The Arg-Gly-Asp-containing peptide, rhodostomin, inhibits in vitro cell adhesion to extracellular matrices and platelet aggregation caused by Saos-2 human osteosarcoma cells

H-S Chiang¹, R-S Yang² and T-F Huang¹

¹Pharmacological Institute and ²Department of Orthopaedics, College of Medicine, National Taiwan University, Taipei, Taiwan.

Summary Saos-2 cells, derived from a primary human osteosarcoma, caused dose-dependent platelet aggregation in heparinised human platelet-rich plasma. Saos-2 tumour cell-induced platelet aggregation (TCIPA) was completely inhibited by hirudin but unaffected by apyrase. The cell suspension shortened the plasma recalcification times of normal, factor VIII-deficient and factor IX-deficient human plasmas in a dose-dependent manner. However, the cell suspension did not affect the recalcification time of factor VII-deficient plasma. Moreover, a monoclonal antibody (MAb) against human tissue factor completely abolished TCIPA. Flow cytometric analysis using anti-integrin MAbs as the primary binding ligands demonstrated that the integrin receptors α1β1, α5β1, and α6β4, were present on the surface of Saos-2 cells, which might mediate tumour cell adhesion to extracellular matrix. Rhodostomin, an Arg-Gly-Asp (RGD)-containing snake venom peptide which antagonises the binding of fibronectin to platelet membrane glycoprotein IIb IIIa, prevented Saos-2 TCIPA as well as tumour cell adhesion to vitronectin, fibronectin and collagen type I. Likewise, the synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) showed a similar effect. On a molar basis, rhodostomin was about 18,000 and 1000 times, respectively, more potent than GRGDS in inhibiting TCIPA and tumour cell adhesion.

Keywords: osteosarcoma; platelet aggregation; tissue factor; integrin; extracellular matrix; Arg-Gly-Asp-containing peptide

The formation of a metastatic lesion is the result of a complex series of events called the ‘metastatic cascade’. Following intravasation, circulating tumour cells interact with a variety of host cells such as effectors of the cellular immune responses, endothelial cells and platelets. The interaction of tumour cells and host platelets may promote the metastatic process (Honn et al., 1992). Indeed, there is evidence that the incidence of lymph node metastasis caused by ten cell lines, from rat renal carcinoma, in vivo correlates well with their ability to induce platelet aggregation in vitro (Pearlstein et al., 1980).

Platelets may enhance tumour cell adhesion to endothelial cells and subendothelial surfaces during the haematoogenous metastasis process. The cell–cell or cell–extracellular matrix (ECM) interactions of both normal and tumour cells have been proven to be mediated by a variety of plasma membrane receptors including the integrin family (Hynes et al., 1987). Integrins are transmembrane proteins which link the ECM with the cell cytoskeleton. Such linkage enables cellular attachment to substrata and forms focal adhesion plaques in distinct plasma membrane regions which establish contact with the matrix (Grunnicke, 1990). The interaction of integrins with adhesion proteins has been shown to be partially mediated by binding of integrin to Arg-Gly-Asp (RGD), the short hydrophilic amino acid sequence within adhesion proteins (Pierschbacher and Rouslahti, 1984; Rouslahti and Pierschbacher, 1987).

Purified components from snake venoms have been widely studied and found to affect platelet function, including trigramin-like anti-platelet peptides (Huang et al., 1987a, 1991a,b; Shebuski et al., 1989; Rucinski et al., 1990). Trigramin, an RGD-containing peptide purified from venom of the Trimeresurus gramineus snake, is a specific antigen of platelet membrane glycoprotein IIb IIIa (Huang et al., 1987a, 1989). Rhodostomin, another RGD-containing peptide purified from the venom of the Malayan pit viper Agkistrodon rhodostoma, also inhibits platelet aggregation by antagonism of the Gp IIb IIIa–fibronogen interaction (Huang et al., 1987b, 1990). These peptides all contain the RGD sequence, are rich in cysteine and bind with high affinity to the surface of platelets.

The in vitro metastatic characterisation of osteosarcoma is not yet well understood. In the present study, we examined in vitro the TCIPA caused by Saos-2 human osteosarcoma cells. We probed Saos-2 TCIPA with a variety of inhibitors and monoclonal antibodies in order to characterise fully the mechanism of this phenomenon. Rhodostomin was found to inhibit potently both TCIPA and tumour cell adhesion to ECM (i.e. fibronectin, vitronectin, collagen type I), which is closely related to the binding characterisation of RGD-dependent rhodostomin to the integrins expressed on the Saos-2 cell surface.

Materials and methods

Materials
Saos-2 human osteosarcoma cells were obtained from ATCC (American Type Culture Collection) Laboratory. Crude venom of Agkistrodon rhodostoma (or Calloselasma rhodosoma) was purchased from Latoxan (Rosans, France) and stored at −20°C. Rhodostomin was purified from venom of A. rhodostoma as previously described (Huang et al., 1989, 1990). GRGDS was purchased from Peninsula Laboratories, CA, USA. Gly-Arg-Gly-Ser (GRGES) was synthesised by the Biochemical Institute, College of Medicine, National Taiwan University. Asparyse (grade III), heparin, hirudin (grade IV from leeches), fibronectin (from bovine plasma), vitronectin (from human plasma), laminin (from basement membrane of mouse sarcoma), collagen type I (from calf skin) and type IV were obtained from Sigma (St Louis, MO, USA). Tissue thromboplastin reagent was Simplastin Excel standard (Organan Teknika, NC, USA). Coagulation factor-deficient human plasmas (deficient in factor VII, VIII or IX) were obtained from Sigma.

Monoclonal antibodies (MAbs) 7E, and 10E, raised against the glycoprotein (GP) IIb IIIa complex were kindly supplied by Dr B Coller (State University of New York, Stony Brook, NY, USA). MAb to human tissue factor was
obtained from Biogenesis (Bournemouth, UK). MCA757 (anti-α, β), MCA698 (anti-α, β), MCA699 (anti-α, β) and MCA794 (anti-α, β) were purchased from Serotec (Bicester, UK). Goat anti-mouse IgG-FITC was from Boehringer (Mannheim, Germany). Cell culture reagents and materials, including Dulbecco’s modified Eagle medium (DMEM) and fetal calf serum (FCS) were from Gibco (Grand Island, NY, USA). FCS was heat inactivated at 56°C for 30 min prior to use.

Cell culture
Saos-2 cells were cultured in a 95% air–5% carbon dioxide atmosphere using tissue culture-grade plastic flasks. Cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). Confluent monolayers were harvested from culture flasks with brief 0.1% trypsin–1 mM EDTA treatment. They were washed three times to remove residual FCS and finally resuspended in phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium hydrogen phosphate, 1.4 mM potassium phosphate, pH 7.25). Based on trypan blue exclusion studies, cell viability was greater than 95%.

Aggregation studies
Human blood was anticoagulated with heparin (final concentration 1 U ml⁻¹). 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 about 3 × 10⁷ platelets ml⁻¹. Platelet aggregation 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, monoclonal antibody or peptide was added at various times before addition of 20 µl of cell suspension (3 × 10⁶ cells ml⁻¹, final concentration). The reaction was allowed to proceed for at least 10 min and the degree of aggregation expressed as changes in light transmission.

Measurement of procoagulant activity
Procoagulant activity of the cell suspension was measured by plasma recalcification time (Sheu et al., 1992). PPP was prepared from whole blood, collected from healthy human volunteers and mixed with 3.8% (w/v) sodium citrate (9:1 v:v). In brief, 100 µl of either fresh normal citrated PPP or human factor-deficient plasmas (deficient in factor VII, VIII or IX) was incubated with 100 µl of cell suspension containing various concentrations of tumour cells for 2 min at 37°C. Thereafter, 100 µl of prewarmed 25 mM calcium chloride was added, and the plasma clotting time determined by a fibrometry (Coag-a-mate, Organon Teknika, NC, USA). Tissue thromboplastin was used as a positive control for activating the extrinsic coagulation pathway.

Flow cytometric analysis
Flow cytometric studies were performed to quantify surface expression of integrins (Grossi et al., 1989). Cells were detached (using 0.5 mM EDTA), washed free of serum proteins with Hanks' balanced salt solution (HBSS, pH 7.25) containing 2 mM Ca²⁺ and 2 mM Mg²⁺, then finally suspended at a concentration of 10⁶ cells per sample. Cells were fixed with paraformaldehyde (0.07%, 10 min) prior to labelling for surface integrins. The fixed cells were blocked with normal goat serum (1:2) for 25 min, and labelled with MAbS (25 µg ml⁻¹) for 1 h. After washing cells were finally relabelled with goat anti-mouse IgG-FITC. FITC signals were detected and digitised in logarithmic configuration and the data collected on a EPICS computer system. Data were collected at 256-channel resolution and 10 000 cells were counted per experimental group. Fluorescence intensity was directly proportional to the fluorescein label present on the tumour cell surface. The control fluorescence intensity was obtained with cell suspension in which primary antibodies were omitted. All experiments were repeated at least four times.

Adhesion studies
2',7'-Bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein acetoxymethyl (BCECF-AM) has been used in fluorescence-based viability assessment in adherent cell cultures (Vaporciyan et al., 1993). In our study, cells (5 × 10⁵) were incubated with fluorescent dye (2 µg ml⁻¹) in HBSS for 30 min at 37°C. Following incubation, cells were washed once in PBS, and finally resuspended in serum-free DMEM H/F 1:2 containing 0.5% of the medium supplement ITS +. Plates (96-well, Costar, USA) were coated overnight at 4°C with 50 µl of fibronectin (30 µg ml⁻¹), vitronectin (15 µg ml⁻¹), laminin (15 µg ml⁻¹) and collagen type I and type IV (80 µg ml⁻¹) in PBS. Cells were treated with rhodostomin or GRGDS (15 min at 25°C) before adhesion assay. Control or treated cells (2.5 × 10⁶) were added to each well and incubated for 24 h at 37°C. The non-adherent cells were removed by aspiration and the plates read with a CytoFluor 2300 fluorescence plate reader (Millipore, Bedford, MA, USA).

Results
Characterisation of platelet aggregation induced by Saos-2 cells
The Saos-2 cell suspension was a potent inducer of irreversible platelet aggregation in heparinised human PRP (Figure 1). This TCIPA was dose dependent and preceded by a lag phase. Cells at concentrations of more than 5 × 10⁶ cells ml⁻¹ caused aggregation. The tracings of aggregation induced by at least 5 × 10⁶ cells ml⁻¹ were interrupted by delayed fibrin clot formation (Figure 1), which was also grossly evident. The lag phase preceding aggregation became progressively shorter with increasing tumour cell concentrations.

Cell suspension at 3 × 10⁶ cells ml⁻¹ was used for the following platelet aggregation studies. Pretreatment with the ADP scavenger apyrase (0.5 U ml⁻¹, final concentration) in PRP did not inhibit TCIPA (Figure 2), indicating that ADP is essentially not involved. However, the lag phase preceding TCIPA was prolonged. Hirudin (5 U ml⁻¹), a specific thrombin inhibitor, completely inhibited the aggregation response (Figure 2), suggesting that formation of thrombin is required for Saos-2 TCIPA.

Effect of Saos-2 cells on the plasma recalcification time
As shown in Table I. Saos-2 cell suspension shortened the one-stage recalcification time of normal human citrated PPP in a concentration-dependent manner. The clotting times of factor VIII- and factor IX-deficient plasmas were similarly shortened. However, cell suspension did not shorten the recalcification time of factor VII-deficient plasma. A similar pattern of results was obtained with control thromboplastin. These data indicated that the procoagulant activity of Saos-2 cells is via activation of factor VII in the extrinsic coagulation pathway, leading to the activation of the common pathway.

Effect of rhodostomin and monoclonal antibodies on Saos-2 TCIPA
The binding of fibrinogen to its specific receptor is mainly through the peptide sequence RGD in fibrinogen (Plow et al., 1986). Pretreatment of platelets with rhodostomin, an RGD-containing snake venom peptide (0.5 µg ml⁻¹), completely inhibited Saos-2 TCIPA. This inhibition was also observed with synthetic peptide GRGDS (500 µg ml⁻¹), while GRGES (1 mg ml⁻¹) had no significant effect. Either snake venom
peptide or GRGDS inhibited TCIPA in a dose-dependent manner (Figure 3). On a molar basis, rhodostomin (IC\textsubscript{50} = 0.03 μM) is a 18,000-fold more potent than GRGDS (IC\textsubscript{50} = 0.56 mM). Pretreatment with MAb 7E\textsubscript{3} (25 μg ml\textsuperscript{-1}) against the platelet membrane GP IIb IIIa complex completely inhibited TCIPA (Figure 2). In addition, complete inhibition was obtained after pretreatment of cells with MAb against human tissue factor at 37°C for 20 min.

Integrin expression on Saos-2 cells

Integrins are a superfamily of cell-surface glycoproteins that mediate cell–cell and cell–ECM interactions. To determine whether integrins are expressed on the surface of Saos-2 cells, an indirect immunofluorescence measurement using flow cytometry was performed by utilising MAb specific for α\textsubscript{ii}β\textsubscript{3} (10E\textsubscript{7}), α\textsubscript{v}β\textsubscript{3} (MCA757), α\textsubscript{v}β\textsubscript{1} (MCA698), α\textsubscript{v}β\textsubscript{1} (MCA699) and α\textsubscript{v}β\textsubscript{1} (MCA794) as the primary antibodies. 10E\textsubscript{7} does not cross-react with the vitronectin (α\textsubscript{v}β\textsubscript{3}) or fibronectin (α\textsubscript{v}β\textsubscript{3}) receptors (Coller et al., 1983; Grossi et al., 1988), and therefore may be used to distinguish between these immunologically related glycoproteins. As shown in Figure 4, Saos-2 cells express various integrin molecules (i.e. α\textsubscript{v}β\textsubscript{3}, α\textsubscript{v}β\textsubscript{1} and α\textsubscript{v}β\textsubscript{1}) as verified by the increment in relative fluorescence quantified by flow cytometry. This increment in fluorescence intensity was more marked with the cells labelled with the MAb against α\textsubscript{v}β\textsubscript{3} and α\textsubscript{v}β\textsubscript{1} than those cells labelled with MAb against α\textsubscript{v}β\textsubscript{1}. An increase in the number of highly fluorescent cells within the population was also observed (from 4.7% to 94.8% and 98.1% for cells labelled with MAb against α\textsubscript{v}β\textsubscript{3} or α\textsubscript{v}β\textsubscript{1}, respectively; Figures 4 and 5). However, Saos-2 cells do not express integrins α\textsubscript{v}β\textsubscript{3} and α\textsubscript{v}β\textsubscript{1} since no significant increment in fluorescence intensity was found.

Effect of rhodostomin on adhesion of Saos-2 cells to ECM

The tripeptide sequence RGD is present in a number of adhesion proteins, including fibronectin, fibrinogen, vitronectin, collagen type I and thrombospondin (Ruoslahti and Pierschbacher, 1987). Therefore we examined the effect of rhodostomin on Saos-2 cell adhesion to ECM. As shown in Figure 6, adhesion of tumour cells to ECM was inhibited by

| Table 1 | Recalcification clotting times of normal and coagulation factor-deficient plasma in the presence of Saos-2 cells. Data presented as means ± s.e.m. (n = 4–5) |
|------------------|------------------|------------------|------------------|
|                  | Plasma recalcification times (s) |                  |                  |
|                  | Normal            | Factor IX        | Factor VIII      |
|                  | factor-deficient  | factor-deficient | factor-deficient |
| PBS buffer       | 168 ± 4           | 168 ± 4          | 170 ± 5          | 169 ± 3 |
| Thromboplastin   | 17 ± 1            | 18 ± 1           | 17 ± 1           | 35 ± 2  |
| Saos-2 cells     |                  |                  |                  |
| 2 × 10^5 cells ml\textsuperscript{-1} | 79 ± 2           | 83 ± 3           | 78 ± 4           | 166 ± 3 |
| 2 × 10^6 cells ml\textsuperscript{-1} | 60 ± 4           | 63 ± 1           | 62 ± 3           | 163 ± 5 |
| 6 × 10^5 cells ml\textsuperscript{-1} | 43 ± 2           | 45 ± 3           | 42 ± 2           | 167 ± 5 |
| 2 × 10^6 cells ml\textsuperscript{-1} | 34 ± 2           | 32 ± 2           | 36 ± 3           | 145 ± 5 |

Figure 3 Effect of rhodostomin, GRGD and GRGES on Saos-2 TCIPA. A 400 μl aliquot of heparinised PRP was preincubated with varied concentrations of rhodostomin, GRGD or GRGES at 37°C for 2 min, followed by addition of 20 μl of Saos-2 cells (3 × 10^6 cells ml\textsuperscript{-1}, arrow) to induce platelet aggregation. Data presented as means ± s.e.m. (n = 6).
rhodostomin in a dose-dependent manner. At 0.2 μM, rhodostomin inhibited cell adhesion to collagen type I, vitronectin and fibronectin by approximately 90%. However, even at higher concentration (0.3 μM), rhodostomin only slightly inhibited cell adhesion to laminin (less than 20%), and it had no inhibitory effect on cell adhesion to collagen type IV. Synthetic peptide GRGDS showed a similar inhibitory pattern (Figure 6). On a molar basis, rhodostomin is 1000-fold more potent than GRGDS in inhibiting Saos-2 cell adhesion to RGD-dependent ECM.

Discussion

Numerous studies using experimental tumour models have suggested that host platelets may act as causative agents in the formation of successful metastatic foci (Honn et al., 1992). It has been suggested that aggregation reactions are involved in the process of blood-borne metastasis (Jamieson et al., 1987). In previous reports (Gasic et al., 1968; Pearlstein et al., 1984), an adequate platelet number was necessary for metastasis since induction of thrombocytopenia was associated with a decrease in the number of metastatic lesions. Moreover, many anti-platelet agents have potent anti-metastasis effects (Al-Mondhiry et al., 1984). In clinical practice, osteosarcoma is metastasis prone, which is

Figure 4 Flow cytometric analysis of integrin expression on Saos-2 cells. Saos-2 cell suspensions were labelled with MAbs (b) MCA757, (c) MCA698 or (d) MCA699, then secondarily labelled with goat anti-mouse IgG-FITC. Cells (b, c and d) showed a significant increase in fluorescence intensity relative to control cells (a) without the prior addition of primary antibodies. Ten thousand cells were counted per experimental group. HPCV, histogram per cent coefficient of variation.

Figure 5 Quantification of flow cytometry of integrins expression on Saos-2 cells. Immunolabelling of surface integrins was performed as described in the legend to Figure 4. Labelled cells showed an increase in mean fluorescence intensity (FI) relative to control and a percentage increase in the number of cells labelled with MAbs ( ). Data presented as means ± s.e.m. (n = 4).
concentrations higher than $5 \times 10^6$ cells ml$^{-1}$. This event occurs after aggregation since platelet aggregation requires less thrombin than does fibrinogen proteolysis in PRP. Additionally, this TCIPA is observed in heparinised PRP but not in citrated PRP, and the lag phase preceding platelet aggregation is markedly prolonged by raising the concentration of heparin from 1 to 6 units ml$^{-1}$ (data not shown), suggesting that the event is mediated by thrombin formation. Furthermore, the shortening of recalcification time in factor VIII-and factor IX-deficient plasmas but not in factor VII-deficient plasma and the complete inhibition of TCIPA by MAb against human tissue factor confirm that the procoagulant activity of Saos-2 cells is via the expression of tissue factor.

Our results indicate that Saos-2 TCIPA is thrombin dependent and that ADP is not fundamentally involved. During the lag period of platelet aggregation, the accumulation of thrombin sufficient to trigger aggregation was required. This is consistent with the observation reported elsewhere that thrombin inhibitors prolong the lag period in a dose-depen-

dent manner, but do not influence the maximum response of aggregation once platelets begin to aggregate (Pearlstein et al., 1981). Although Saos-2 TCIPA was essentially thrombin dependent, apyrase was found to prolong the lag phase preceding TCIPA. This is also observed with other human colon adenocarcinoma lines, colo 205 and colo 397 (Scarlett et al., 1987). The reason may reside in the rate or amount of thrombin generation. Low concentrations of thrombin are thought to mediate platelet aggregation in part by triggering platelet release of ADP, whereas higher concentrations of thrombin will produce aggregation independent of ADP release (Kinlough-Rathbone et al., 1977).

Integrin-mediated cell adhesion has been demonstrated for several RGD-containing proteins found in the mineral compartment of bone (Weiss and Reddi, 1980; Oldberg et al., 1986; Gehron et al., 1989). RGD also appears to be the active epitope in most disintegrins (Gould et al., 1990). In this study, we detected various integrins (i.e. $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$) expressed on Saos-2 cells by flow cytometric analysis. Saos-2 cells do not express integrin $\alpha_2\beta_1$, unlike human colon adenocarcinoma cells (SW-480) and prostate adenocarcinoma cells (PC-3) (data not shown). We showed that rhodostomin inhibits adhesion of Saos-2 cells to several ECM (i.e., fibronectin, vitronectin and collagen type I), probably by interfering with several epitopes of the RGD-dependent integrin receptors. The synthetic peptide GRGDS has a similar inhibitory effect on adhesion to these ECM, further confirming that the tripeptide sequence Arg-Gly-Asp is important for receptor recognition. In the study of TCIPA, both rhodostomin and GRGDS and the MAb raised against GP IIb IIIa had an inhibitory effect through blockade of fibrinogen binding to its platelet surface receptor. A growing body of evidence strongly suggests that platelets are indispensable in enabling metastasis. In this study, we showed that Saos-2 human osteosarcoma cells act as a potent inducer of platelet aggregation and that thrombin-dependent TCIPA resulting from tissue factor (TF) activity expression and tumour cell adhesion to several ECM is inhibitable by RGD-containing peptides, particularly the strikingly potent venom peptide rhodostomin. Therefore, multiple possible avenues exist for biological and pharmaceutical intervention in the management of neoplastic disease. Because TCIPA and the adhesion process are seemingly critical steps in tumour metastasis, anti-TF compounds, proteinase inhibitors and integrin receptor antagonists such as rhodostomin might have utility as adjunct therapeutic agents in preventing cancer metastases. Such novel interventions might be relevant in bone cancer, as suggested in our in vitro studies of osteosarcoma cells.

**Abbreviations**

TCIPA, tumour cell-induced platelet aggregation; MAb, monoclonal antibody; RGD, Arg-Gly-Asp; ECM, extracellular matrix; PRP, platelet-rich plasma; FITC, fluorescein isothiocyanate; TF, tissue factor.

**Acknowledgements**

This programme was financially supported by grants from National Science Council of Taiwan (NSC-82-0412-B002-086) and a research grant from National Taiwan University Hospital (NTUH-8353-B07).

**References**

AL-MONDHIRY H. (1984). Tumour interaction with haemostasis: the rationale for the use of platelet inhibitors and anticoagulants in the treatment of cancer. *Am. J. Hematol.*, **16**, 193–202.

COLLER BS, PEERSCHKE EV, SCLUDER LE AND SULLIVAN CA. (1983). Murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthen-like state in normal platelets and binds glycoprotein Ib and GpIIa. *J. Clin. Invest.*, **72**, 326–338.

GASIC GJ, GASIC TB AND STEWARD CC. (1968). Antimetastatic effects associated with platelet reduction. *Proc. Natl Acad. Sci. U.S.A.*, **61**, 46–52.

GEHRON ROBEY P, YOUNG MF, FISHER LW AND MCCLAIN TD. (1989). Thrombospondin is an osteoblast-derived component of mineralized extracellular matrix. *J. Cell Biol.*, **108**, 719–727.

GOULD RJ, POLOKOFF MA, FRIEDMAN PA, HUANG TF, HOLT JC, COOK JJ AND NEWGAROWSKI S. (1990). Disintegrins: a family of integrin inhibitors proteins from viper venoms. *Proc. Soc. Exp. Biol. Med.*, **185**, 169–171.

**Figure 6** Dose–response relationship of rhodostomin (a) and GRGDS (b) on Saos-2 cell adhesion to immobilised ECM, i.e. fibronectin 30 μg ml$^{-1}$ (●), vitronectin 15 μg ml$^{-1}$ (△), laminin 15 μg ml$^{-1}$ (Ο), type I (●) and type IV collagen 80 μg ml$^{-1}$ (△). Adhesion of Saos-2 cells to each ECM was performed for 24 h at 37°C. Inhibition of cell adhesion is shown as a percentage of the control. Data presented as means ± s.e.m. (n = 5–6).
PEARLSTEIN E. AMBROGIO C. GASIC G AND KARPATHKIN S. (1981). Inhibition of the platelet-aggregating activity of two human adenocarcinomas of the colon and anaplastic murine tumor with a specific thrombin inhibitor: dansylarginine N4-ethyl-1.5-pentanediylamide. *Cancer Res.*, 41, 4533–4539.

PEARLSTEIN E. AMBROGIO C AND KARPATHKIN S. (1984). Effect of antiplatelet antibody on the development of pulmonary following injection of CT26 colon adenocarcinoma. Lewis lung carcinoma, and B16 amelanotic melanoma tumor cells into mice. *Cancer Res.*, 44, 3884–3887.

PIERSCHBACHER MD AND ROUSLAHTI E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*, 309, 30–33.

PLOW E. GINSBERG MH AND MARGUERIE GA. (1986). In *Platelet Membrane Glycoprotein*. Philips DR and Schuman MS (eds) pp. 255–256. Plenum Press: New York.

RODAN SB. MAI Y. THIEDE MA. WESOLOWSKI G. THOMPSON D. BAR-SHAVIT Z. HULL S. MANN K AND RODAN GA. (1987). Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. *Cancer Res.*, 47, 4961–4969.

ROUSLAHTI E AND PIERSCHBACHER MD. (1987). New perspectives in cell adhesion: RGD and integrins. *Science*, 238, 491–497.

RUCINSKI B. NIEWIAROWSKI S. HOLT JC. SOSEKA T AND KNUDSEN KA. (1990). Batroxostatin, an Arg-Gly-Asp-containing peptide from *Bothrops atrox*, is a potent inhibitor of platelet aggregation and cell contact with fibronectin. *Biochim. Biophys. Acta.* 1054, 257–262.

SCARLETT JD. THURLOW PJ. CONNELLAN JM AND LOUIS CJ. (1987). Plasma-dependent and -independent mechanisms of platelet aggregation induced by human tumor cell lines. *Thromb. Res.*, 46, 715–726.

SHERUSKI RJ. RAMJIT DR. BENCEN GH AND POLOKOFF MA. (1989). Characterization and platelet inhibitory activity of Batistatin, a potent arginine-glycine-aspartic acid-containing peptide from the venom of the viper *Bothis atrum*. *J. Biol. Chem.*, 264, 21550–21556.

SHEU JR. LIN CH. CHUNG IL. TENG CM AND HUANG TF. (1992). Triflavin, an Arg-Gly-Asp containing snake venom peptide, inhibits aggregation of human platelets induced by human heparin. *Cancer Lett.*, 66, 679–691.

VAPORCIYAN AA. JONES ML AND WARD PA. (1993). Rapid analysis of leukocyte-endothelial adhesion. *J. Immunol. Methods*, 159, 93–100.

WEISS RE AND REDDI AH. (1980). Synthesis and localization of fibronectin during collagenous matrix–mesenchymal cell interaction and differentiation of cartilage and bone in vivo. *Proc. Natl Acad. Sci. USA*, 77, 2074–2078.