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Original Paper

Characterisation of Platelet Aggregation Induced by PC-3 Human Prostate Adenocarcinoma Cells and Inhibited by Venom Peptides, Trigramin and Rhodostomin

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PC-3 cells, a metastatic human prostate adenocarcinoma line, caused dose-dependent platelet aggregation in heparinised human platelet-rich plasma (PRP). PC-3 tumour cell-induced platelet aggregation (TCIPA) was completely inhibited by hirudin (5 U/ml) and limited by increasing concentrations of apyrase. This TCIPA was unaffected by cysteine proteinase inhibition with E-64 (10 μM), but was limited by cell pretreatment with phospholipase A_2. PC-3 cell suspension caused marked, dose-dependent decreases in plasma recalcification times using normal, Factor VIII-deficient and Factor IX-deficient, but not Factor VII-deficient, human plasma. This effect was potentiated in cell lysates, but was inhibited in intact cells preincubated with sphingosine. Overall, these data suggest that PC-3 TCIPA arises from PC-3 tissue factor activity expression. Trigramin and rhodostomin, RGD-containing snake venom peptides which antagonise the binding of fibrinogen to platelet membrane glycoprotein IIb–IIIa, prevented PC-3 TCIPA. Similarly, synthetic peptide GRGDS as well as monoclonal antibodies against platelet membrane glycoproteins IIb–IIIa and Ib prevented PC-3 TCIPA, which was unaffected by control peptide GRGDS. On a molar basis, trigramin (IC_{50}, 0.11 μM) and rhodostomin (IC_{50}, 0.03 μM) were approximately 5000 and 18000 times, respectively, more potent than GRGDS (IC_{50}, 0.56 mM).

Key words: prostate adenocarcinoma, platelet aggregation, metastasis, tissue factor, glycoprotein IIb–IIIa, thrombin, Factor VII, snake venom peptide, fibrinogen, Arg–Gly–Asp
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INTRODUCTION

THE TENDENCY of tumour cells to metastasise, a critical feature of their malignant potential, may be linked to interactions between tumour cells and platelets [1, 2]. Studies with murine tumour cell lines show that platelets can facilitate all the intermediate steps of haematogenous metastasis, from initial tumour cell arrest in the microvasculature to tumour cell proliferation after arrest, tumour cell interactions with the subendothelial matrix and tumour cell extravasation [3–6]. Arrest of intraveneously injected tumour cells in the microcirculation is accompanied by formation of platelet aggregates around these cells [7–9]. Therefore, aggregation of host platelets by tumour cells may play an important role in the process of metastasis.

The participation of platelets in metastasis probably arises either from direct platelet binding to tumour cells or from release reaction products following platelet aggregation induced by tumour cells, or both [10]. With an *in vivo* metastasis model, the number of pulmonary metastatic nodules correlates with the ability of a given tumour cell line to induce platelet aggregation *in vitro* [11, 12]. TCIPA is gaining acceptance as a key intermediate step in the process of blood-borne metastasis [13]. In previous reports [12, 14, 15], an adequate platelet number was necessary for metastasis since induction of thrombocytopenia was associated with a decrease in the number of metastatic lesions; reconstitution of metastasis was noted upon platelet repletion. Moreover, many antiplatelet agents have potent antimetastatic effects [16–18]. These

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observations have provided persuasive evidence that platelets play an important role in facilitating cancer metastasis.

Purified components from snake venoms, including trigramin-like antiplatelet peptides [19, 20], have been found to affect platelet function. Trigramin and rhodostomin are Arg–Gly–Asp (RGD)-containing peptides purified from venoms of the snake *Trimeresurus gramineus* and the Malayan pit viper *Agkistrodon rhodostoma*, respectively [21–24], which inhibit platelet aggregation by antagonising the binding of fibrinogen and platelet membrane glycoprotein IIb–IIIa (i.e. integrin $\alpha_{IIb}\beta_3$). Recently, there is evidence that cells derived from solid tumour possess a receptor immunologically related to platelet $\alpha_{IIb}\beta_3$ [25, 26] in addition to other RGD-related integrins. These integrin receptors have been shown to play a critical role in adhesive and migratory activities of tumour cells [26]. The trigramin-like peptides all contain the epitope RGD, are rich in cysteine, and were already shown to inhibit tumour cell adhesion and migration via binding to RGD-related integrins (i.e. $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$) expressed on tumour cells [27, 28].

Prostate cancer is quite common in males and its proclivity for metastasis, especially to bone, well recognised. However, few data exist regarding mechanisms of prostate cancer metastasis. Here, we show that PC-3 cell suspension is a potent inducer of platelet aggregation via thrombin formation. The platelet-aggregating and procoagulant activity of the PC-3 cell line, which we probed with a variety of inhibitors, is expressed via tissue factor-like activity. We also evaluated the effects of trigramin and rhodostomin on PC-3 TCIPA in heparinised human platelet-rich plasma, and compared their action with that of synthetic peptides and monoclonal antibodies.

MATERIALS AND METHODS

PC-3 human prostate adenocarcinoma cells were provided by the Department of Bacteriology, College of Medicine, National Taiwan University. *Trimeresurus gramineus* venom and *Agkistrodon rhodostoma* (or *Calloselasma rhodostoma*) venom was purchased from local merchants and from Latoxan (France), respectively, and stored at −20°C. Trigamin and rhodostomin were purified from venoms of *T. gramineus* and *A. rhodostoma*, respectively, as previously described [26, 28]. Gly–Arg–Gly–Asp–Ser (GRGDS) was purchased from Peninsula Laboratories, California, U.S.A. Gly–Arg–Gly–Glu–Ser (GRGES) was synthesised by the Biochemical Institute, College of Medicine, National Taiwan University. Apyrase (grade III), heparin, hirudin (grade IV from leeches), D-sphingosine, human thrombin, phospholipase A_2 (from *Laticauda semifasciata*) and 1-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) were obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. Tissue thromboplastin reagent was Simplastin Excel standard (Organon Teknika Corp. Durham, North Carolina, U.S.A.). Human plasmas deficient in coagulation factors VII, VIII or IX were obtained from Sigma Monoclonal antibodies (MAbs) $7E_3$ and $10E_5$, raised against the glycoprotein (GP) IIb–IIIa complex, were kindly supplied by Dr B. Coller (State University of New York, Stony Brook, New York, U.S.A.). MAb AP_1, raised against platelet membrane GP Ib and AP_3, raised against GP IIIa were kindly supplied by Drs P. Newman and R. Montgomery (Milwaukee Blood Center, Milwaukee, Wisconsin, U.S.A.). Cell culture reagents and materials, including Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS), were obtained from Gibco, Grand Island, New York, U.S.A.

Cell culture

PC-3 human prostate carcinoma cells were grown in 95% air–5% CO_2 in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Confluent monolayers were harvested from tissue culture flasks by brief treatment with trypsin (0.25%, w/v) and EDTA (1 mM). They were washed 3 times to remove residual FCS and finally resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.25). A Trypan blue exclusion test was performed for cell viability assessment, which was more than 95% in these cultures.

Aggregation studies

Human blood was anticoagulated with heparin (final concentration 1 U/ml). Platelet-rich plasma (PRP) was prepared by centrifugation at 120*g* for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared from the remaining blood by additional centrifugation at 500*g* for 10 min. PRP was adjusted with PPP to contain 3.0×10^8 platelets/ml. Platelet aggregation of PRP was measured turbidimetrically with a Lumi-aggregometer (Chrono-log). PRP (400 μl) was prewarmed at 37°C for 2 min in a silicone-treated glass cuvette. Each inhibitor, snake venom peptide, monoclonal antibody or peptide, was added at indicated intervals before addition of 35 μl of PC-3 cell suspension (5×10^4 cells/ml, final concentration). In some experiments, PC-3 cell suspensions were pretreated with either snake venom peptide or monoclonal antibody at 37°C for 20 min, washed and followed by addition of the same concentrations of cells to induce platelet aggregation. The reaction was allowed to proceed for at least 10 min and the degree of aggregation expressed as change in light transmission.

Measurement of procoagulant activity

Procoagulant activity of the tumour cells were measured by plasma recalcification time [29]. Platelet-poor plasma (PPP) was prepared from whole blood, collected from healthy human volunteers and mixed with 3.8% (w/v) sodium citrate (9:1, v/v). One hundred microlitres of fresh normal citrated PPP or human plasmas deficient in Factors VII, VIII or IX were incubated with an equal volume of cell suspension at various dilutions for 2 min at 37°C. Therefore, 100 μl of prewarmed 25 mM $CaCl_2$ was added, and the plasma clotting time was determined. Tissue thromboplastin was used as a positive control for activating the extrinsic coagulation pathway. Cell lysate was prepared by immersion of the cell suspension into 95% ethanol and dry ice, followed by thawing at 37°C. This was repeated twice. D-sphingosine solution was prepared as described by Conkling and associates [30]; sphingosine was delivered with albumin in all cases because it minimises toxicity of this compound [31]. Cells were cultured alone or with sphingosine (100 μM final concentration) and incubated overnight (18 h) at 37°C. Recalcification times with cells or cell lysates were then measured.

RESULTS

Platelet aggregation induced by prostate carcinoma PC-3

Human prostate carcinoma PC-3 induced dose-dependent, irreversible, platelet aggregation in heparinised human PRP

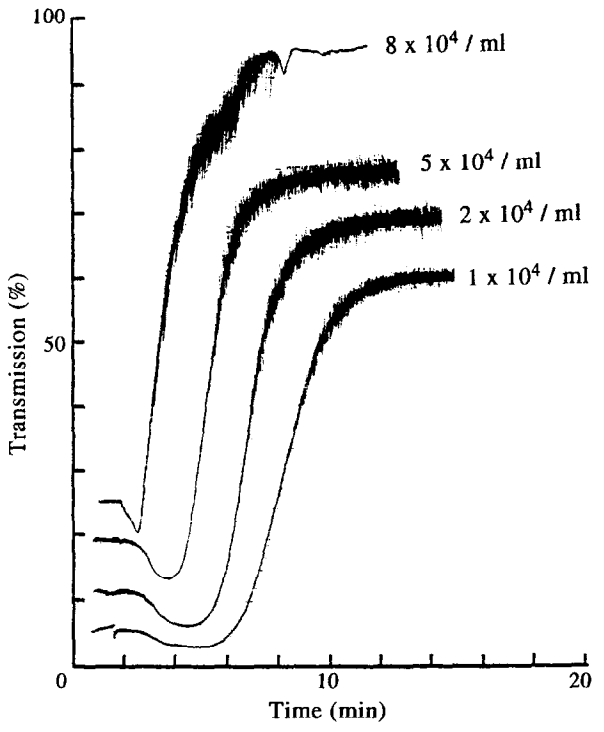

Figure 1. Concentration effect on platelet aggregation induced by human prostate carcinoma PC-3 in human heparinised PRP. PC-3 cell suspension was added to heparinised PRP to trigger platelet aggregation (upward tracing).

(Figure 1). Concentrations of more than 1×10^4 cells/ml caused aggregation. The tracings of aggregation induced by at least 8×10^4 cells/ml of PC-3 cells were interrupted by fibrin clot formation (Figure 1), which was also grossly evident. The lag phase preceding aggregation became progressively shorter as tumour cell concentration was increased. However, inter-donor variability was seen in the duration of the lag phase as well as in the rate and amplitude of the aggregatory response to a given concentration of tumour cells.

Effects of apyrase and hirudin on PC-3 TCIPA

PC-3 cells at a concentration of 5×10^4 cells/ml were used for the following platelet aggregation studies. Pretreatment with the ADP scavenger, apyrase (0.5 U/ml final concentration), in PRP did not inhibit PC-3-induced platelet aggregation (Figure 2a) but the lag phase preceding TCIPA was slightly prolonged. Furthermore, increasing the concentration of apyrase in PRP from 0.5 to 1 U/ml produced a marked increase in the lag time and a partial inhibition of the aggregation, while at 1.5 U/ml apyrase inhibited PC-3 TCIPA in a 20-min reaction period (data not shown). Hirudin, a specific thrombin inhibitor (5 U/ml), completely blocked the aggregation response (Figure 2b). Thus, thrombin formation is required for PC-3 TCIPA.

We obtained serum-free 12- and 24-h-conditioned medium from confluent PC-3 cultures. Each conditioned medium was added in equivalent volume (35 μl) or more to platelets. Conditioned medium did not induce platelet aggregation; this suggests that PC-3 TCIPA is not triggered by any product secreted by PC-3 cells.

Effects of cysteine proteinase inhibition and phospholipase A_2 on PC-3 TCIPA

To determine the source of thrombin generated in PC-3 TCIPA, we used E64 (1-trans-epoxysuccinyl-leucylamino(4-guanidino)butane) (10 μM), a highly specific and irreversible cysteine proteinase inhibitor. No significant inhibitory effect on PC-3 TCIPA was observed (Figure 2c). Thus, PC-3 cell-induced thrombin generation was not dependent upon cysteine proteinases which can activate coagulation factors [32].

Pretreatment of PC-3 cells with phospholipase A_2 (50 μg/ml) at 37°C for 30 min almost completely blocked PC-3 TCIPA (Figure 2d). Intact phospholipid is thus required for the platelet-aggregatory activity of PC-3 cells.

Effect of PC-3 suspension on plasma recalcification time

As shown in Table 1, PC-3 cell suspension shortened the one-stage recalcification time of normal human citrated PPP in a concentration-dependent manner. In addition, the clotting times of Factor VIII- and Factor IX-deficient plasmas were shortened to a similar extent by the tumour cell suspension. However, PC-3 suspension did not greatly shorten the recalcification time of Factor VII-deficient plasma. Similar results were obtained with rabbit brain tissue thromboplastin (Table 1). When cells were lysed by three freeze-thaw cycles before the assay, the recalcification time of normal PPP relative to untreated cell suspension was further shortened (Figure 3). When cells were preincubated with sphingosine (100 μM) for 18 h at 37°C, there was a marked prolongation of recalcification time (Figure 3).

Effects of trigramin, rhodostomin, synthetic peptides and MAbs on PC-3 TCIPA

The binding of fibrinogen to its receptor is mainly through the peptide sequence RGD in fibrinogen [33]. Since trigramin and rhodostomin are both RGD-containing antiplatelet peptides, it is reasonable to predict that either should inhibit PC-3

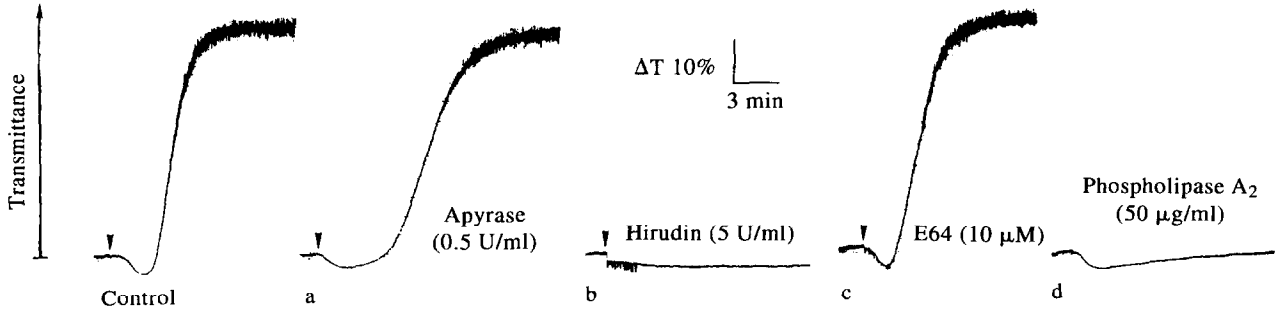

Figure 2. Effect of (a) apyrase, (b) hirudin, (c) E64 and (d) phospholipase A_2 on PC-3 TCIPA.

Table 1. *Recalcification clotting times of normal and coagulation factor-deficient plasmas in the presence of PC-3 cells. Data are presented as median and range* (n = 3–7)

	Plasma recalcification times (s)			
	Normal	Factor IX-deficient	Factor VIII-deficient	Factor VII-deficient
PBS buffer				
Median	366	>600	>600	354
Range	351–379			332–370
Thromboplastin				
Median	17	16	17	39
Range	16–17	15–17	16–17	37–42
PC-3 cells				
2×10^4 cells/ml				
Median	211	218	214	345
Range	205–216	213–224	208–221	337–351
2×10^5 cells/ml				
Median	98	103	101	308
Range	91–103	98–107	97–105	299–315
6×10^5 cells/ml				
Median	60	63	62	292
Range	54–62	60–67	58–65	288–297
2×10^6 cells/ml				
Median	47	48	47	275
Range	43–51	45–51	43–49	268–279

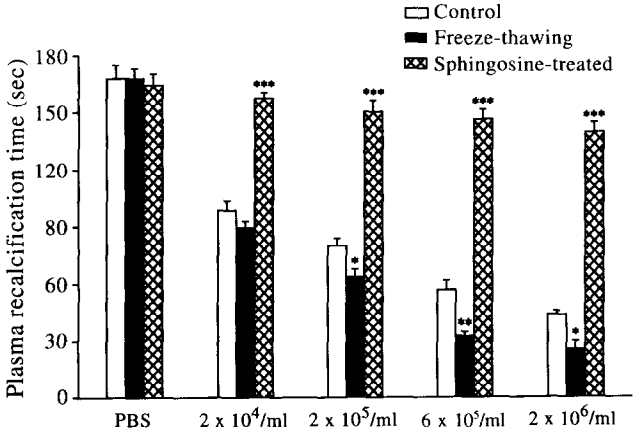

Figure 3. Plasma recalcification time of intact cells (control), freeze-thawing cell lysate and sphingosine-treated cells in normal human citrated plasma. For detailed experimental procedures, see Materials and Methods. Data are presented as mean ± S.E.M. as compared to control, at Student's *t*-test ($n = 4$–7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

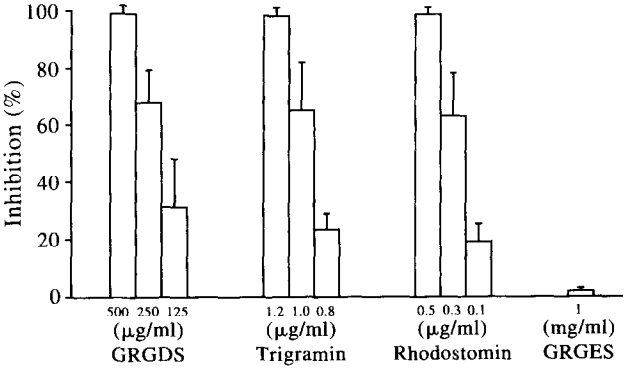

Figure 4. Effect of trigramin, rhodostomin and GRGDS on PC-3 TCIPA. The data are presented as mean ± S.E.M. ($n = 6$).

TCIPA. Pretreatment of platelets with trigramin (1.2 μg/ml), rhodostomin (0.5 μg/ml) or GRGDS (500 μg/ml) completely inhibited PC-3 TCIPA while GRGDS peptide (up to 1 mg/ml) had no significant effect. PC-3 TCIPA was inhibited dose-dependently either by snake venom peptide or by GRGDS (Figure 4). On a molar basis, trigramin (IC_{50}, 0.11 μM) and rhodostomin (IC_{50}, 0.03 μM) are 5000- and 18000-fold more potent, respectively, than GRGDS (IC_{50}, 0.56 mM).

Pretreatment with monoclonal antibodies $7E_3$ or $10E_5$ (at 25 μg/ml) completely inhibited PC-3 TCIPA (Figure 5). A marked inhibition was also observed with monoclonal antibody AP_1 (25 μg/ml) whereas no significant inhibitory effect was observed after pretreatment with AP_3 (25 μg/ml). However, no inhibition of TCIPA was observed when PC-3 cells were pretreated with either snake venom peptide (3.5 μg/ml), monoclonal antibodies $7E_3$, $10E_5$ (both at 25 μg/ml) or AP_1 (25 μg/ml), and washed before the addition of these PC-3 cells (data not shown).

DISCUSSION

We present here the first evidence for platelet aggregation being triggered by a human prostate tumour cell line. Human prostate carcinoma PC-3 cell suspension is a potent inducer of aggregation for human platelets, and this ability is a property it shares with certain other tumour cell lines of known metastatic potential [2, 13, 34]. TCIPA is not a universal property of tumour cells, in general, nor of prostate adenocarcinoma cells in particular. For example, the DU-145 human prostate adenocarcinoma line is completely inactive with regard to TCIPA (unpublished observations). Rather, the ability to cause TCIPA is a feature of many metastasis-competent tumour cell types. The PC-3 line is derived from a lumbar metastatic lesion [35]. TCIPA effectiveness *in vitro* appears to correlate with metastatic potential *in vivo* [11, 12].

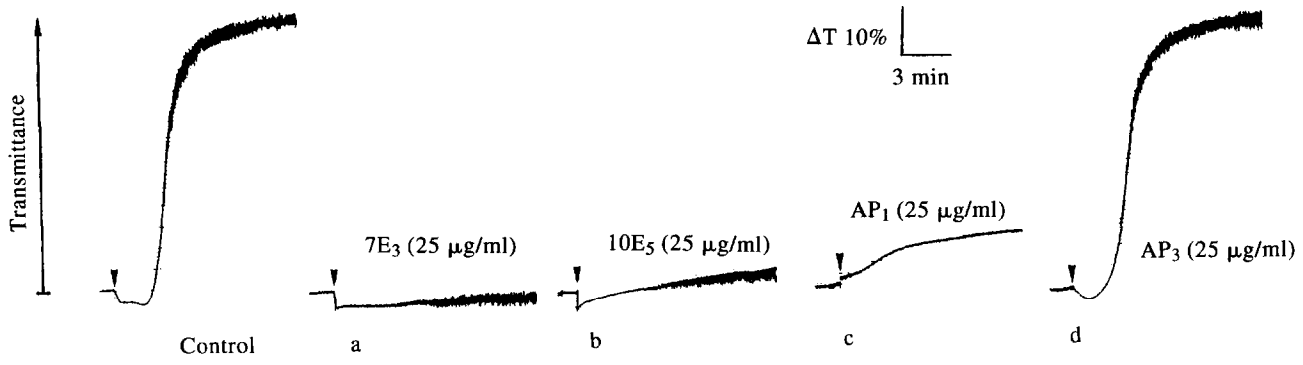

Figure 5. Effect of monoclonal antibodies, (a) $7E_3$, (b) $10E_5$, (c) AP_1 and (d) AP_3 on PC-3 TCIPA.

However, the mechanism by which platelet aggregation is induced varies among tumour cell lines. Several factors can be classified as soluble stimulators shed by circulating tumour cells. Some tumour cells cause aggregation via thrombin-like proteinases or by initiating formation of thrombin itself [13, 34]. Some release cathepsin B, which gives rise to thrombin [32] or to ADP [36], a platelet agonist. Other mechanisms include interaction between tumour cells and platelets via a trypsin-sensitive protein on the tumour cells [37]. The formation of a sialolipoprotein complex possibly participates in the interaction between some tumour cells and platelets [38].

We have shown that the PC-3 line causes irreversible TCIPA, following a lag phase which was dependent on tumour cell concentrations (Figure 1). PC-3 cell suspension was approximately 10-fold more potent than human hepatoma cells [29] in causing TCIPA. PC-3 cells also afforded clot formation at tumour cell concentrations of more than 8×10^4 cells/ml, which always followed the initial phase of aggregation, since platelet aggregation requires less thrombin than fibrinogen proteolysis in PPP. PC-3 TCIPA was observed in heparinised PRP but not in citrated PRP, and the lag phase preceding irreversible platelet aggregation was markedly prolonged by raising the concentration of heparin (data not shown), suggesting that PC-3 TCIPA is mediated by thrombin formation.

Selective inhibitors of platelet agonists were used to characterise the mechanism of PC-3 TCIPA, which appears to be thrombin-dependent (Figure 2). As reported elsewhere, some tumour cell lines cause TCIPA through generation of thrombin by activation of the coagulation cascade [39–42]. We interpret the lag phase preceding platelet aggregation as an accumulation of sufficient thrombin to trigger aggregation. This is consistent with the observation elsewhere that thrombin inhibitors prolonged the TCIPA lag period in a dose-dependent manner, but did not influence the maximal aggregation response once platelets began to aggregate [39]. Although PC-3 TCIPA was essentially thrombin-dependent, apyrase was found to prolong the lag phase preceding TCIPA (Figure 2). Furthermore, very high concentrations of apyrase even blocked TCIPA. Such TCIPA co-dependence on thrombin and ADP has been reported for other tumour cell lines, such as human colon carcinoma Colo 205 and Colo 397 [43]. The reason may reside in the rate or amount of thrombin generation. Low doses of thrombin are thought to mediate platelet aggregation, in part by triggering platelet release of ADP, whereas thrombin at higher concentrations will produce aggregation independent of ADP release [44].

The shortening of recalcification time discloses the procoagulant activity of PC-3 cells. At least five different types of tumour cell procoagulants have been described: (a) a Factor VII-dependent tissue factor-like substance [39, 40, 43, 45]; (b) proteinase activating Factor X to Xa [46]; (c) a cysteine proteinase isolated from malignant tissues [47]; (d) platelet-activating/procoagulant activity purified from murine and rat tumour cells [48]; and (e) HLA-DR (a class II MHC molecule) expressed on some tumour cells [49]. Our studies showed that E-64 had no significant effect on PC-3 TCIPA, suggesting that PC-3 cell-induced thrombin generation was not caused by cysteine proteinase activity (Figure 2). However, it may be mediated by tissue factor-dependent thrombin generation as PC-3 TCIPA was blocked by phospholipase A_2 pretreatment (Figure 2). It has been speculated that the tissue factor (TF)-like activity may be located on tumour cell surfaces [43]. This may reflect the fact that phospholipids are required for the expression of TF-like activity of PC-3 cells, consistent with other reports regarding some tumour cell lines [39, 40, 43]. PC-3 procoagulant activity is dependent upon the presence of Factor VII but not that of Factors VIII or IX (Table 1). Tissue thromboplastin behaves very similarly to PC-3 cells in shortening recalcification times.

Thus, PC-3 TCIPA is seemingly mediated by thrombin generated through TF-like activity of PC-3 cells. This is corroborated by our observations with cell lysate recalcification times, which are even shorter than those of suspensions of intact cells. Le and associates have demonstrated the importance of anionic phospholipids, such as phosphatidylserine, for full manifestation of TF activity [50]; such phospholipids are typically present almost exclusively on the cytoplasmic leaflet of the membrane bilayer. In their study, as in ours, cell lysates had enhanced TF activity. We further probed the apparent PC-3 TF activity with sphingosine, which has been shown to inhibit blood coagulation initiated by lipopolysaccharide-stimulated human monocytes by inhibition of cell-associated TF activity [30]. Sphingosine may be inserted into membrane phospholipid, perturbing the ability of phospholipid to promote TF–Factor VII–VIIa binding [30]. When we preincubated intact PC-3 monolayers with sphingosine (100 μM), the cell suspension prepared thereafter was ineffective at shortening plasma recalcification time (Figure 3).

Trigramin and rhodostomin are RGD-containing antiplatelet peptides which directly impair the interaction of fibrinogen with its specific receptor associated with the glycoprotein IIb–IIIa complex [25–28]. The fibrinogen binding to the activated platelets is a final common pathway of platelet aggregation

induced by ADP, epinephrine, thrombin, collagen and prostaglandin endoperoxides. It is well established that the platelet receptor recognition site on human fibrinogen involves the RGD epitope [51]. As shown in Figure 4, PC-3 TCIPA was completely inhibited by either snake venom peptide or by GRGDS. This result is similar to that with monoclonal antibodies against glycoprotein IIb–IIIa (Figure 5). Trigramin and rhodostomin are approximately 5000- and 18000-times, respectively, more potent than GRGDS in inhibiting PC-3 TCIPA. Rhodostomin is 3–5 times more potent than trigramin in inhibiting ADP-induced platelet aggregation in human PRP as well as fibrinogen binding to activated platelets (unpublished data), correlating with relative potency in inhibiting PC-3 TCIPA. Thus, a similar inhibitory mechanism for PC-3 TCIPA among RGD-containing peptides and anti-GP IIb–IIIa MAb is through blockade of fibrinogen binding to its receptor on the platelet surface membrane. We attribute the partial inhibitory effect of anti-GP Ib MAb (Figure 5) to the fact that thrombin binding to GP Ib causes platelet activation [52]. In contrast, anti-GP IIIa MAb had no inhibitory effect on PC-3 TCIPA (Figure 5). Furthermore, blockade of integrin receptors on PC-3 cells by pretreatment with each antagonist (i.e. snake venom peptides or monoclonal antibodies) did not affect TCIPA, suggesting that the inhibitory effects shown in our study were not due to blockade of integrin receptors expressed on PC-3 cells.

The thrombin-dependent PC-3 TCIPA due to PC-3 TF activity expression and inhibited by platelet glycoprotein IIb–IIIa antagonists, particularly the strikingly potent venom peptides trigramin and rhodostomin, suggests a variety of possible avenues for biological and pharmaceutical intervention in the management of human neoplastic disease. Because TCIPA is seemingly a critical step in tumour metastasis, proteinase inhibitors, sphingosine-related anti-TF compounds and fibrinogen receptor antagonists, such as trigramin and rhodostomin, might have utility as adjunct therapeutic agents in preventing some cancer metastases.

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