment with FAA (150 mg/kg) alone or in combination with CQ (1 or 2 mg/kg) with or without HT (43°C, 15 min) are

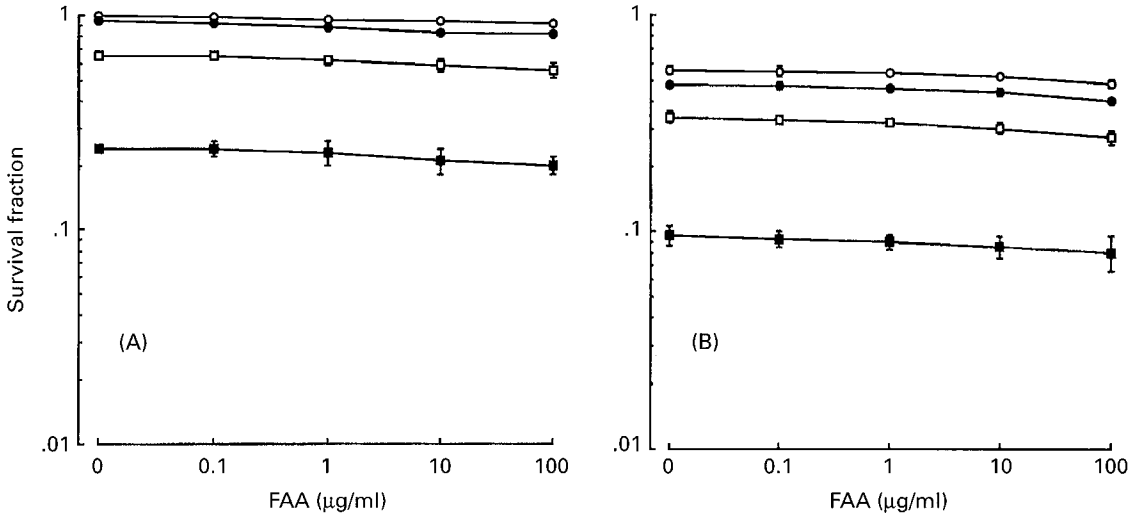

Figure 1. Cytotoxicity of FAA combined with CQ on inhibition of colony formation of B16 melanoma cells at 37°C (A) and at 43°C (B). The concentrations of CQ were: 0 μg/ml (○), 0.01 μg/ml (●), 0.05 μg/ml (□) and 0.1 μg/ml (■). Data are presented as means ± SD from three experiments.

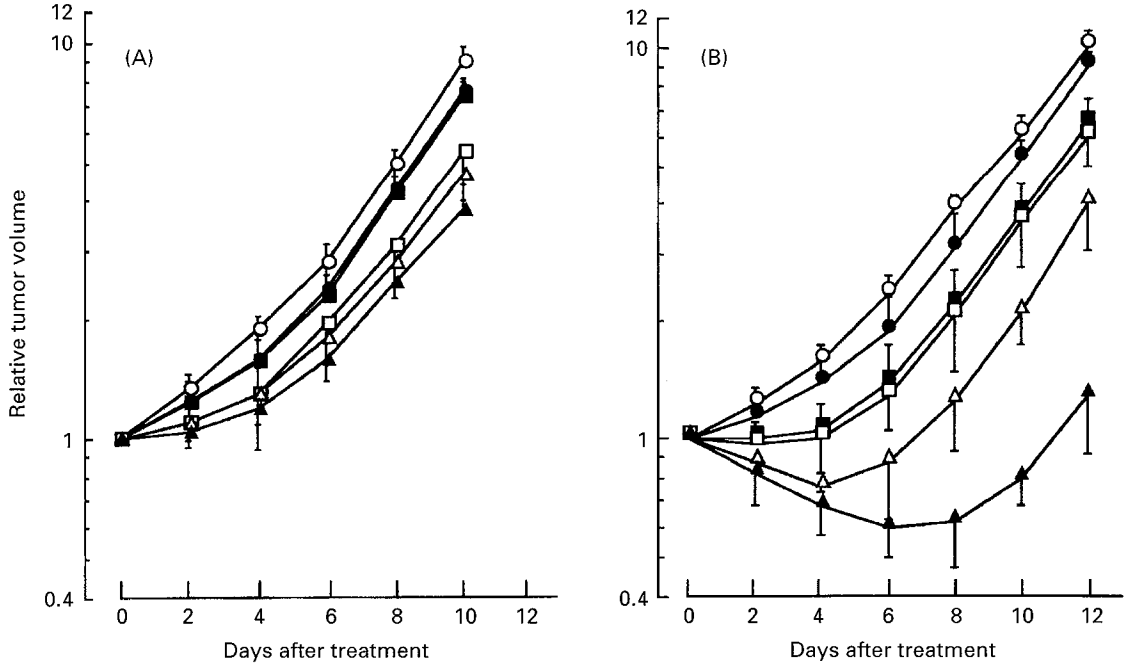

Figure 2. The growth curves of B16 melanoma tumors in C57/6NCrj male mice treated with various combinations of CQ and/or FAA under normothermic (A) or hyperthermic conditions (B). Relative tumor volume was expressed as the ratio of tumor volume on every second day to initial tumor volume at time of treatment. Symbols: control (○), 1 mg/kg CQ (●), 2 mg/kg CQ (□), 150 mg/kg FAA (■), 1 mg/kg CQ+150 mg/kg FAA (△) and 2 mg/kg CQ+150 mg/kg FAA (▲). Data are presented as means ± SD of tumor measurements from seven mice.

Table 1. Treatment group, TGT, TGD and body weight changes after treatment with FAA, CQ and HT, either alone or in combination

Treatment	TGT (days)[a]	TGD (days)[b]	Body weight change[c] (%)
NaCl	4.3±0.5	0	
CQ (1 mg/kg)	5.0±0.5	0.7	98.3
CQ (2 mg/kg)	6.1±0.7	1.9	97.3
FAA (150 mg/kg)	5.1±0.5	0.8	102.3
CQ (1 mg/kg)+FAA (150 mg/kg)	6.5±1.0	2.2	99.5
CQ (2 mg/kg)+FAA (150 mg/kg)	7.1±1.2	3.8	96.8
NaCl+HT (43°C, 15 min)	5.2±0.3	0.9	98.7
CQ (1 mg/kg)+HT (43°C, 15 min)	6.2±0.5	2.0	101.2
CQ (2 mg/kg)+HT (43°C, 15 min)	8.0±0.9	3.8	99.5
FAA (150 mg/kg)+HT (43°C, 15 min)	7.6±0.6[d]	3.3	98.1
CQ (1 mg/kg)+FAA (150 mg/kg)+HT (43°C, 15 min)	9.8±1.4[d]	5.5	97.2
CQ (2 mg/kg)+FAA (150 mg/kg)+HT (43°C, 15 min)	13.4±1.4[d]	9.2	96.0

[a] TGT was calculated as time required to reach a tumor volume twice that of the initial tumor volume.
[b] TGD was calculated by subtracting the tumor growth time of the control group from that of the treated group.
[c] The mean body weights on day 7, expressed as a percentage of that on day 0 in each group.
[d] Significant differences resulting from the same dosage without hyperthermia ($p<0.05$).

shown in Figure 2. The inhibition was the greatest when FAA (150 mg/kg) plus CQ (2 mg/kg) in combination with HT were combined. TGT data for FAA and CQ with or without HT are summarized in Table 1. TGT was 6.1±0.7, 5.1±0.5 and 7.1±1.2 days in CQ (2 mg/kg) alone, FAA (150 mg/kg) alone and CQ (2 mg/kg) plus FAA, respectively. In combination with HT, TGT was 8.0±0.9, 7.6±0.6 and 13.4±1.4 days in CQ (2 mg/kg) plus HT, FAA plus HT and CQ (2 mg/kg) plus FAA with HT, respectively. The combination treatment of FAA plus HT, FAA plus CQ (1 mg/kg) with HT and FAA plus CQ (2 mg/kg) with HT significantly delayed tumor growth time ($p < 0.05$). The greatest enhancement of the antitumor effect of CQ was observed in combination with FAA and HT. Mean body weight at 7 days after treatment in all treated groups revealed no significant difference from that of the control group. The mice tolerated these combined modality therapies of FAA plus CQ with HT well and there was no obvious toxicity or acute death.

Discussion

The presence of hypoxic cells in a solid tumor is a major problem in cancer chemotherapy, because these hypoxic cells are relatively resistant to chemotherapeutic agents.[23] In this study, we regard this disadvantageous hypoxia as a possible route for planning tumor-specific therapies, since hypoxic cells in a solid tumor are sensitive to bioreductive drugs such as CQ. Indirect cancer therapy via vascular-mediated cell death such as our proposed chemo-hyperthermia treatment protocol appears to be a feasible and attractive approach.

Vascular disruption in tumors has been shown to be attributable to the cytotoxic action of FAA toward solid tumors[7] and, in this experiment system, we have already reported that administration of FAA can reduce tumor blood flow without reducing blood flow to normal tissues.[21] Several proposed mechanisms of FAA-induced reduction of tumor blood flow have been reported. These include vascular failure by induction of a tumor necrosis factor,[24] an increase in intravascular coagulopathy,[25] a change in endothelial barrier function leading to increased vascular permeability[26] and an alteration of platelet function by inhibition of platelet adhesion.[27]

In hypoxic conditions, the cell-killing effect of HT was known to be enhanced, when compared to aerobic conditions,[28] because of the combined effects of acidosis-induced primary activation of lysosomal activity and heat-induced cell membrane damage.[29,30] The cytotoxicity of CQ has also been enhanced toward a hypoxic condition.[12] CQ has three biologically active groups, aziridinyl, carbamoyloxy and quinonyl, and requires reductive transformation. Its chemical structure is similar to that of MMC,[31,32] which was enhanced toward hypoxic subpopulations within solid tumors by requiring anaerobic and NADPH-generating systems.[33] This structural resemblance is reminiscent of the bioreductive activation of CQ, especially in hypoxic cells.

Our results clearly demonstrated that the cytotoxicity of CQ was significantly enhanced by administration of FAA in combination with HT in *in vivo* tumor systems but not in *in vitro*. TGD in the treatment of CQ and FAA was greater than that achieved with either therapy alone. This may have resulted from CQ being trapped and maintained within the tumor. The reduced tumor blood flow produced by the administration of FAA tended to optimize the metabolism of CQ in active cytotoxic species and enhance cell killing under FAA-mediated hypoxia. In combination with HT, since HT is known to diminish tumor vascular volume and blood flow,[34,35] the temperature of tumors was increased as a result of a decrease of the cooling effect, and the tumor cells became more hypoxic than with FAA-mediated hypoxia alone, because of decreasing both oxygen supply and lactic acid removal; nevertheless the FAA-induced reduction of tumor blood flow was transient.[21] Under these circumstances, the cytotoxicity of CQ was significantly enhanced, when compared to the treatment of CQ and FAA.

The combination of FAA plus CQ with HT might be uniquely synergistic, as well as the combination of FAA plus MMC and HT. The enhanced antitumor efficacy of the combined treatment may represent a novel approach to selective therapy in human solid tumors including hypoxic subpopulations.

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