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Flavone Acetic Acid Potentiates the Induction of Endothelial Procoagulant Activity by Tumour Necrosis Factor

J. Clifford Murray, K. Anne Smith and David M. Stern

Treatment of human umbilical vein endothelial cells with flavone acetic acid (FAA) at 800 μg/ml for 4 h resulted in a 3–11-fold increase in procoagulant activity. This increase was due to enhanced tissue factor expression on the endothelial cell surface, as evidenced by the blocking of the enhanced clotting with antibody to tissue factor, by substitution of normal with factor VII deficient plasma, or by simultaneous treatment of the endothelial cells with cycloheximide or actinomycin D. FAA was not toxic to endothelial cell at concentrations up to 1.6 mg/ml over 4 h. Combined treatment with FAA and tumour necrosis factor α (TNF-α) (100 pg/ml) produced a 675-fold (range 160–1980) increase in tissue factor activity, compared to 5-fold and 50-fold increases for the individual agents respectively. Northern blotting of total RNA from cells treated with the combination of agents or either agent alone, followed by probing with a cDNA to human tissue factor demonstrated a synergistic increase in tissue factor mRNA after combination treatment. *In vivo*, the combination of FAA and TNF-α could be shown to induce greater growth delay in two murine tumours than would be predicted on the basis of the activity of either agent alone.
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INTRODUCTION

TUMOUR NECROSIS factor alpha (TNF-α) causes rapid regression in a number of murine tumour models [1]. Although the mechanism of action of TNF-α is poorly understood, nutrient deprivation due to occlusion of the tumour vasculature is thought to be an important component of its activity [2, 3]. *In vivo*, TNF-α treatment of tumour-bearing mice causes the deposition of insoluble fibrin within the vasculature of the tumour with the formation of occlusive thrombi [2], while *in vitro* treatment of human umbilical vein endothelial cells (HUVEC) or bovine aortic endothelial cells with TNF-α enhances tissue factor expression and suppresses the protein C anticoagulant pathway, thus promoting clot formation [4]. Recent studies have indicated that the tumour vasculature may also represent a target for the novel anticancer agent flavone acetic acid (FAA): not only is there a rapid loss of blood flow in the tumour after FAA treatment [5–7] but this is associated with a consumption coagulopathy, which is absent in non-tumour bearing animals [8]. These data suggest that there may be shared components in the mechanisms of action of TNF-α and FAA,

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particularly in terms of their vascular effects. We therefore carried out a series of experiments to determine whether any similarities exist between TNF-α and FAA in terms of their ability to modify the expression of tissue factor on cultured endothelial cells, and how they might interact *in vitro* to promote coagulation, and *in vivo* to inhibit tumour growth.

MATERIALS AND METHODS

Endothelial cell cultures

Human umbilical vein endothelial cells (HUVEC) were prepared as previously described [9] and used in all experiments at passage 2–3. For procoagulant activity assays cells were grown to confluence on 24-well gelatin-coated culture dishes (1.9 cm^2, Costar) and maintained in Medium 199 supplemented with 5% human serum, 15% fetal calf serum, heparin (50 μg/ml, Sigma), and endothelial cell growth factor (7.5 μg/ml, Boehringer Mannheim).

Assay of procoagulant activity

Cell monolayers were washed with RPMI 1640 medium supplemented with 10 mmol/l "Hepes" buffer, 10% fetal calf serum and polymyxin B (0.5 μg/ml), and then all further incubations carried out in the same medium. In some experiments medium was supplemented with flavone acetic acid (obtained as the free acid and dissolved in 0.01 mol/l NaOH), or with recombinant human TNF-α (rhTNF-α, 10^8U/mg). After the appropriate incubation period at 37°C in an atmosphere of 5% CO_2, monolayers were washed once with complete medium. In several experiments cells grown and treated on 30 mm dishes were scraped off wells and assayed for procoagulant activity in suspension, as previously described [10]. This method also gives reproducible clotting times although it is not clear to what extent cellular damage has occurred as a result of the scraping, and therefore the monolayer assay was considered to be superior. Single stage clotting assays were carried out directly on the intact monolayers (or cell suspensions) by adding 100 μl normal citrated human plasma, 100 μl barbital-buffered saline and 100 μl 0.02 mol/l $CaCl_2$, and incubating at 37°C. Clotting time was defined as the time taken for the appearance of the first fibrin strands. Tissue factor equivalence was calculated by reference to a standard curve generated by the addition of known amounts of phospholipid-reconstituted human tissue factor (kindly provided by R. Bach, Mt Sinai School of Medicine) to the clotting mixture. All values represent the mean of at least three replicate incubations carried out simultaneously. In some experiments monolayers were incubated with a monoclonal antibody to human tissue factor (R. Bach, Mt Sinai) for 30 min immediately prior to assay to block tissue factor activity, or were incubated with cycloheximide (2–10 μg/ml) or actinomycin D (5 μg/ml) for varying periods to inhibit protein synthesis or RNA synthesis respectively. In some experiments normal human citrated plasma was replaced by factor VII deficient plasma in the clotting assay. To determine whether FAA might induce direct activation of factor X, one experiment was carried out in which endothelial cells were incubated with FAA at 800 μg/ml for 4 h, the dishes washed, and fresh medium supplemented with purified factor X (100 μg/ml) added. The incubation was continued, and at timed intervals aliquots of medium were removed and assayed for procoagulant activity with plasma deficient in factors VII and X.

Expression of tissue factor mRNA

Total RNA was extracted from confluent cultures of HUVEC (approx. 6×10^7 cells) after incubation for 4 h with TNF-α (100 pg/ml), FAA (800 μg/ml), or the combination of agents, by the guanidinium isothiocyanate procedure [11]. RNA (approx. 20 μg) was subjected to electrophoretic separation on 1% agarose [12], and then transferred onto nitrocellulose. cDNA probes to human tissue factor (TF) and thrombomodulin (generously provided by J.E. Sadler, Washington University) were labelled with ^{32}P by the random hexamer technique, and hybridised to the nitrocellulose-bound RNA by incubating overnight at 42°C [13]. The nitrocellulose was then washed under conditions of high stringency [14], dried, and exposed to Kodak X-Omat film at −80°C. The cDNA probe to tissue factor detects a major mRNA species of 2.2 kb in most human tissues as well as a minor component of 3.2 kb which may be the result of either incomplete or alternative RNA splicing [15]. The probe to thombomodulin detects a single mRNA species of 3.8 kb.

In vivo studies with TNF-α/FAA

Experiments were carried out on CBA/HtBSVS strain male mice bearing the CaNT tumour, a moderately differentiated

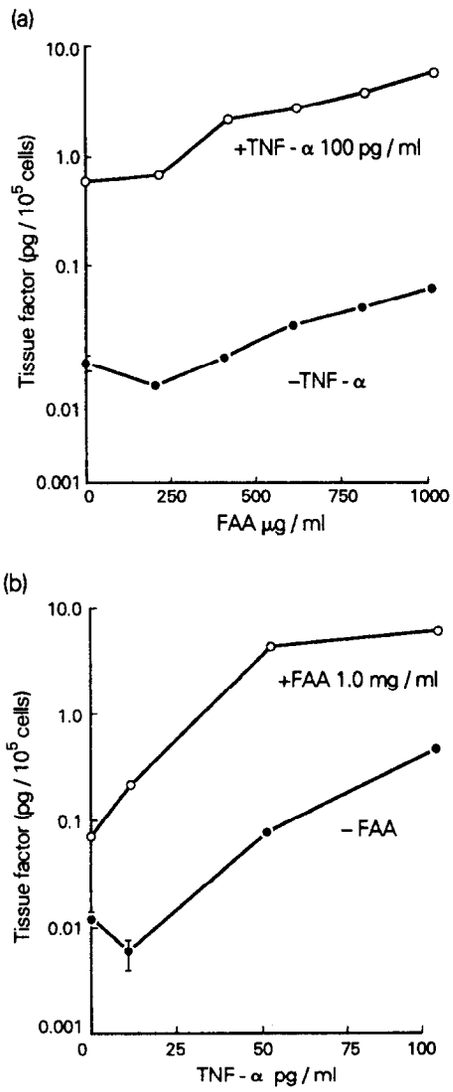

Fig. 1. (a) Effect of FAA on tissue factor expression in confluent monolayers of HUVEC. Values represent the mean (S.E.) of triplicate determinations from a representative experiment. In some cases the error bars are smaller than the symbol. (b) Effect of varying TNF-α concentration on tissue factor expression.

Table 1. Effect of antibodies to human tissue factor on TNF-α-induced and FAA-induced endothelial procoagulant activity

	TF activity pg/10^5 cells		
Treatment	Medium only	Normal* IgG	AntiTF*
Control	0.003 (0.001)	0.006 (0.001)	ND
TNF-α (100 pg/ml)	0.350 (0.030)	0.600 (0.010)	0.010 (0.005)
FAA (800 μg/ml)	0.010 (0.005)	0.010 (0.005)	ND
TNF-α + FAA	5.880 (0.290)	6.280 (0.240)	0.020 (0.005)

Mean (S.E.).
ND = not detectable.

adenocarcinoma, and on WHT/GyBSVS male mice bearing the FibT tumour, a poorly differentiated fibrosarcoma, implanted subcutaneously onto the back. Tumours were measured in three orthogonal directions 2–3 times per week and geometric mean diameters (GMD) calculated. Treatments were initiated when the tumours had reached a GMD of 6–7 mm. FAA, dissolved in sterile saline, was administered by intraperitoneal injection at an appropriate concentration to allow a constant volume (0.01 ml/g) to be administered at each drug dose. TNF-α (10^8U/mg, Hoffman La Roche) was dissolved in sterile saline and injected intravenously via the tail vein. All animal manipulations were carried out in accordance with UK Home Office regulations.

RESULTS

Effects of FAA and TNF-α on procoagulant activity

Figure 1a shows the effect of FAA at different doses on procoagulant activity after 4 h incubation with confluent HUVEC, in the presence or absence of TNF-α at 100 pg/ml. The results are from a representative experiment. FAA alone at 800 μg/ml induced an average 5-fold increase in procoagulant activity (8 experiments, range 3–10.5), and had no effect on cell viability at levels up to 1.6 mg/ml and exposure times of 4 h as assessed by trypan blue exclusion (data not shown). Treatment with TNF-α alone at 100 pg/ml under similar incubation conditions led to a 50-fold increase in cell surface-associated procoagulant activity (6 experiments, range 31–116).

The combination of TNF-α at 100 pg/ml and FAA at 1 mg/ml produced a 600-fold increase in procoagulant activity compared to controls (Fig. 1a). In 5 experiments this combination produced an average 675-fold increase in procoagulant activity (range 160–1980). Figure 1b demonstrates the dose dependence of the FAA effect on TNF-α concentration, and once again demonstrates synergy for the combination of agents.

Table 2. Procoagulant activity of TNF-α-treated and FAA-treated HUVEC measured in the presence of normal or factor VII-deficient human plasma

	TF activity pg/10^5 cells	
Treatment	Normal	Factor VII deficient
Control	0.003 (0.001)	ND
TNF-α (100 pg/ml)	0.110 (0.005)	0.021 (0.004)
FAA (800 μg/ml)	0.012 (0.001)	0.002 (0.001)
TNF-α + FAA	0.477 (0.015)	0.085 (0.001)

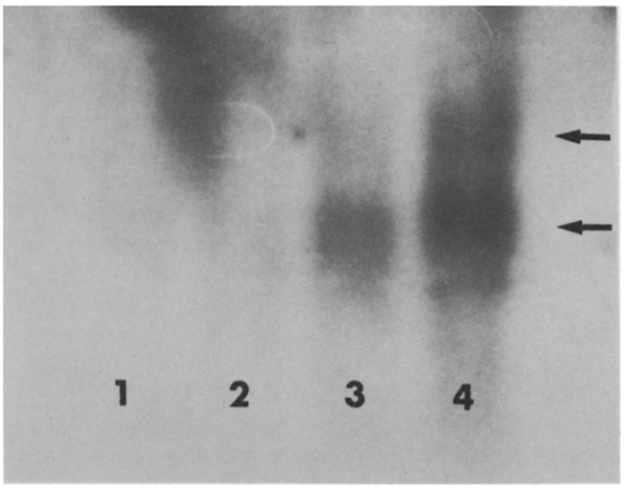

Fig. 2. Autoradiogram of a northern blot of total RNA extracted from HUVEC treated with TNF-α, FAA or the combination of agents for 4 hours, and probed with a ^{32}P-labelled cDNA for human tissue factor. 20 μg RNA was run per lane. Lane 1, control; lane 2, FAA alone; lane 3, TNF-α alone; lane 4, FAA + TNF-α. Arrows indicate regions of 2.2 and 3.2 kb RNA.

Although the apparent procoagulant activity was generally higher than that observed in monolayer procoagulant assays, in two experiments carried out with HUVEC cells in suspension, a similar response to the two agents was seen; FAA alone induced a 3-fold increase in procoagulant activity, TNF-α alone a 58-fold increase, and the combination a 300-fold incease compared to controls (data not shown).

Enhanced procoagulant activity is due to tissue factor

Table 1 shows the results of experiments confirming that the increase in procoagulant activity seen in HUVEC monolayers after TNF-α and/or FAA treatment was due to enhanced expression of TF. Pretreatment with monoclonal antibody to human TF almost completely abolished procoagulant activity in all groups. Pretreatment with normal mouse immunoglobulins at the same concentration had little effect on the FAA/TNF-α stimulated cells, although there was a small but significant drop in procoagulant activity in the TNF-α alone treated cells. Similar experiments where the clotting assay was carried out with factor VII deficient plasma also indicated that the enhanced clotting observed in the presence of FAA and TNF-α was dependent upon the expression of TF by the cells (Table 2). Simultaneous incubation of the HUVEC cultures with TNF-α and/or FAA and cycloheximide (2 μg/ml) or actinomycin D (5 μg/ml) also reduced TF activity by 95% (data not shown).

FAA-treated cells were also unable to activate purified factor X in the absence of factor VII, as determined by a two-stage assay in which factor X was exposed to FAA-treated cells and supernatants then assayed at timed intervals for clotting activity in factor VII deficient plasma (data not shown), again suggesting that induction of TF by FAA was involved.

Northern blotting and hybridisation with a cDNA for tissue factor

Figure 2 shows an autoradiograph of a northern blot of total RNA from HUVEC treated with FAA, TNF-α or the combination of agents for 4 h, to which a ^{32}P-labelled cDNA probe for TF was hybridised. Lanes 1 and 2 (control and 800 μg/ml FAA respectively) showed faint bands in the expected

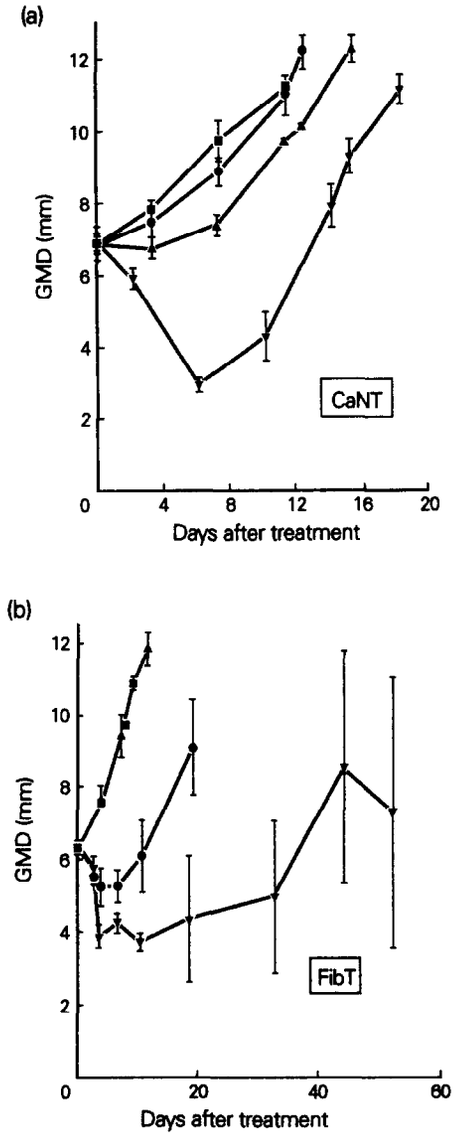

Fig. 3. Regrowth curves for (a) the CaNT tumour and (b) the FibT tumour after different treatment regimes. (■) Control; (●) TNF-α, 5 μg/mouse; (▲) FAA, 150 mg/kg; and (▼) TNF-α and FAA combined. TNF-α was injected intravenously via a tail vein in sterile saline, and FAA was injected intraperitoneally. Values are given as mean (S.E.) (n = 5).

regions, corresponding to 2.2 and 3.2 kb, indicating very little mRNA for tissue factor. Lane 3 (100 pg/ml TNF-α) showed the presence of slightly more TF mRNA, while lane 4 (FAA and TNF-α together) demonstrated the greatest enhancement of TF mRNA, with very dark bands in both the 2.2 and 3.2 b regions. Probing identical nitrocellulose blots with cDNA to thrombomodulin indicated no significant change in amounts of mRNA in any group compared to control (data not shown).

In vivo studies

Figure 3 shows regrowth data for the CaNT and FibT tumours after treatment with single doses of TNF-α (5 μg), FAA (150 mg/kg), or the combination of agents. The CaNT showed a growth delay, measured as time to grow to treatment size plus 3 mm, of 2 days in response to TNF-α, while the FibT showed no growth delay. Both tumours responded to FAA, giving 4.1 and 12.3 days of growth delay in the CaNT and FibT, respectively. In both tumours the combination of TNF-α and FAA had a supra-additive effect, producing mean growth delays of 8.6 days in the CaNT, and 31.2 days in the FibT.

DISCUSSION

FAA, an agent with potent activity against solid murine tumours, induces a small but significant increase in TF expression on HUVEC, leading to increased procoagulant activity. This functional observation was confirmed by the blocking of procoagulant activity by preincubation with an antibody to TF. The effect was also blocked by actinomycin D, suggesting that modulation may occur at the transcriptional level, although northern blotting did not demonstrate increased TF mRNA; presumably the change is below the limit of detection for this probe. With TNF-α alone there was a small but detectable increase in TF mRNA. These results confirm a degree of similarity between FAA and TNF-α in terms of their ability to promote procoagulant activity through increased TF expression on the endothelial cell surface. More surprising perhaps is the observation that TNF-α and FAA act synergistically to increase total procoagulant activity of HUVEC *in vitro*. This appears to be the result of greatly enhanced functional TF expression, and in this case the effect is coupled with a significant increase in steady-state levels of TF mRNA. Such an increase could be the result of increased mRNA synthesis, or stabilisation of the mRNA to prevent rapid degradation, or both. As actinomycin D was added simultaneously with FAA and TNF-α we cannot rule out either possibility.

The mechanism of action of FAA, although the subject of intense investigation, is poorly understood. It has been suggested that FAA may act as a biological response modifier. This hypothesis was first put forward to explain the similarities in histological changes seen in murine solid tumours treated with FAA and TNF-α [16]. Further work revealed that FAA augments splenic natural killer (NK) cell activity in mice [17], and peripheral blood NK activity in patients [18], probably mediated by the induction of interferon alpha or beta (IFN-α/β) [18]. In addition it was shown that FAA acted synergistically with IL-2 both in inducing IFN-α/β production and eliciting cures of a murine renal cell carcinoma [19].

More recently it has been reported that FAA up-regulates the expression of a battery of cytokine genes in splenic leucocytes, including those for IFN-α, IFN-γ and TNF-α, and that this up-regulation correlates in dose terms with the therapeutic efficacy of FAA against a murine renal cell carcinoma [20]. It has also been reported that vascular shutdown induced in murine solid tumours by FAA can be inhibited by simultaneous administration of antibodies to TNF-α, suggesting that at least some of the effects of FAA are mediated through TNF-α [21]. These results support the view that FAA exerts its antitumour effects indirectly, through a complex cytokine network.

Our results, on the other hand, indicate that a synergistic interaction occurs between TNF-α and FAA, and these findings are difficult to reconcile with a model in which FAA induces increased expression of TNF-α *per se*, although this possibility cannot be ruled out, as we have not carried out similar experiments with antibodies to TF. FAA may render endothelial cells more sensitive to the effects of TNF-α, perhaps by altering the affinity or number of exposed receptors on the endothelial cell surface, although based upon the clotting assay results alone, where we found a wide range of values for the enhancement, it is not possible to say which is the case. Linearisation of the data in Fig. 1b (data not shown) indicates a highly significant

difference in the slopes, suggesting that TNF concentration-dependence is altered by the presence of FAA. It has recently been demonstrated that the association constant for TNF-α receptors on the surface of endothelial cells may vary significantly according to the growth state of the cells; thus actively proliferating, motile cells, or confluent cells treated with microtubule poisons to disrupt the cytoskeleton, express high affinity TNF-α binding sites (K_d 0.1 nmol/l) while postconfluent, unperturbed cells express only lower affinity binding sites (K_d 1.8 nmol/l) [22]. The results of a limited number of assays with suspended HUVEC, which must be considered perturbed, show a similar synergistic interaction between FAA and TNF-α, and therefore tend to argue against a receptor-mediated effect.

FAA appears to be pleiotropic in its actions on endothelial cells, altering barrier function [23] as well as procoagulant activity of endothelial cell monolayers, which we have demonstrated here. FAA does not activate coagulation directly, based upon the inability of FAA-treated cells to activate factor X in the absence of factor VII, and the sensitivity of the procoagulant effect to actinomycin D. Potentiation of procoagulant activity in cultured endothelial cells may therefore occur via two possible routes; a direct, although relatively small, effect on the expression of TF, and a synergistic interaction with TNF-α to increase TF mRNA, in part due to enhanced steady-state levels of TF mRNA.

The results of experiments with tumour-bearing mice indicate that the synergy between the two agents is expressed both in terms of enhanced tumour response and changes in *ex vivo* clotting times of plasma. In clotting assays carried out on plasma from these mice, FAA and TNF-α significantly lowered clotting times at 15 min and raised them above control levels at 240 min, as previously demonstrated for FAA alone [8], however only at 240 min did the combination produce a significant change compared to individual agents alone (data not shown). These data provide corroborating, but not direct, evidence of a role for endothelial-mediated vascular effects in the mode of action of FAA.

We believe the "vascular" effect of FAA in murine solid tumours to be a major component of its antitumour action. In addition we hypothesise that vascular occlusion results from a combination of events; changes in endothelial barrier function leading to increased vascular permeability [23], and increased endothelial procoagulant activity potentiated by TNF-α, both observed *in vitro* with human endothelial cells. We have also confirmed the occurrence of changes in vascular permeability and a systemic coagulopathy after FAA administration to tumour-bearing mice *in vivo* [23, 8]. Finally, it seems that the focal nature of these effects, producing occlusion within the tumour vascular bed alone, may be conferred by newly identified tumour factors such as the Meth-A factor [10], which potentiates the effects of TNF-α. We have also recently described a 25kD factor produced by a human melanoma cell line which in combination with FAA or TNF-α potentiates endothelial procoagulant activity *in vitro* [24], and which bears many similarities to a factor recently described in supernatants from a human bladder carcinoma cell line [25].

The use of immunohistochemistry with antibodies to TF, and *in situ* hybridisation with TF probes will help to confirm or deny the relevance of tumour procoagulant activity as a potential component of antitumour therapy. Nevertheless, the existence of factors produced by tumour cells such as those just described, or by accompanying host cells, suggests that a complex interplay of several biological mediators may render endothelial cells associated with tumours particularly susceptible to chemical modifiers such as FAA, and suggest a novel approach to tumour therapy based upon the endothelial cell as target.

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Expression of the Breast Cancer Associated Gene pS2 and the Pancreatic Spasmolytic Polypeptide Gene (hSP) in Diffuse Type of Stomach Carcinoma

Birgit Theisinger, Cornelius Welter, Gerhard Seitz, Marie-Christine Rio, Rich Lathe, Pierre Chambon and Nikolaus Blin

Expression of the pancreatic spasmolytic peptide (hSP) gene and pS2 (a gene isolated from oestrogen-induced breast carcinoma cells) were analysed in 36 samples of human stomach carcinoma. 17 tumours were investigated at the RNA level (by northern blots) as well as at the gene product level (by immunochemistry). Since pS2 had been shown to be expressed in normal stomach mucosa its activity in carcinoma samples was expected. Surprisingly, strong pS2 immunoreactivity was noted in the diffuse carcinoma type, whereas the intestinal type displayed weak reactivity. The tumour samples showing strong immunostaining expressed the regular 0.6 kb pS2 RNA band and weak staining was paralleled by aberrant transcripts. Additionally, only in tumour samples with regular pS2 transcription was the typical 0.7 kb hSP RNA band seen; samples with aberrant pS2 bands did not express hSP at all. This is the first demonstration of hSP gene activity in a human tumour.
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INTRODUCTION

THE HUMAN pS2 gene, transcriptionally controlled by oestrogen in a subclass of oestrogen receptor positive breast carcinomas [1] is also expressed, independently of oestrogen, in normal stomach mucosa [2]. Additionally, it has been noted to be active in pancreatic carcinoma but not in the corresponding healthy tissue (B.T. *et al*). The gene's partial homology to the pancreatic spasmolytic polypeptide gene [2, 3] suggests similar function of both proteins in affecting cell metabolism. Since cDNAs for both sequences, pS2 and hSP, are available [4, 5] we initiated a comparison of gene organisation and expression of both sequences in healthy tissue of the stomach in addition to primary stomach carcinomas and some metastases. Activity of hSP was examined whenever RNA from tumours was available, in particular in those tumour samples which displayed altered pS2 expression as judged at the mRNA level (by northern blots) and protein level (by immunostaining), and finally in some rare cases when primary tumours and metastases were available.

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MATERIAL AND METHODS

Tissue samples

The tumour and stomach mucosa specimens were frozen in N_2 within 30 minutes of operation. All samples were examined histologically. Histopathological classification was according to Lauren [6]. In addition to primary carcinomas of the stomach (n = 33), 3 metastases were obtained.

DNA and RNA analyses

Simultaneous isolation of DNA and RNA from all tissues was performed as described [7]. Gene organisation and copy number in tumour cells was monitored by the restriction pattern using restriction endonucleases and by the intensity of DNA bands in