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Platelet Proaggregating Activity of Human Colorectal Tumour Cell Lines Does Not Correlate with the Degree of Differentiation

P. Ferroni, F. Guadagni, A. Pavan, E. Martinico, M.R. Torrisi and P.P. Gazzaniga

Six human colorectal tumour cell lines with various degrees of differentiation were studied. Each of the cell lines studied showed an *in vitro* platelet proaggregating activity, represented by the induction of typical aggregation waves. This activity was dose-dependent and probably related to a thrombin-dependent mechanism. However, the degree of cell differentiation did not correlate with the proaggregating activity. In fact, significant differences were observed between the two well differentiated cell lines, while a comparison between well and poorly differentiated cell lines did not reveal any difference. These results were confirmed by the ultrastructural observations, indicating that similar platelet–tumour cell interaction may be found in all the cell lines studied. The present results suggest that platelet proaggregating activity of the human colorectal tumour cell lines under investigation is unrelated to their degree of differentiation.
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INTRODUCTION

THE INVOLVEMENT of platelets in tumour cell metastasis has been a subject of interest since the initial observation [1] that cells from a variety of tumours can induce platelet aggregation *in vitro*. Subsequently, several reports [2, 3] have suggested that platelets are involved in the haematogenous metastasis of tumour cells from the primary tumour site. To explain the nature of such interaction(s) different mechanisms are now accepted, including: (a) release of ADP from tumour cells [4–6]; (b) generation of thrombin by tumour cells [7–9]; (c) generation and release from tumour cell membranes of a sialolipoprotein proaggregating material (PAM) [10, 11]; (d) surface interaction between GpIb and GpIIb/GpIIIa-like glycoproteins shared by tumour cells and platelets [12–14]; (e) release of different types of proteinases from tumour cells [15, 16]. Activation of platelets through one of the above reported mechanisms will be ultimately responsible for trapping of tumour cells within a platelet mass, thus leading the cells to escape immunological surveillance within the circulation and providing them with the possibility of attachment.

These observations raise interesting questions regarding a possible correlation between *in vitro* platelet proaggregating activity and *in vivo* metastatic potential of tumour cells. In this context, Pearlstein *et al.* [17] reported that the metastatic potential of PW20 rat renal sarcoma sublines significantly correlated with their ability to aggregate platelets. This correlation was subsequently confirmed using subpopulations isolated from the murine melanoma B16 cell line which differed in their platelet-aggregating and lung colony-forming abilities [18]. However, a different study employing the low metastatic (F1) and highly metastatic (F10) variants of B16 cells failed to show such a correlation, suggesting that the ability of tumour cells to promote platelet aggregation is unrelated to the metastatic potential [19].

More recently, Seyfert *et al.* [20] provided some evidence against the idea that platelet aggregation plays a major role in tumour cell invasion and metastasis. These authors found a range from 15 to 57% platelet aggregation when platelets from colon cancer patients were stimulated with their own tumour cells. However, no relationship was found between clinical and histopathological findings and platelet aggregation patterns; nevertheless, the follow-up was too short for exact long-term studies concerning the development of metastases.

The present study was designed to evaluate the ability of six human colorectal tumour cell lines, with various degrees of differentiation, to induce *in vitro* platelet aggregation. The mechanism responsible for their proaggregating activity was also analysed. A correlation was attempted between the degree of differentiation and *in vitro* proaggregating activity of the six tumour cell lines under investigation.

MATERIALS AND METHODS

Cell lines and culture conditions

Six human colorectal tumour cell lines demonstrating various degrees of differentiation were studied: the well differentiated GEO [21] and LS174T [22] cell lines, the moderately differentiated HT-29 [23] and WiDr [24] cell lines and the poorly differentiated DLD-1 [25] and MIP [26] cell lines. All cell lines were kindly supplied by Dr J. Schlom (National Cancer Institute, Bethesda, Maryland, U.S.A.). Cell lines were routinely grown in RPMI 1640 medium supplemented with (1×) glutamine, 50 μg/ml gentamycin, and 10% heat-inactivated fetal bovine serum (FBS). All established cell lines were routinely passaged every 5 days.

Tumour cells were harvested by decanting the culture medium, washing the monolayer twice with RPMI 1640

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medium, and then treating them for 5 min with 1% trypsin EDTA at 37°C. The cell suspension was centrifuged at 300 *g* for 10 min, the supernatant solution was removed, and the cell pellets were washed twice with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (pH 7.2). Tumour cells were finally resuspended in PBS without Ca^{2+} and Mg^{2+} at a final concentration of 1×10^7 cells/ml. Viability was determined by exclusion of trypan blue. The range of viable cells was always greater than 95%.

Platelet aggregation studies

Blood samples were obtained from healthy volunteers by venipuncture of the antecubital vein, collected in sodium citrate 3.8% (1:9 v:v) and immediately centrifuged at 120 *g* for 15 min to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained after removal of PRP and further centrifugation at 2000 *g* for 10 min. In all cases PRP was adjusted to a final count of $3.5 \times 10^5/\mu l$ by dilution with autologous PPP (Platelet Counter PL100 Sysmex TOA).

Platelet proaggregating activity of the tumour cell suspension was evaluated by use of a four sample Menarini 3210 Aggrecorder, using siliconised glass cuvettes, at 37°C and under continuous stirring at 1000 rpm. In the dose-dependency studies different amounts of tumour cell suspensions, ranging from 50 to 200 μl, were added to 300 μl of PRP. In all cases the final volume of the tumour cell/platelet suspension was adjusted to 500 μl by addition of PBS without Ca^{2+} and Mg^{2+} (pH 7.2). As a control the same PRP (300 μl plus 200 μl PBS without Ca^{2+} and Mg^{2+}) was stimulated by the addition of ADP at a final concentration of 2 μmol/l.

Inhibition studies

In some experiments PRP samples were preincubated with an ADP scavenging system to evaluate the role of ADP release from tumour cells in promoting platelet aggregation. Apyrase (V grade, Sigma Chemical Co., St Louis, Missouri, U.S.A.) (approximately 3.4 U/mg of protein) was added to PRP at a final concentration of 130 μg/ml, immediately prior to the addition of tumour cells. To evaluate the role of a thrombin-dependent mechanism in tumour cell-induced platelet aggregation, tosyl-arginine-methyl-ester (TAME) was added to PRP at a final concentration of 1 mmol/l and incubated for 5 min at 37°C.

Adenine nucleotide release

ATP release following tumour cell/platelet interaction was analysed by use of LKB Luminometer 1251. Fifty microlitres of a solution containing luciferin (80 μg/ml) and luciferase (8000 U/ml) were added to 300 μl of PPP or normal human serum (NHS), and ATP luminescence was measured after addition of 200 μl of tumour cell suspensions in PBS without Ca^{2+} and Mg^{2+} (pH 7.2) (2×10^7 cells/ml). The increase in luminescence obtained by addition of a pyruvate kinase (PK) (0.75 U/ml)/phospho-enolpyruvate (PEP) (0.8 mmol/l) system, which completely converts ADP into ATP, indicated the amount of ADP released.

Electron microscopy

Samples of PRP plus tumour cells were resuspended in PBS without Ca^{2+} and Mg^{2+} (pH 7.2) after 10 min of tumour cell/platelet interaction, and fixed by direct addition of glutaraldehyde (final concentration 2%) followed by a 1-h incubation at 4°C. Samples were then postfixed for 2 h at 4°C in a solution of osmium tetroxide 1% in veronal acetate buffer (pH 7.4). Following dehydration in acetone the samples were embedded in EPON 812, stained with uranyl acetate and lead hydroxide. Ultrathin sections were examined with a transmission electron microscope CH10 (Philips, Eindhoven, The Netherlands).

RESULTS

In vitro *proaggregating activity of tumour cell suspensions*

Each of the six colorectal tumour cell lines examined showed an *in vitro* platelet proaggregating activity, represented by the induction of typical aggregation waves. This activity was strictly dependent on cell number. Dose-dependency of tumour cell/platelet interactions is shown in Fig. 1. Different amounts of tumour cells, ranging from 5×10^5 to 2×10^6 cells were incubated with 300 μl of PRP (approximately 10^9 platelets) for 10 min. At the highest concentrations the six cell lines were all capable of inducing an irreversible platelet aggregation, characterised by the appearance of a monophasic or diphasic wave. Conversely, at the lower concentration (5×10^5) not all cell lines induced an aggregatory response. As shown, WiDr and HT-29 were still able to promote an irreversible diphasic wave, while LS174T, GEO and MIP only induced a monophasic reversible aggregation wave; the addition of 5×10^5 DLD-1 cells did not induce any aggregatory response. Increasing cell concentration above 2×10^6 cells/300 μl of PRP did not result in any augmentation of the percentage of platelet aggregation.

The percentage of *in vitro* platelet aggregation obtained with a cell concentration of 2×10^6/300 μl of PRP is reported in Table 1. In this table, the proaggregating activity of the six cell lines is presented as mean percentage ± S.E., obtained from 20 different

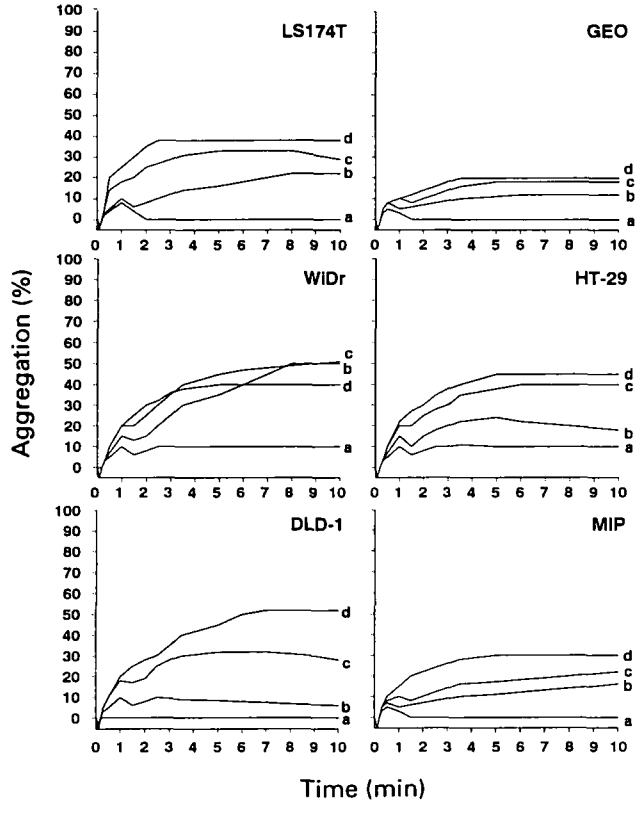

Fig. 1. Dose-dependent effect of the six human colorectal cell lines under investigation on *in vitro* platelet aggregation. (a) 0.5×10^6 cells/10^9 platelets; (b) 1×10^6 cells/10^9 platelets; (c) 1.5×10^6 cells/10^9 platelets; (d) 2×10^6 cells/10^9 platelets.

Table 1. Comparison of platelet proaggregating activity in different established human colorectal cell lines. Effects of two different inhibitors

Cell line	Degree of differentiation	Cell concentration ($\times 10^6$)	% of platelet aggregation (mean ± S.E.)*	Inhibition	
				Apyrase	TAME
GEO	Well	2	16.9 ± 2.82	2nd wave only	Complete
LS174T	Well	2	38.5 ± 9.68	2nd wave only	Complete
HT-29	Moderate	2	39.0 ± 3.68	2nd wave only	Complete
WiDr	Moderate	2	34.6 ± 3.82	2nd wave only	Complete
DLD-1	Poor	2	31.6 ± 4.45	2nd wave only	Complete
MIP	Poor	2	28.5 ± 7.83	2nd wave only	Complete

*Calculated on the basis of 20 different experiments.

experiments performed using PRP samples obtained from 10 different donors. As shown, LS174T, HT-29, WiDr, DLD-1 and MIP cell lines all induced a remarkable *in vitro* platelet aggregation, with percentages ranging from 39% (HT-29) to approximately 29% (MIP), while the GEO cell line was only capable of inducing a slight aggregatory response (approximately 17%) (see also Fig. 1). A statistical analysis, performed using the Student's *t*-test, demonstrated the lack of significant differences between the two moderately differentiated tumour cell lines, the two poorly differentiated tumour cell lines, as well as among all four cell lines. Conversely, significant differences were observed between GEO cell line and LS174T ($P = 0.01$), HT-29 ($P < 0.001$), WiDr ($P < 0.001$), and DLD-1 ($P = 0.006$) cell lines. The comparison between GEO and MIP cell lines failed to show any significant difference.

Similar results were obtained when platelet aggregation assays were performed on PRP samples obtained at different times from a single blood donor. Table 2 shows the percentages of *in vitro* platelet aggregation (mean ± S.E.) induced by the six tumour cell lines. A strict correlation can be observed between the intra-individual and the inter-individual variability of platelet proaggregating activity of the different cell lines, as indicated by the lack of significant differences between the mean ± S.E. observed in PRP preparations obtained from different donors and the mean ± S.E. observed in PRP preparations obtained from a single donor (Tables 1 and 2).

Table 2. Platelet proaggregating activity of different human colorectal cell lines in PRP samples obtained from a single blood donor

Cell line	% range	% of platelet aggregation (mean ± S.E.)*
GEO	0–40†	13.6 ± 2.85
LS174T	44–53	48.0 ± 2.65
HT-29	14–54	42.1 ± 3.08
WiDr	4–61‡	40.9 ± 4.18
DLD-1	11–62	31.2 ± 5.07
MIP	11–69	26.4 ± 8.72

*Calculated on the basis of 13 different experiments.
†A 40% *in vitro* platelet aggregation was observed only in 1 case.
‡A 4% *in vitro* platelet aggregation was observed only in 1 case.

Inhibition studies

Trypan blue exclusion tests, performed after the interactions, consistently showed a cell viability ranging from 90 to 95%, thus suggesting that no adenine nucleotide release had occurred due to cell death. These results, in addition to the luminometric finding of a lack of significant ATP/ADP secretion from tumour cells, support the hypothesis that mediators other than ADP are responsible, in our system, for the early events in tumour cell/platelet interaction. Moreover, the preincubation of PRP with apyrase (Table 1, Fig. 2) did not affect the primary response induced by tumour cell addition, but caused a complete inhibition of the secondary aggregation wave, due to platelet components.

Conversely, the preincubation of PRP with TAME, a thrombin inhibitor, resulted in the complete suppression of both primary and secondary aggregation waves in all cell lines under investigation. A summary of the inhibitory effects obtained on all cell lines is reported in Table 1. Figure 2 shows in detail the aggregometric patterns obtained with HT-29 cell line.

Ultrastructural observations

Ultrastructural observation was performed after 10 min of platelet/tumour cell interaction, and platelet aggregation was

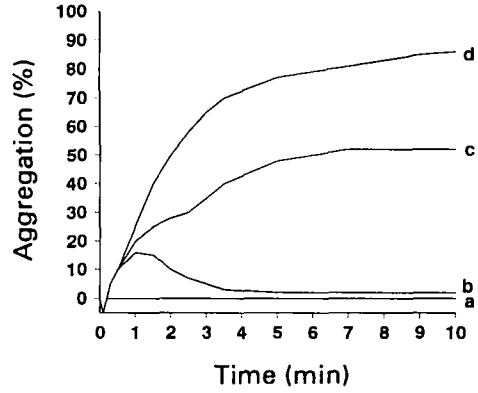

Fig. 2. Inhibition studies. Effects of tosyl-arginine-methyl-ester (a) and apyrase (b) on *in vitro* platelet aggregation promoted by 1.5×10^6 HT-29 tumour cells (c). (d) Control aggregation induced by ADP 2 μmol/l.

recorded during the whole period. An accurate examination of the sections obtained from electron microscopy specimens showed a strict correlation with the aggregometric patterns. As expected, the presence of larger aggregates of tightly adherent platelets, partially or completely degranulated, was observed in pellets recovered from aggregation assays after interaction with those tumour cell lines that were capable of inducing the highest percentages of aggregation, while the size and number of aggregates were lower when the interaction was carried out with the GEO or MIP cell lines (data not shown).

Interestingly, close contacts between tumour cell membrane and platelets were observed in all samples, without major differences among the different cell lines. Figure 3 represents an example of the electron microscopy findings obtained with the GEO (Fig. 3a), WiDr (Fig. 3b) and MIP (Fig. 3c) cell lines. The percentages of platelet aggregation obtained with the three cell lines in this single experiment were 13, 40 and 15%, respectively. Figure 3a shows a platelet aggregate in close contact with a restricted portion of the cytoplasmic plasma membrane of a GEO tumour cell (arrows). The platelets at the periphery of the aggregate are tightly adherent to the cell membrane, whose villi appear to protrude through the platelet mass. Figure 3b represents an interaction of platelets with WiDr tumour cells. Close contacts are less evident than in Fig. 3a, but activated platelets, partially or completely degranulated and displaying several pseudopodia, are surrounding a tumour cell. Moreover, a cytoplasmic portion of a tumour cell (Cy) appears to be captured within the platelet aggregate, and to exhibit close contacts with an activated and partially degranulated platelet (arrow). Finally, Fig. 3c shows an interaction between platelets and MIP tumour cells. In this case platelets exhibit fewer signs of activation and, despite the presence of several pseudopodia, the degranulation is infrequent or absent. Still, it is possible to observe a partially activated platelet in tight contact with the cell membrane (arrow).

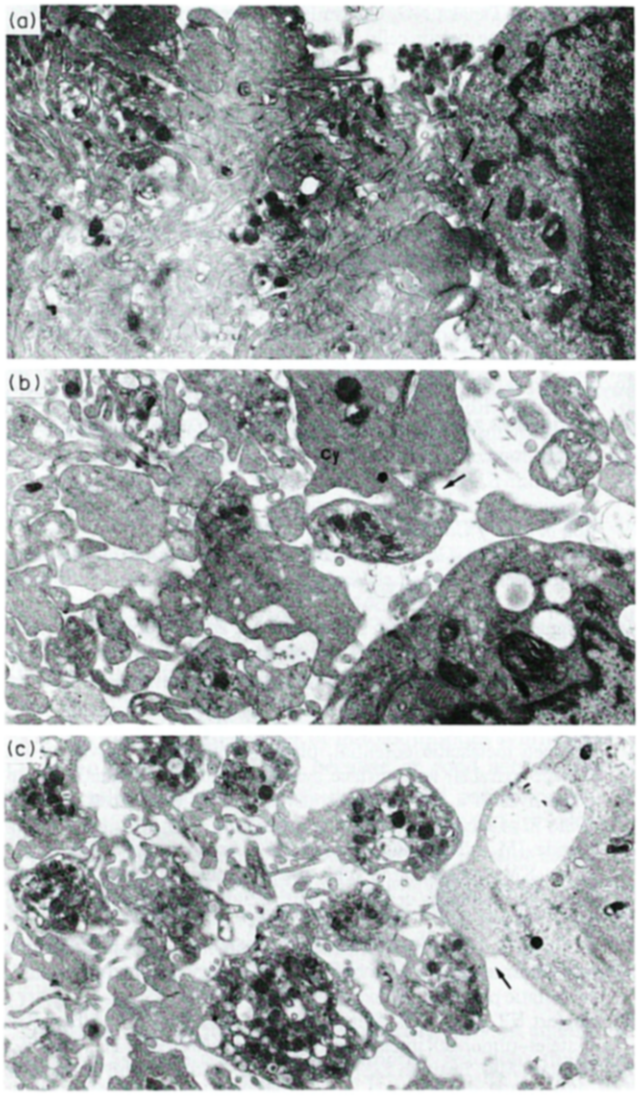

Fig. 3. Ultastructural observations on pellets from aggregometric assays after 10 min interaction with GEO (a), WiDr (b) and MIP (c) colorectal tumour cell lines. Arrows in (a–c) indicate close contacts between platelet and tumour cell membranes; Cy in (b) indicates WiDr cell cytoplasm. For details see Results. (a) × 12000; (b) × 13000; (c) × 11000.

CONCLUSIONS

Malignant tumours may show a wide pattern of cell differentiation and exhibit various degrees of anaplasia. In turn, anaplasia may be considered as a prognostic index, for both local recurrence and metastasis. In addition, whether or not neoplasms are unicellular or multicellular in origin, most are heterogeneous and contain many subpopulations of cells with differing phenotypes and biological behaviour. During the metastatic process only few cells survive; to establish metastases, tumour cells have to complete all of the steps involved in the metastatic process, and enhanced performance in one step does not compensate for an inability to complete a subsequent step.

Therefore, a lot of effort has been undertaken to identify the factors that may affect and regulate the metastatic potential. Under this point of view, the platelet proaggregating activity of tumour cells may be a rate limiting factor during a haematogenous dissemination, and tumour cell-induced platelet aggregation has been shown to correlate with the metastatic ability in many [17, 18] though not all [19, 20, 27] cell lines.

Platelet proaggregating activity of human tumour cell lines has been related to various mechanisms, including the generation of thrombin by tumour cells [7–9]. A mechanism involving the production and release of tumour-associated cysteine proteinases has also been proposed to play a major role in both proaggregating and procoagulant activities of colon carcinoma cells [20]. In the present study the incomplete response to apyrase and the lack of significant ATP/ADP secretion from tumour cells (the amount of ADP released is lower than the physiological threshold concentrations capable of inducing platelet activation and primary aggregation) support the hypothesis that, in our system, mediators other than ADP are responsible for the early events in tumour cell/platelet interaction. The present results suggest that the interaction between platelets and the six colorectal tumour cell lines under investigation may involve a thrombin-related mechanism, as demonstrated by the consistent lack of proaggregating activity after incubation with TAME.

These results are in partial disagreement with a previous report [6] stating that the HT-29 colorectal tumour cells induce a biphasic platelet aggregation, which is completely blocked in the presence of apyrase (125 μg/ml), but not of hirudin (100 U/ml), thus suggesting a possible ADP-related mechanism of action. The discrepancies observed may be explained by differences in the type and grade of apyrase used and by the consideration that hirudin can be considered a more specific inhibitor of thrombin at the level of the coagulative cascade, while being less

effective in inhibiting the α- and γ-thrombin-induced platelet aggregation [28]. In fact, complexing of hirudin to α-thrombin is postulated to begin at the fibrin(ogen)-recognition exosite, and hirudin binding to α-thrombin completely inhibits the fibrinogen clotting activity, while it is not crucial for other enzymatic properties, such as factor VIII or V activation. Moreover, the fibrin(ogen)-recognition exosite is essentially deleted in γ-thrombin, which still retains platelet activating properties [28]. Furthermore, in utilizing carcinoma cell lines, it was reported that the antigenic phenotype of certain tumour cell lines changed with extended passages *in vitro*, a phenomenon termed antigenic drift [29], thus suggesting that the cell lines used in 1982 experiments may be somehow different from the one used in the present study.

Nevertheless, the primary aim of the present study was to investigate whether a correlation exists between the platelet proaggregating activity of human colorectal tumour cell lines and the degree of cell differentiation, using homologous systems. It is worth noting that a great body of evidences supporting, or arguing against, the correlation between platelet activating properties and metastatic potential has been obtained using murine tumour cell lines [17–19, 27]. However, in a more recent study, no relationship was found between clinical and histopathological findings and aggregation patterns when platelets from colon cancer patients were stimulated with their own tumour cells [20].

In the present study the degree of cell differentiation did not correlate with the platelet proaggregating activity of six different human colorectal tumour cell lines. In fact, significant differences were observed between the well differentiated GEO and LS174T cell lines, while the comparison between GEO and the poorly differentiated MIP cell lines failed to show any significant difference. Similar results were obtained when comparing the well differentiated LS174T cell line with the moderately differentiated HT-29 and WiDr, and the poorly differentiated DLD-1 cell lines.

These results were fully confirmed by the ultrastructural observations, indicating that platelet/tumour cell interaction may be found in all the cell lines under investigation, independently from the percentage of *in vitro* platelet aggregation reached with the single cell lines. In fact, tight membrane contacts could be observed with the well differentiated GEO cell line, as well as with both the moderately and poorly differentiated cell lines.

The correlation between the degree of differentiation and the *in vivo* invasive and metastatic potential of the six tumour cell lines under investigation has been previously suggested by data showing that the growth kinetics *in vivo* for each cell type were inversely proportional to the degrees of differentiation [30]. For example, the poorly differentiated MIP cell line grew rapidly *in vivo*, as subcutaneous tumours in athymic mice, and formed poorly differentiated colorectal carcinomas. In contrast, the GEO cells, which show a proaggregating activity similar to that observed for the MIP cell line, grew much more slowly and produced highly differentiated tumours [30].

In conclusion, the present results suggest that platelet proaggregating activity of the six human colorectal tumour cell lines under study, as well as their ability to closely interact with platelet membranes, is unrelated to the degree of cell differentiation. However, the debate about the role of platelets in haematogenous metastasis is still open.

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Potentiation of EO9 Anti-tumour Activity by Hydralazine

Michael C. Bibby, N. Ruth Sleigh, Paul M. Loadman and John A. Double

EO9[3-hydroxy-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol] has been selected for phase I evaluation in Europe. Activity has been seen previously in a highly refractory, necrotic mouse adenocarcinoma (MAC 16) but EO9 is shown here to be inactive against early tumours (MAC 15A and MAC 13) and a well vascularised, well-differentiated established adenocarcinoma (MAC 26). EO9 becomes active against MAC 26 tumours when hydralazine (10 mg/kg) is administered 1 min after EO9. Co-administration of hydralazine decreases EO9 plasma clearance and increases plasma area under the curve values (0.053 to 0.115 μg h/ml). These pharmacokinetic changes are accompanied by anti-tumour activity but no increase in bone marrow toxicity so this therapeutic gain may be due, at least in part, to microenvironmental changes resulting from hydralazine induced tumour vascular shutdown.
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INTRODUCTION

THE INDOLOQUINONE EO9 [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)-prop-β-en-α-ol] has been selected for phase I evaluation in Europe under the auspices of the EORTC New Drug Development Coordinating Committee and Office. EO9 was chosen from a series of indoloquinones synthesised by Oostveen and Speckamp [1] because of good activity against human solid tumour cell lines *in vitro* and promising activity against solid murine and human tumour xenografts *in vivo* [2].

Bioreductive alkylation is thought to play a major role in the mechanism of action of EO9 with the activation being catalysed by the two-electron donating flavoenzyme DT-diaphorase [NAD(P)H: (quinone-acceptor) oxidoreductase, EC1.6. 99.2]. The two-electron reduction of EO9 via DT-diaphorase generates DNA damaging species *in vitro* [3].

Hypoxic cells within a solid tumour mass exist in an environment that is more conducive to reductive reactions than their well-oxygenated counterparts [4]. It is likely then that EO9 might be more active against established solid tumours than against ascitic tumours or early tumour deposits, assuming that drug delivery to the hypoxic fraction of solid tumours is not impaired. Previous studies in this laboratory have shown activity against a normally refractory tumour (MAC 16) [5] which becomes highly necrotic [6, 7]. Another way to test the "bioreductive potential" of EO9 *in vivo* might be to utilise an initially well-vascularised tumour which can be rendered hypoxic by the use of a vasoactive agent. Hydralazine has been shown to enhance the effectiveness of other bioreductive agents [8, 9]. The present study examines the *in vivo* activity of EO9 against three members of a panel of murine colon adenocarcinomas (MAC tumours) comprising an ascitic tumour and two solid tumours of varying growth characteristics and morphology. The investigation aims to compare activities against ascites, recently implanted and advanced tumours, and also to examine the influence of co-administration of hydralazine on the activity of EO9 against a well vascularised tumour in which the vasculature has already been shown to respond to hydralazine [10]. Since in a previous study we have demonstrated that hydralazine causes significant changes to the pharmacokinetics of the anti-cancer agent tauromustine [11] this investigation will also examine the influence of hydralazine on mouse plasma pharmacokinetics of EO9 and on mouse bone marrow toxicity.

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