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Changes in coagulation and permeability properties of human endothelial cells *in vitro* induced by TNF-α or 5,6 MeXAA

ME Watts, S Arnold and DJ Chaplin

Tumour Microcirculation Group, Gray Laboratory Cancer Research Trust, PO Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK.

Summary 5,6 dimethyl xanthenone acetic acid (5,6 MeXAA), an analogue of flavone acetic acid (FAA), has been shown to be more active against murine tumours than FAA. As both drugs have a vascular component in their mechanism of action similar to that observed for TNF-α, we have studied the effects of 5,6 MeXAA alone and in combination with TNF-α on endothelial function *in vitro*. The changes induced by the drugs on procoagulant activity and permeability were determined under tumour-simulated conditions of low oxygen tension and the presence of tumour-secreted factors. Procoagulant activity was assayed by measuring the time taken for human umbilical vein endothelial cells (HUVECs) to clot normal human plasma, increased activity resulting in reduced clotting times. HUVECs incubated under aerobic conditions were more sensitive to TNF-α than cells incubated at ≤0.2% oxygen. Culture medium conditioned by the human breast adenocarcinoma cell line MDA-MB-231 strongly upregulated procoagulant activity under both aerobic and hypoxic conditions; clotting times were further reduced by TNF-α. Both 5,6 MeXAA and FAA potentiated the effect of TNF-α on normal hypoxic endothelial cells; however, under all other conditions, neither drug in combination with TNF-α upregulated clotting activity. The presence of tumour-secreted factors had a far greater effect on upregulating procoagulant activity than did oxygen tension. In contrast to procoagulant activity, permeability was insensitive to TNF-α and low concentrations of 5,6 MeXAA also caused no change in permeability.

Keywords: hypoxia; tumour microenvironment; human umbilical vein endothelial cell; MDA MB 231 cell

It is well-known that tumour cells can produce factors which activate the coagulation cascade and alter endothelial permeability (Sun *et al.*, 1979; Rickles and Edwards, 1983; Collins *et al.*, 1993). In addition, it is well-established that the low oxygen levels found in solid tumours profoundly influence the outcome of many forms of therapy (Thomlinson and Gray, 1955; Freitas and Baronzio, 1991; Workman and Stratford, 1993). There is also indirect evidence from cryospectrophotometry studies that even endothelial cells within tumours may be subjected to periods of hypoxia (Mueller-Klieser *et al.*, 1981). The predominantly macrophage-produced cytokine tumour necrosis factor (TNF-α) is known to induce deposition of fibrin within tumour blood vessels, resulting in vascular occlusion, nutrient deprivation and haemorragic necrosis (Carswell *et al.*, 1975; Nawroth *et al.*, 1988).This observation stimulated interest in the potential benefit which might be obtained by deliberately inducing coagulation in tumours. However, although injecting TNF-α intravenously produces dramatic tumour shrinkage in many animal models (Haranaka *et al.*, 1984), clinical trials with TNF-α have been disappointing (Saks and Rosenblum, 1991). The synthetic flavonoid, flavone acetic acid (FAA), exhibits many similarities to TNF-α in terms of its anti-tumour activity. It causes a profound and rapid drop in blood flow in murine tumours (Bibby *et al.*, 1989; Hill *et al.*, 1989) and activates coagulation (Murray *et al.*, 1989). Evidence also exists that FAA induces the *de novo* synthesis of TNF-α *in vivo* and it has been suggested that the vascular effects of FAA *in vivo* are mediated through TNF-α (Mace *et al.*, 1990). Response rates to FAA in clinical trials have been disappointing when used either as a single agent (Kerr *et al.*, 1987; Olver *et al.*, 1992) or when combined with interleukin-2 (IL-2) (Holmlund *et al.*, 1995). Structure activity studies on compounds related to FAA showed 5,6 dimethyl xanthenone acetic acid (5,6 MeXAA) to be more effective by 10-15-fold against murine tumours than FAA on a concentration basis (Rewcastle *et al.*, 1991). Later work with 5,6 MeXAA (Ching *et al.*, 1994; Laws *et al.*, 1995) showed it to have both vascular and immunological mechanisms of action similar to those seen with FAA (Futami *et al.*, 1991). In the present study, we have investigated the effect of 5,6 MeXAA alone or in combination with the biological modifier TNF-α on human endothelial cell function *in vitro* under low oxygen tension and in the presence of factors secreted by a human tumour cell line; for comparative purposes, FAA was included in the study. The cellular functions studied were effects on procoagulant activity and permeability of endothelial monolayers as both FAA and TNF-α are known to affect both these properties (Murray *et al.*, 1991; Watts and Woodcock, 1992; Burke-Gaffney and Keenan, 1993).

Correspondence: ME Watts

Materials and methods

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffe *et al.* (1973), characterised as endothelial by staining positively for the presence of von Willebrand factor and positively for uptake of acetylated low density lipoprotein. They were grown on gelatin (0.2%)-coated tissue culture grade plasticware in M199 supplemented with 20% fetal calf serum (FCS), 20 μg ml^{-1} endothelial growth supplement and 17.5 U ml^{-1} heparin and used from first to third passage. Each separate experiment was carried out using cells originating from one individual donor. Human breast adenocarcinoma cells (MDA-MB-231) were maintained in Eagle's minimum essential medium (MEM) supplemented with 15% FCS. Tumour-conditioned medium (TCM) was collected from confluent cultures of regularly subcultured cells.

Drugs

5,6 MeXAA was supplied by the Cancer Research Campaign, UK, FAA by LIPHA Pharmaceuticals, France, and TNF-α (1 U = 25 pg) by the National Institute for Biological Standards and Control, UK.

Cytotoxicity studies

The effects of 5,6 MeXAA and FAA on the survival of HUVECs were measured using the neutral red cytotoxicity assay. Briefly, HUVECs were plated at 4×10^3 cells per well in gelatinised 96-multiwell plates. After 18 h, the cells were incubated in a range of concentrations of 5,6 MeXAA and FAA in M199 medium or TCM for 4 h at 37°C. The monolayers were then washed once and incubated in M199 for 7–9 days. When control wells were ~90% confluent, the neutral red assay was performed as described by Borenfreund *et al.* (1990). Absorbance was read at 540 nm using a plate reader and surviving fractions calculated.

Coagulation studies

HUVECs were seeded in gelatin-coated 24-multiwell plates at a density of $\sim 8 \times 10^4$ cells per well. After 18 h incubation, the medium was replaced by fresh M199 or TCM and the cells incubated under air + 5% carbon dioxide or nitrogen + 5% carbon dioxide for 24 h in the absence or presence of TNF-α at 500 U ml^{-1}. 5,6 MeXAA or FAA (both at 0.3 or 3.0 mM) was present for the last 4 h of this incubation period. Clotting activity was assayed by washing the monolayers twice with phosphate-buffered saline, adding 100 μl Owren's buffer and 100 μl normal human plasma; after 2 min at 37°C, clotting was initiated by addition of 100 μl prewarmed 25 mM calcium chloride. Clotting time was defined as the time taken for the appearance of the first fibrin strands.

Permeability studies

Permeability studies were carried out as described by Watts and Woodcock (1992) with HUVECs being seeded on 9 mm diameter, 0.45 μm pore size Falcon cell culture inserts at 10^5 cells per insert. After 24 h, the cells were incubated in 0.15 mM 5,6 MeXAA or 1000 U ml^{-1} TNF-α. At timed intervals, the integrity of the monolayers was assayed by measuring the passage of FITC–dextran through the cell layers.

Results

Cytotoxicity

Using the neutral red cytotoxicity assay 3 mM FAA was found to be non-toxic to HUVECs in M199 or TCM; this result is in agreement with previous toxicity data obtained using a limiting dilution method (Watts and Woodcock, 1992). 5,6 MeXAA was also non-toxic at 3 mM in M199; however, in TCM survival was reduced to approximately 50%.

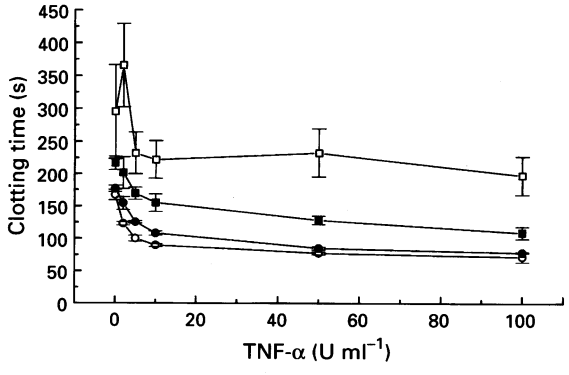

Figure 1 Effect of TNF-α on clotting times of HUVECs. ■, aerobic control; □, hypoxic control; ●, aerobic TCM-treated; ○, hypoxic TCM-treated. Each data point represents the mean of three observations ± s.e.m.

Induction of procoagulant activity

Control monolayers of quiescent HUVECs had long clotting times, reflecting the expression of low levels of tissue factor. Exposure of HUVECs to a range of concentrations of TNF-α for 24 h resulted in upregulation of procoagulant activity manifested as reduced clotting times. From Figure 1, it can be seen that this increase in activity was dependent on both TNF-α concentration and oxygen tension, aerobic cells in M199 being more sensitive than hypoxic ones, with concentrations as low as 5 U ml^{-1} causing significant ($P<0.5$) reductions in clotting times. Cells conditioned with TCM exhibited greatly reduced clotting times which were further reduced in a dose-dependent manner by TNF-α (Figure 1). In contrast to control cells, aerobic and hypoxic TCM-treated cells were equally sensitive to TNF-α at concentrations greater than 50 U ml^{-1} ($P<0.05$).

Neither 5,6 MeXAA (0.3 mM) nor FAA (0.3 mM) reduced the clotting times of aerobic or hypoxic control cells; similar results were seen at 3.0 mM (data not shown). Combining either drug with 500 U ml^{-1} TNF-α under aerobic conditions caused no further increase in activity than that induced by TNF-α alone. In contrast, both 5,6 MeXAA and FAA potentiated the effect of TNF-α on the relatively resistant hypoxic cells, increasing their sensitivity to that of aerobic cells treated with TNF-α alone or the drug combination (Figure 2).

Neither 5,6 MeXAA nor FAA caused further reductions in the clotting times of TCM-treated cells over and above the reduction induced by TCM alone (Figure 3). Combining either drug with TNF-α did not potentiate the effect of TNF-α.

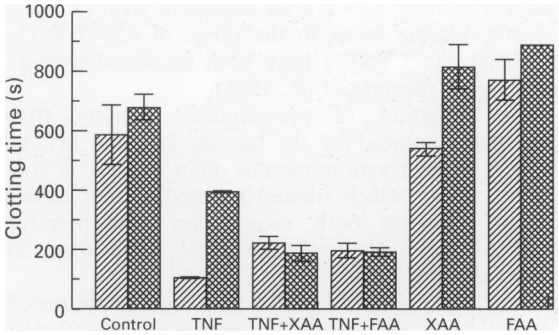

Figure 2 Effect of 500 U ml^{-1} TNF-α, 0.3 mM 5,6 MeXAA and 0.3 mM FAA on the clotting times of HUVECs in normal medium. TNF-α was present for 24 h with 5,6 MeXAA or FAA present for the last 4 h of this incubation time. Each bar represents the mean of three observations ± s.e.m. ▨, air; ▩, hypoxia.

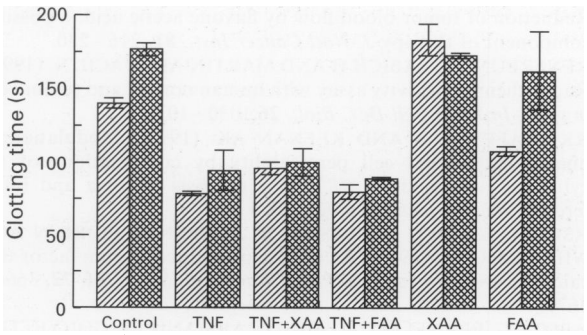

Figure 3 Effect of TNF-α, 0.3 mM 5,6 MeXAA and 0.3 mM FAA on the clotting times of HUVECs conditioned by TCM. TNF-α was present for 24 h with 5,6 MeXAA or FAA present for the last 4 h of this incubation time. Each bar represents the mean of three observations ± s.e.m. ▨, air; ▩, hypoxia.

Permeability studies

In contrast to procoagulant activity, permeability was insensitive to TNF-α at the time intervals studied for up to 6 h, remaining unchanged by a concentration of 1000 U ml^{-1} Incubation with 5,6 MeXAA also caused no significant change in permeability of HUVECs monolayers (data not shown).

Discussion

Initial studies focused on both permeability and coagulation properties of endothelial cells and how they changed with addition of TNF-α. However, the lack of effect on permeability of even the highest concentration of TNF-α led us to concentrate on the effects on coagulation parameters. As TNF-α production in tumours may be a key process in many of the actions of 5,6 MeXAA and FAA, the effects of TNF-α were evaluated over a range of concentrations.

The equal cytotoxicities of 5,6 MeXAA and FAA observed *in vitro* contrast with the far greater toxicity seen with 5,6 MeXAA *in vivo* when compared with FAA. This difference in toxicity may be due to the influences of immune cells in *in vivo* studies.The similar cytotoxicities of 5,6 MeXAA and FAA *in vitro* enabled us to compare equal concentrations of the two agents. Studies at the higher concentration of 3 mM enabled 5,6 MeXAA to be compared directly with earlier *in vitro* results obtained with FAA, whereas the lower concentration of 0.3 mM was chosen as it was deemed to be more relevant to concentrations used *in vivo*. The incubation time of 4 h was chosen for this study as FAA has been shown to increase plasma levels of TNF-α significantly in mice 3–4 h after treatment (Hill *et al.*, 1995). In patients, plasma levels in the range of 450–650 U ml^{-1} (200–300 pg ml^{-1}) TNF-α have been measured over a 24 h infusion period (Spriggs *et al.*, 1985).

The upregulation of procoagulant activity (reduced clotting times) induced by the tumour cell line MDA-MB-231 is in agreement with numerous other *in vitro*, *in vivo* and clinical studies in which tumour-secreted factors have been shown to influence both coagulation and permeability properties of endothelium (Senger *et al.*, 1986; Claus *et al.*, 1990; Rickles *et al.*, 1992).

Hypoxia has been reported to both upregulate procoagulant activity and to modulate permeability (Gertler *et al.*, 1991; Ogawa *et al.*, 1990). However, in the present study, no significant effect of hypoxia was evident on the clotting activity of control or TCM-treated HUVECs. The oxygen concentration in our studies was *c.* 0.2%, based on the radiobiological measurements conducted by Chapman *et al.* (1970) on cells plated on polystyrene Petri dishes, whereas that in the studies of Ogawa *et al.* (1990) and Gertler *et al.* (1991) were higher. Oxygen tension does, however, influence the cytotoxicity of TNF-α; studies by Park *et al.* (1992) and Sampson and Chaplin (1994) showed a reduced effect of TNF-α on hypoxic murine fibroblasts and, in the present study, we have shown that the procoagulant activity of control HUVECs is also less sensitive to TNF-α under hypoxic conditions.

The absence of a direct effect of 5,6 MeXAA and FAA on the upregulation of procoagulant activity lends supporting evidence to other studies that have demonstrated that many of the effects of FAA and 5,6 MeXAA are mediated via induction of TNF-α and other cytokines by activated macrophages (Futami *et al.*, 1991; Chabot *et al.*, 1993; Ching *et al.*, 1994). Although no effect of 5,6 MeXAA or FAA on endothelial cells in the absence of macrophages may be expected, there was, however, an increased direct effect of these drugs on HUVECs in normal medium when combined with TNF-α under hypoxia (Figure 2). A similar effect was observed with 100 U ml^{-1} TNF-α (data not shown). FAA has been shown to induce TNF-α mRNA in murine cells only, whereas 5,6 MeXAA is equally potent in upregulating the gene in both mice and humans (Ching *et al.*, 1994), although this may not be translated into TNF-α protein. Chabot *et al.* (1993) measured TNF-α in the plasma of mice, but not of patients, after administration of FAA. These results indicate that FAA is species-specific whereas 5,6 MeXAA does not show such specificity and therefore may be expected to be more clinically active.

In conclusion, our studies demonstrate that tumour-secreted factors upregulate procoagulant activity equally under both aerobic and hypoxic conditions and, in the absence of these factors, hypoxic cells are more resistant to TNF-α than aerobic ones. 5,6 MeXAA and FAA are equally toxic to HUVECs and have little, if any, direct effect on clotting activity, indicating that any effects on procoagulant activity *in vivo* would probably be mediated by their ability to induce TNF-α release. Both drugs are equally effective in potentiating the effect of TNF-α on hypoxic control HUVECs. Under all other conditions, there was no potentiation of TNF-α by these drugs. The presence of tumour-secreted factors has a far greater effect on upregulation of procoagulant activity than does oxygen tension.

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