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Flavone Acetic Acid Induced Changes in Human Endothelial Permeability: Potentiation by Tumour-conditioned Medium

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Flavone acetic acid (FAA) causes significant regression of larger established tumours in murine *in vivo* systems. This *in vivo* effect of FAA has been shown to include a vascular component. In an effort to elucidate the mechanism of action of FAA, we have studied the effects of FAA on the permeability of human endothelium *in vitro*. Monolayers of human umbilical vein endothelial cells (HUVEC) grown on polycarbonate filters were incubated in 1 mg/ml FAA for 120 min at 37°C. During the first 60 min, there was a 6–8-fold increase in permeability; this was followed by a return to control levels even in the continued presence of FAA. In contrast, in the presence of tumour conditioned medium, FAA caused a rapid 6-fold increase in permeability which did not subsequently return to control levels. The permeability changes which occurred under the latter conditions were accompanied by a rapid contraction of the cytoskeleton. The permeability of monolayers of human melanoma cells was unaffected by FAA.
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INTRODUCTION

FLAVONE ACETIC ACID (2-phenyl-8-(carboxymethyl) benzopyran-4-one) (FAA) is an antitumour agent which has undergone clinical trials in Europe and the USA. Although it causes significant regression of larger, solid tumours in mice, it has little or no effect on ascites tumours, leukaemias, small lung nodules and human tumour xenographs in nude mice [1–6]. In addition, *in vitro* studies have shown that high concentrations of FAA or long exposure times are necessary for direct cytotoxicity (cell death) to occur [7, 8]. This evidence strongly suggests that the anti-tumour effects of FAA are indirect and may include vascular and immunological components [5, 9, 10].

FAA activity in mouse tumours is dependent upon an established tumour vasculature [11] and manifests itself as a rapid onset of haemorrhagic necrosis together with a rapid reduction in tumour blood flow [12–17]. FAA has also been shown to reduce plasma coagulation times *ex vivo* and induce endothelial procoagulant activity *in vitro* [18, 19], and to cause a rapid increase in permeability in tumours and livers of tumour-bearing mice [20, 21]. These vascular effects of FAA imply that the endothelium, particularly that of tumours, may be a primary target for this agent.

Although results from clinical trials have thus far indicated poor antitumour activity for FAA when given as a single agent]22–25], FAA continues to generate interest due to its novel mode of action. The present study investigates, *in vitro*, the effects of FAA on the permeability of human endothelial monolayers and the influence of factors secreted by tumour cells on these permeability changes.

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MATERIALS AND METHODS

Cells

Human umbilical vein endothelial cells (HUVEC) were isolated according to the method of Jaffe *et al.* [26]. Cells from each cord were cultured separately on tissue culture grade plastic-ware coated with 0.2% gelatin. They were maintained in medium M199 supplemented with 5% pooled human serum, 15% fetal calf serum, 15 μg/ml endothelial cell growth supplement (Boehringer Mannheim) and 90 μg/ml heparin (Sigma), and used from first to third passage. The cells were characterised as endothelial by staining positively for the presence of von Willebrand factor, and positively with lectin from *Ulex europaeus*. Each experiment was carried out with cells originating from a single cord. Human melanoma cells (RPMI-7951), purchased from the American Type Culture Collection, were maintained in Eagle's minimal essential medium supplemented with 1% non-essential aminoacids, 1% sodium pyruvate and 10% fetal calf serum, and were used from passage 24 to 35. Tumour conditioned medium was collected from confluent cultures of these cells.

Cytotoxicity studies

The effect of 1 mg/ml FAA on the survival of both HUVEC and melanoma cells was measured using a limiting dilution method suitable for anchorage dependent cells of a migratory nature which do not form compact colonies [27]. HUVEC were plated at 7.5×10^5 cells onto 5 cm diameter fibronectin coated Petri dishes; 18 h later, the cells had formed a confluent monolayer. The monolayers were then incubated at 37°C in fresh HUVEC medium or 100% tumour conditioned medium with or without FAA for 120 min. The cells were then washed free of FAA and trypsinised. After counting, cells were plated into fibronectin coated 96-well multiwell plates at 5, 2 or 0.5 cells per well. Three weeks later, cells were stained with 0.2% crystal violet in 70% ethanol: distilled water, and survival was calculated as described previously [27]. The survival of melanoma cells incubated in fresh medium containing 1 mg/ml FAA was meas-

ured as described for HUVEC except that 2, 1 or 0.5 cells per well were plated into non-fibronectin coated wells.

Permeability studies

For permeability studies 10^5 HUVEC were seeded on 6.5 mm diameter Transwell-COL (Costar Corp.) filter inserts, pore size 3 μm, coated with 3 μm/cm² bovine fibronectin (1 h at room temperature) in 24-well plates. Melanoma cells were seeded on uncoated filter inserts.

Briefly, this system consists of two compartments separated by a membrane on which the cell monolayers are grown. Growth medium is present in both the luminal (insert) and abluminal (well) chambers. After 4 h incubation at 37°C under 5% CO_2/95% air, non-attached cells were removed. After a further 18 or 42 h incubation, the filters were examined microscopically to ensure growth was confluent. The filter inserts were then incubated in FAA (supplied by Lipha Pharmaceuticals) at 0.5 or1.0 mg/ml in full growth medium at 37°C for a maximum of 120 min. HUVEC were incubated in fresh M199 medium or Eagle's medium conditioned by the melanoma cells, and melanoma cells were incubated in fresh Eagle's medium. The permeability of the monolayers was assayed at timed intervals by removing FAA from the filter insets and replacing it with medium containing 0.8 mg/ml fluorescein isothiocyanate conjugated dextran (average molecular weight 20 000) and FAA. After 10 min incubation at 37°C, aliquots were removed from the wells and fluorescence measured using a LS-5 fluorescence spectrometer (Perkin-Elmer Ltd). Control filters were treated the same way, except FAA was absent. All experiments were carried out in the absence of pressure gradients and all solutions used were pre-warmed to 37°C. Sufficient early passage cells from each individual cord were available to enable two filters to be used for each time point per experiment. This small number of filters for each data point precluded statistical analyses of each time point within individual experiments; however, each experiment was repeated a number of times.

Cytoskeletal studies

Cells grown on glass cover-slips until almost confluent were incubated at 37°C in the presence or absence of 1 mg/ml FAA. At timed intervals, cover-slips were washed twice with Dulbecco's phosphate buffered saline (PBS), fixed in 3% formaldehyde for 20 minutes at room temperature and washed with PBS. The cells were then permeabilised with 0.1% Triton X-100 in PBS for 3.5 min, washed in PBS and stained with 55 μmol/ml rhodamine phalloidin (phalloidin–TRITC, Sigma), which is specific for F-actin filaments of the cytoskeleton, for 40 min. After thorough rinsing in PBS, the cover-slips were mounted cell side down in a 1:1 solution of glycerol and PBS containing 1 mg/ml *o*-phenylenediamine [28]. Photomicrographs were taken using a Nikon Optiphot microscope equipped with epi-fluorescence, filters for rhodamine excitation and emission, and Kodak Ektachrome 400 ASA film.

RESULTS

Cytotoxicity studies

Using colony formation as the endpoint, 1 mg/ml FAA was not cytotoxic to HUVEC or melanoma cells under any of the experimental conditions tested. The surviving fractions measured are shown in Table 1. Those for HUVEC under test conditions were not significantly different from the control ($P > 0.45$); nor were the two surviving fractions for melanoma cells significantly different from each other ($P > 0.13$).

Table 1. Surviving fractions for HUVEC and melanoma cells after 120 min incubation in FAA

	Medium			
	Fresh		Tumour conditioned	
Cells	Control	1 mg/ml FAA	Control	1 mg/ml FAA
HUVEC	0.13 (0.06) $n = 15$	0.17 (0.1) $n = 6$	0.10 (0.02) $n = 3$	0.12 (0.05) $n = 6$
Melanoma	0.44 (0.05) $n = 8$	0.40 (0.05) $n = 4$	—	—

Mean (S.D.).

Permeability studies

The present experiments were designed to measure changes in the permeability of cell monolayers using the tracer molecule dextran–fluorescein isothiocyanate (FITC). The passage of dextran-FITC through both seeded and unseeded filters was linear with time for a period of 20 min (Fig. 1). The percentage of dextran–FITC passing through HUVEC monolayers in 10 min was mean (S.D.) 0.28 (0.18%) ($n = 6$) and for melanoma monolayers 1.94 (0.62%) ($n = 24$). For filters without cells, either coated with fibronectin or uncoated, the values were 1.83 (0.48%) ($nn = 6$) and 2.61 (0.51%) ($n = 8$), respectively. The data indicate that HUVEC provide a tight barrier against diffusion of dextran–FITC, whilst melanoma cells are relatively leaky.

The permeability of cell monolayers was estimated as the ratio of fluorescence in the well to the amount of initial fluorescence added to the insert. The value for cells treated with FAA was then compared to the value for untreated control cells.

Results from typical experiments with HUVEC incubated in 1 mg/ml FAA are shown in Figs 2a and b and 3a. During the first 60 min, there is an increase in permeability followed by a return to control levels, even in the continued presence of FAA,

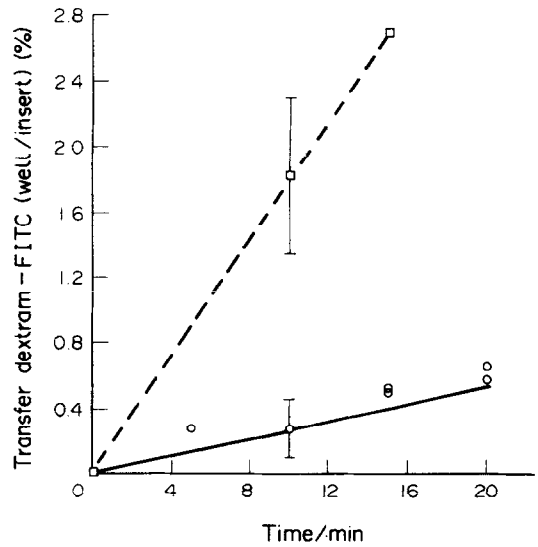

Fig. 1. Passage of dextran–FITC through fibronectin coated filters. □ unseeded control filters, ○ HUVEC seeded filters. Individual data points shown except at 10 minutes, where the means (S.D.) ($n = 6$) are shown.

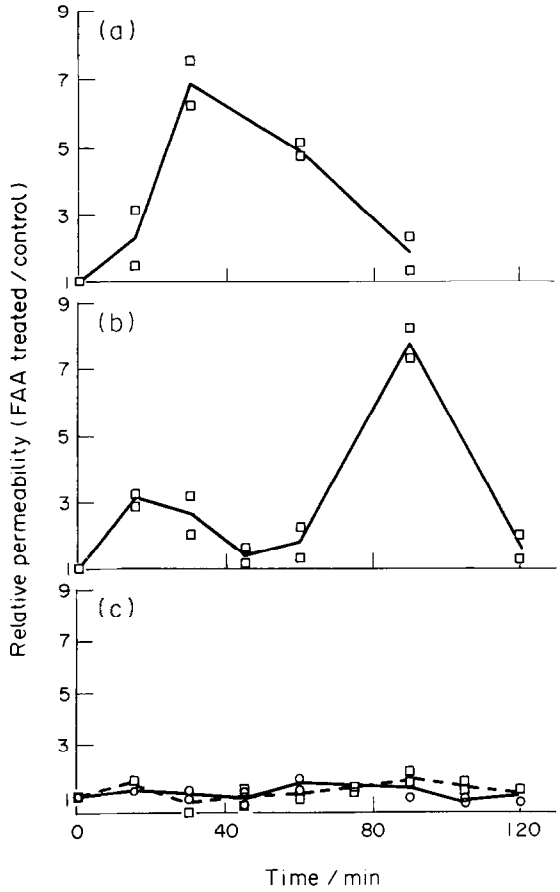

Fig. 2. Time course for the permeability of cell monolayers incubated in 1 mg/ml FAA, (a) and (b) time course for HUVEC, (c) time course for melanoma cells. Permeability is expressed as the percentage of dextran–FITC accumulating in the abluminal (well) chamber to the amount of dextran–FITC added to the luminal (insert) chamber. The values for cells treated with FAA compared with the value for untreated control cells are then expressed as a ratio.

drug treated monolayers being 6–8-fold more permeable than untreated control monolayers. In some cases, there was a second increase in permeability (Fig. 2b). In contrast, the permeability of monolayers of melanoma cells was not affected by FAA (Fig. 2c). The effect of tumour conditioned medium on the permeability of HUVEC monolayers is shown in Fig. 3. Conditioned medium alone (100%) had no effect on permeability (data not shown); however, the presence of 1 mg/ml FAA (Fig. 3a) caused a rapid 6-fold increase in permeability, which remained at this level for the duration of the experiment. The lower concentration of 0.5 mg/ml FAA had a small effect on the permeability of HUVEC monolayers in normal medium and only a modest enhancement of effect in the presence of tumour conditioned medium was observed (Fig. 3b). Table 2 summarises results from repeat experiments showing the long lasting increase in permeability seen in the presence of FAA and tumour conditioned medium. In general, at 20 min the ratio of relative permeability in tumour conditioned medium vs. normal growth medium is low, as FAA increases the permeability in both media. At longer times, permeability in tumour conditioned medium remains elevated, whereas that in normal growth medium returns to control levels, resulting in an increase in the ratio for the two media.

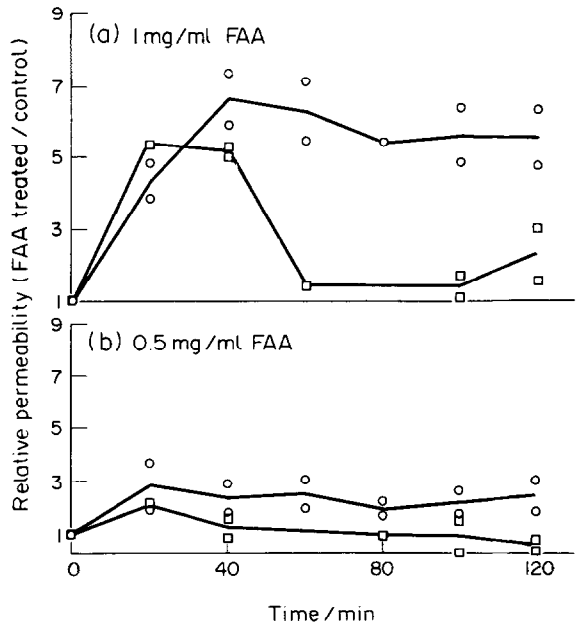

Fig. 3. Time course for the permeability of HUVEC monolayers incubated in normal growth medium or tumour conditioned medium containing FAA. (a) Cells incubated in 1 mg/ml FAA, (b) cells incubated in 0.5 mg/ml FAA, □ normal growth medium, ○ tumour conditioned medium. Individual data points are shown.

Cytoskeletal studies

For clarity, HUVEC monolayers were treated before reaching complete confluency, hence a small number of gaps in the monolayers can be seen in the photomicrographs (Fig. 4). After 120 min incubation, control HUVEC in either HUVEC medium or tumour conditioned medium showed a fine network of microfilaments extending across the cells and a denser staining band around the cell peripheries (Fig. 4a). The cytoskeleton of HUVEC incubated in normal medium was unaffected by 1 mg/ml FAA (Fig. 4b); however, in contrast, the cytoskeleton of HUVEC in tumour conditioned medium containing FAA showed a densely staining contracted cytoskeleton by 15 min, with no further changes observed during 120 minutes (Fig. 4c). Melanoma cells, although staining positively for the presence of

Table 2. Effect of FAA on relative permeability of HUVEC monolayers

		Time (min)		
Expt. no.	FAA (mg/ml)	20	60	100
1	1	4.33 : 5.39 (0.80)	6.30 : 1.43 (4.41)	5.65 : 1.44 (3.92)
2	1	3.27 : 1.83 (1.79)	3.12 : 2.18 (1.43)	2.26 : 1.64 (1.38)
3	0.5	5.91 : 3.83 (1.54)	5.29 : 1.69 (3.13)	3.07 : 0.58 (5.29)
4	0.5	2.76 : 2.07 (1.33)	2.50 : 1.09 (2.29)	2.13 : 0.89 (2.39)

Results expressed as ratio of permeability in tumour conditioned medium: permeability in normal control medium. Numbers in brackets represent ratio.

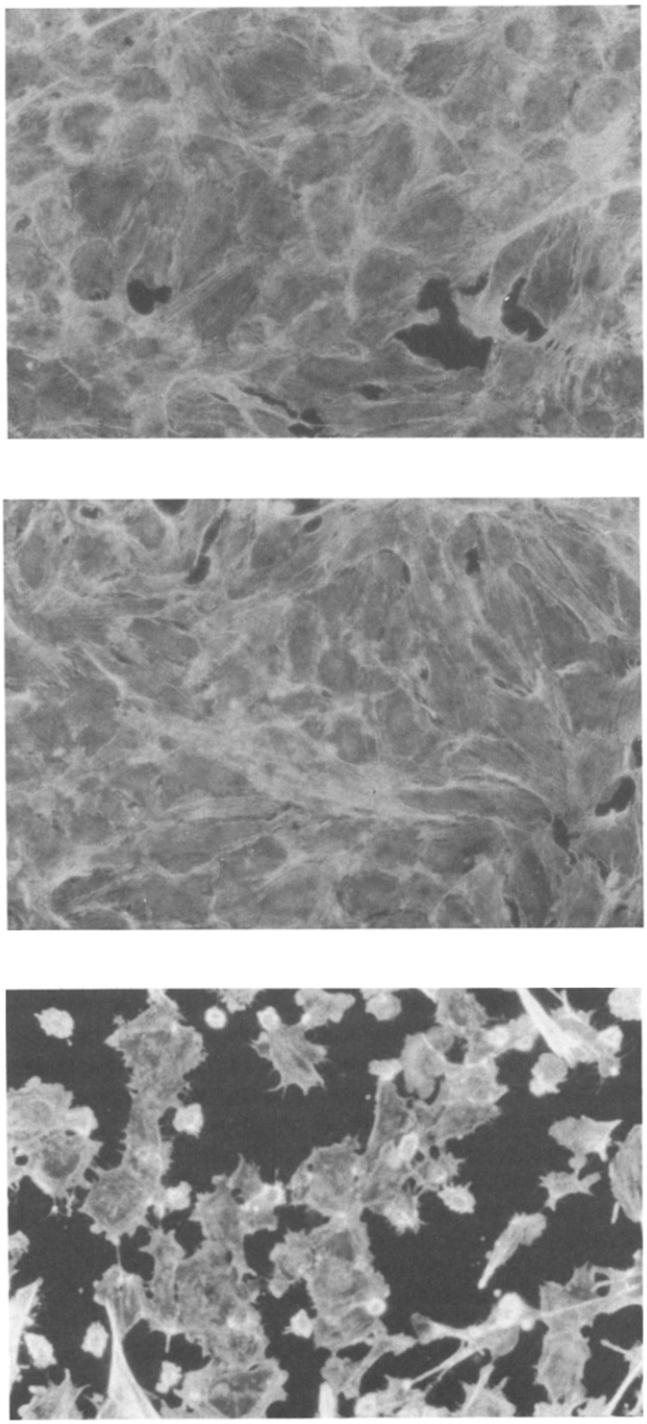

Fig. 4. HUVEC grown on glass coverslips and stained with rhodamine phalloidin to visualise the actin filament system after 120 min incubation in (a) fresh HUVEC medium, (b) fresh HUVEC medium containing 1 mg/ml FAA, and (c) tumour conditioned medium containing 1 mg/ml FAA.

F-actin, showed no distinct microfilaments and FAA had no observable effect on the cytoskeleton.

DISCUSSION

The absence of cell kill of HUVEC or melanoma cells by FAA over a period of 120 min is in agreement with previously published results which demonstrated that FAA had a significant effect on a murine tumour *in vivo*, but only limited toxicity to murine tumour cells and endothelial cells *in vitro* [7]. Earlier studies also showed lack of sensitivity *in vitro* compared to marked effects *in vivo* [3, 5]. Thus the permeability changes observed in the present study are most probably not due to endothelial cell death.

The present study demonstrates that FAA can increase the permeability of cultured HUVEC monolayers to the macromolecule dextran. This change may be due to the disruption of the relatively strong intercellular connections between HUVEC. Maintenance of the endothelial barrier has been correlated with the maintenance of the endothelial cytoskeleton in an extended conformation [29, 30]. Although endothelial cell monolayers formed on membrane filters such as those used in this study are more permeable than the endothelial layer within the vascular wall, they have provided a useful model for examining the response of the endothelium to FAA [31, 32]. The transient increase in permeability observed with HUVEC incubated in fresh medium containing FAA may have been due to a temporary modest disruption of the cytoskeleton, although little change was seen in the rhodamine phalloidin-staining pattern of the cytoskeleton. The cause of the variable time course of the response is unknown; however, it may be related to the characteristics of the individual cords used. In contrast, HUVEC incubated in tumour conditioned medium containing FAA showed an irreversible increase in permeability that was accompanied by cell retraction and a collapse or condensation of the cytoskeleton. These gaps between the cells provided a route for the passage of dextran across the monolayers.

This sub-lethal effect on endothelial permeability, which is enhanced by the presence of tumour conditioned medium, may relate to effects seen *in vivo*. FAA has been shown to cause a rapid increase in the retention of albumen in the livers and tumours of tumour-bearing mice: in contrast, no increase in albumen retention was seen in the livers of non-tumour-bearing mice [20]. In a similar manner, FAA induces a significant reduction in plasma clotting times which is more profound in tumour-bearing animals than those which are non-tumour-bearing [18]. Finally, FAA is more toxic to tumour-bearing mice than to those which are non-tumour-bearing [14, 33]. These studies suggest that the presence of a tumour may have a marked local and, in some cases, more distant effect on the vasculature, perhaps through the release of tumour-specific factors. Indeed, factors released by tumours have now been partly characterised which modify the procoagulant phenotype of the endothelial cell [34–37] as well as the permeability of tumour vasculature [38–41].

The FO-1 factor, isolated from human melanoma cell conditioned medium, enhances endothelial cell procoagulant activity [37]; this effect is potentiated by FAA, suggesting a possible mechanism whereby the anti-vascular action of FAA is localised to tumour blood vessels. Vascular permeability factor (VPF), a protein secreted by a variety of tumour cell lines, has diverse effects on endothelial cells, including local transient changes in endothelial permeability [38–41]. The effects of FAA we observed here on endothelial cultures pretreated with tumour-conditioned medium, might be attributable to the presence in that medium of a VPF-like substance whose activity is more fully expressed when in combination with FAA. An alternative explanation for this phenomenon is that a factor present in the tumour-conditioned medium inhibits endothelial cell "recovery" from the effects of FAA, perhaps by blocking reorganisation of the actin cytoskeleton.

Whatever their molecular basis, these observations may be physiologically significant, as, *in vivo*, rapid collapse after FAA

occurs only in tumours; few effects on normal tissue vasculature are seen, either in control or tumour-bearing mice. Therefore, while FAA may increase permeability throughout the systemic vasculature, recovery is rapid and the barrier function is restored; whilst in tumours barrier function is not restored, leading to irreversible vascular collapse. Finally, these data may suggest a novel application of FAA and similar agents, enhancing the delivery of conventional cytotoxins to tumour cells through this selective effect on endothelial barrier function.

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