Interaction of human platelets with laminin and identification of the 67 kDa laminin receptor on platelets

Narendra N. TANDON,*§ Errol A. HOLLAND,*† Urszula KRALISZ,*
Hynda K. KLEINMAN,† Frank A. ROBEY† and G. A. JAMIESON*

*Cell Biology Laboratory, American Red Cross, Rockville, MD 20855, and †National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, U.S.A.

A microtitre adhesion assay has been developed to define parameters affecting the adherence of washed platelets to laminin. Adherence was optimally supported by Mg^{2+} and was inhibited by Ca^{2+} and by anti-laminin Fab fragments, but significant adhesion (75–90 % of control) was found both in heparinized plasma containing physiological levels of bivalent cations and in plasma anti-coagulated with EGTA. Adherence was unaffected by platelet activation with ADP but was decreased by 50 % by treatment with α -thrombin (1 unit/ml, 5 min). Adherence was unaffected by monospecific polyclonal antibodies to glycoprotein (GP) Ib and GPIV, and was normal with platelets from two patients with Glanzmann's thrombasthenia, indicating that GPIb, the GPIIb/IIIa complex and GPIV are not involved in platelet–laminin interaction. Affinity chromatography of Triton-solubilized membranes on laminin–Sepharose followed by elution with 0.2 M-glycine/HCl (pH 2.85) identified a major band with a molecular mass of 67 kDa in the reduced and of 53 kDa in the unreduced form. This protein gave a positive reaction on Western blotting with a monospecific polyclonal antibody raised against the high-affinity laminin receptor isolated from human breast carcinoma tissue. The adhesion of platelets to laminin was inhibited by two monoclonal IgM antibodies specific to the LR-1 domain of the 67 kDa receptor. The binding protein was surface-oriented, as shown by flow cytometry and by the fact that it could be iodinated in intact platelets, but it was not labelled by the periodate–borotritide procedure, suggesting that it did not contain terminal sialic acid. The laminin-derived peptides Tyr-Ile-Gly-Ser-Arg and Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂, which constitute a complementary binding domain in laminin for the 67 kDa receptor, themselves supported platelet adhesion, bound to the receptor and inhibited the adhesion of platelets to laminin. In addition, Fab fragments of anti-Tyr-Ile-Gly-Ser-Arg antibody inhibited platelet adhesion to laminin. These results demonstrate that the high-affinity 67 kDa laminin receptor previously identified in a range of normal and transformed cells and its complementary Tyr-Ile-Gly-Ser-Arg binding domain play an important role in the interaction of platelets with laminin.

INTRODUCTION

The interaction between platelets and adhesive proteins of the extracellular matrix such as von Willebrand factor, collagen, fibronectin and vitronectin has been the subject of intensive investigation (Ginsberg *et al.*, 1988). Laminin is an adhesive glycoprotein which is a major component of the extracellular matrix and promotes attachment of cells in general (Yamada, 1983; Martin & Timpl, 1987), but its possible role in platelet adhesion to sub-endothelium and in thrombogenesis has not been elucidated. Platelets adhere to laminin but, in contradistinction to their attachment to other adhesive proteins such as collagen and von Willebrand factor, platelets adherent to laminin do not spread or undergo other morphological or secretory changes characteristic of activation (Ill *et al.*, 1984).

Laminin is composed of three chains arranged in a cross-shaped structure (Iwamoto *et al.*, 1987). The cell-attachment domain is thought to reside in one of the short arms of the laminin molecule, whereas a second important biological activity, the neurite outgrowth capability, resides in one of the long arms (Edgar *et al.*, 1984; Graf *et al.*, 1987). The neurite outgrowth capability may be important in the repair of injury to nervous tissue, and the cell attachment capability is thought to be important in metastasis: malignant cells attach more readily to laminin than do their normal counterparts, they have more laminin on their surface, and proteolytic fragments of laminin inhibit experimental metastasis in animal models. Platelets are

known to facilitate tumour metastasis (Gasic *et al.*, 1968; Pearlstein *et al.*, 1984) and this is thought to be due, at least in part, to the attachment of mixed platelet/tumour cell aggregates to components of the sub-endothelium (Jamieson *et al.*, 1987). Thus the interaction of platelets with laminin could play a role in tumour metastasis by indirectly conferring adhesive ability on tumour cells which themselves lack the high-affinity laminin receptor.

Several different laminin-binding proteins have been identified in different mammalian cell types. The best-characterized is a 67 kDa protein that has been isolated by affinity chromatography on laminin–Sepharose from human breast carcinoma tissue (Rao *et al.*, 1983), murine fibrosarcoma cells (Malinoff & Wicha, 1983), rodent striated muscle (Lesot *et al.*, 1983) and NG108-15 neuroblastoma/glioma cells (Kleinman *et al.*, 1988) and whose amino acid sequence has been partially deduced from molecular cloning studies (Wewer *et al.*, 1986; Yow *et al.*, 1988; Zeigler *et al.*, 1988): the isolated receptor retains a high affinity for laminin (K_d 2 nM). This 67 kDa receptor is thought to bind to sequences containing the Tyr-Ile-Gly-Ser-Arg pentapeptide in one of the short arms of the laminin molecule (Graf *et al.*, 1987), although other domains may also be involved (Clement *et al.*, 1990).

In addition to this 67 kDa protein, several laminin-binding proteins in the molecular mass range 110–180 kDa have been identified in neuronally derived cell lines, and these may be members of the integrin family of cell adhesion receptors

Abbreviations used: GP, glycoprotein; PRP, platelet-rich plasma; PGE₁, prostaglandin E₁; PBS, phosphate-buffered saline.

† Present address: Groote Schuur Hospital, Capetown, South Africa.

§ To whom correspondence should be addressed.

(Tomaselli *et al.*, 1987; Smalheiser & Schwartz, 1987; Kleinman *et al.*, 1988; Gehlsen *et al.*, 1988; Basson *et al.*, 1988); for example, VLA-3 may have a role in the adhesion of fibrosarcoma cells to laminin, but it has not been established that VLA-3 occurs in platelets (Wayner & Carter, 1987). Moreover, the binding of integrins to laminin is of much lower affinity than its binding to the 67 kDa receptor, and the integrins bind to the long arm of the laminin molecule.

A surface galactosyltransferase from melanoma cells (60 kDa) has also been implicated in cell adhesion and spreading on laminin (Runyan *et al.*, 1988), and a laminin-binding protein has been identified in *Staphylococcus aureus* (Lopes *et al.*, 1985).

The present studies were undertaken to characterize the interaction of platelets with laminin. After completion of this work, it was reported that the monoclonal antibody GoH3 inhibited platelet adhesion to laminin in a microtitre assay system (Sonnenberg *et al.*, 1988). Since this antibody was known to immunoprecipitate the platelet glycoprotein (GP) Ic/IIa complex (VLA-6) from platelet lysates (Sonnenberg *et al.*, 1987), this complex was proposed as mediating platelet adhesion to laminin. Our results demonstrate that platelet adhesion to laminin is also mediated by the 67 kDa laminin receptor.

MATERIALS AND METHODS

Platelets from two patients with Glanzmann's thrombasthenia were obtained through the kind assistance of Dr. Margaret Johnson, Christiana Hospital, Wilmington, DE, U.S.A. Laminin, isolated from the basement membrane of the Engelbreth-Holm-Swarm transplantable murine tumour, and rabbit anti-laminin antibody were obtained from Collaborative Research (Boston, MA, U.S.A.). Human α -thrombin, polyclonal antibodies to glyocalicin (Harmon & Jamieson, 1986) and GPIV (Tandon *et al.*, 1989a) and polyclonal antibody to the laminin receptor of pooled human breast carcinoma tissue (Wewer *et al.*, 1986) had been previously produced in our laboratories. Monoclonal IgM antibodies 3D1 and 6G4 to synthetic LR-1 peptide (Leu-33→His) of the 67 kDa laminin receptor had been prepared as described (Rahman *et al.*, 1989). Fab fragments were prepared using immobilized papain and Protein A according to the protocol of the manufacturer (Pierce Chemical Co., Rockford, IL, U.S.A.). Anti-Tyr-Ile-Gly-Ser-Arg antibodies were obtained as previously described (Graf *et al.*, 1987).

Platelets

For most studies, whole blood anticoagulated with CPD-A1 was obtained, within 3 h of venepuncture, from the American Red Cross Washington Regional Blood Services, Washington, DC, U.S.A. Platelet-rich plasma (PRP) was obtained by low-speed centrifugation at 180 *g* for 10 min at 22 °C. Prostaglandin E₁ (PGE₁) at a concentration of 1 μ g/ml was added to the PRP, the pH was adjusted to 6.5 with citric acid and the platelets were sedimented by centrifugation at 1800 *g* for 10 min. The platelets were resuspended, washed in citrate wash buffer (5.5 mM-glucose, 120 mM-NaCl, 4.26 mM-NaH₂PO₄, 7.46 mM-Na₂HPO₄, 4.77 mM-trisodium citrate and 2.35 mM-citric acid, pH 6.5) supplemented with 0.35% BSA as previously described (Tandon *et al.*, 1983). After two washes, platelets were suspended in Tris/saline buffer (buffer A; 5.0 mM-Tris, 5.5 mM-glucose, 150 mM-NaCl, 2.0 mM-MgCl₂ and 0.5% BSA, pH 7.4) to a concentration of about 1×10^9 platelets/ml.

For studies with heparinized and EGTA-treated plasma, blood was collected from volunteer staff members using heparin (8 units/ml) or EGTA (1.5 mM) as anticoagulant and was then centrifuged (180 *g*, 15 min) to isolate PRP. A small portion of the

PRP was further centrifuged (1800 *g*, 15 min) to obtain platelet-poor plasma. To the remaining PRP, PGE₁ was added to a final concentration of 1 μ g/ml and the pH was adjusted to 6.5 with citric acid. Platelets were recovered by centrifugation (1800 *g*, 10 min) and the pellet was washed as described above for platelets anticoagulated with CPD-A1.

In each case, platelets were labelled with Na₂⁵¹CrO₄ (50 μ Ci/ml) (Amersham Corporation, Arlington Heights, IL, U.S.A.; 250 mCi/mg) by incubation at 25 °C for 1 h in buffer A followed by washing in citrate wash buffer and suspension in buffer A. When required, platelets were surface-labelled with ¹²⁵I utilizing lactoperoxidase-catalysed iodination, as described previously (Bienz & Clemetson, 1989).

Microtitre adhesion assay

The conditions used were generally similar to those previously described for assays using 35 mm Petri dishes (Ill *et al.*, 1984), but were adapted to the micro scale. Microwell modules (Nunc, Kamstrup, Denmark; 6 mm diameter) were coated by incubating with a solution of laminin (routinely 8 μ g/well) for 1 h at 22 °C. Unoccupied sites on the plates were then blocked for 1 h with buffer A, which contains 0.5% BSA, and excess buffer was then removed by aspiration. ⁵¹Cr-labelled platelets [$(2-2.5) \times 10^7$] in 50 μ l of buffer A were added to the wells in triplicate and incubated at 22 °C without rocking. After 30 min, the non-adherent platelets were removed by washing 3 times with buffer A by aspiration. The extent of adhesion was determined by incubating the wells containing ⁵¹Cr-labelled adherent platelets with 2 $\times$ 0.1 ml portions of 2% SDS in water for 30 min each. The lysates were pooled and the ⁵¹Cr content was determined in a β -radiation counter (Beckman LS-2000). Results are expressed as the percentage of added platelets adhering to the substrate.

For studies on the adhesion of platelets to laminin-derived peptides, the synthetic peptides Tyr-Ile-Gly-Ser-Arg and Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ (Graf *et al.*, 1987) in various amounts (0.1–150 μ g) were dried overnight on to the microtitre wells, which were then blocked with albumin and examined in the standard microtitre adhesion assay, as described above.

For the identification of the platelet component(s) binding to the synthetic peptides, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ was coupled to CNBr-activated Sepharose 4B according to the protocol of the manufacturer (Pharmacia, Uppsala, Sweden) at a concentration of 1.4 mg of peptide/ml of the packed beads.

In certain experiments, washed platelets were activated by ADP (10 μ M) or increasing concentrations of thrombin in the presence of EGTA (2 mM) and PGE₁ (100 nM) to prevent aggregation before being evaluated for adhesion in the microtitre assay. In these experiments, ADP was removed after activation by washing the pelleted platelets with citrate buffer. In the case of activation by thrombin, hirudin (10 units/ml) was added to the activated platelets before the final washing.

Flow cytofluorimetry

To avoid possible activation of platelets during the washing procedure, PGE₁ (50 ng/ml) and EDTA (2 mM) were included in both the platelet wash buffer and Hepes-Tyrode's buffer. Washed platelets in Hepes-Tyrode's buffer (2×10^8) were incubated with either anti-(human breast carcinoma laminin receptor) antiserum or normal rabbit serum at a 1:200 dilution for 1 h at 22 °C. Platelets were washed twice with platelet wash buffer, suspended in Hepes-Tyrode's buffer (2×10^8 /ml) and incubated with a saturating concentration of fluorescein isothiocyanate-labelled goat anti-rabbit IgG [ratio of labelled antibody to cell volume was 1:40, as suggested by the manufacturer (Cooper Biomedical, Malvern, PA, U.S.A.)] for 1 h at 22 °C. Platelets were washed

twice with platelet wash buffer, resuspended in phosphate-buffered saline (PBS; 10 mM- Na_2HPO_4 /140 mM- NaCl , pH 7.35) containing 2 mM-EDTA and PGE_1 (50 ng/ml) at a platelet concentration of 4×10^8 /ml, fixed overnight at 4 °C by adding an equal volume of 2 % paraformaldehyde dissolved in resuspension buffer and analysed in a flow fluorocytometer (EPICS; Coulter Corporation, Hialeah, FL, U.S.A.). The fluorescence signal from 5000 platelets was measured for each sample.

Isolation of laminin-binding proteins

Laminin-Sepharose was prepared and the 67 kDa high-affinity laminin-binding protein was isolated from human platelets by methods essentially similar to those previously reported for its isolation from murine tumour tissue (Rao *et al.*, 1983; Malinoff & Wicha, 1983; Lesot *et al.*, 1983). Washed platelets from 20 units of platelet concentrates [where 1 unit is the number of platelets recovered from 1 unit (450 ml) of blood] were suspended in buffer C (50 mM-Tris, 5 mM-EGTA, 2 mM-phenylmethanesulphonyl fluoride, 50 μg of leupeptin/ml) at platelet concentrations of about 2×10^8 /ml. A crude membrane preparation was prepared by 5×20 s bursts of sonication on ice (Bronson Sonifier, setting 7), followed by centrifugation at 10000 *g* for 10 min at 4 °C to remove cell debris and unbroken platelets, and then at 100000 *g* for 90 min at 4 °C to sediment the membrane fraction. The isolated crude membrane preparation was solubilized by rotation for 2 h at 4 °C in buffer B (5 mM-Tris, 150 mM- NaCl , 2 mM- MgCl_2 and 0.1 % Triton X-100, pH 7.4) supplemented with 1 % Triton X-100 (2 ml of buffer/platelet concentrate). The insoluble material was removed by centrifugation at 100000 *g* for 30 min at 4 °C. A 40 ml sample of the solution of solubilized membranes was cycled over a laminin-Sepharose column (5 ml), or 8 ml of solubilized membranes over a Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-Sepharose column (3 ml), at 4 °C for 18 h at a flow rate of about 20 ml/h. The column was then washed with buffer B until the absorbance returned to baseline (about 5 column vol.). Sequential elution was then carried out with 2 column vol. of buffer B containing 1 M- NaCl followed by 2 column vol. of buffer B containing 5 mM-EGTA and finally, 0.2 M-glycine/HCl containing 0.1 % Triton X-100 (pH 2.85). Fractions of 4 ml were collected into tubes containing 75 μl of Tris (1 M) so as to raise the pH of the collected eluate to 7.6. The glycine/HCl eluate was concentrated by ultrafiltration in an Amicon Centricon (PM30) apparatus. Similar experiments were carried out with platelets that had been surface-labelled by lactoperoxidase-catalysed iodination.

Electrophoresis

SDS/PAGE was performed using Tris/glycine buffer. Samples were solubilized in 1 % SDS either with (reduced) or without (non-reduced) 5 % (v/v) 2-mercaptoethanol. Proteins on gels were fixed with trichloroacetic acid (12 %, w/v) for 1 h. Gels were stained for proteins by silver stain, as described by the manufacturer (Bio-Rad).

Protein blotting

The Bio-Rad Trans-Blot cell was used to transfer proteins from polyacrylamide gels to nitrocellulose sheets. After transfer was complete, excess binding sites on the nitrocellulose sheet were blocked by treatment for 1 h with Tween 20 (0.3 %) in PBS containing 2 % BSA. Strips (4–6 mm wide) were cut from the sheet and individual strips were incubated with various dilutions of appropriate antibodies at 4 °C overnight, followed by several washes with PBS/Tween containing BSA (0.1 %). Strips were then incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG followed by four washings with PBS/Tween buffer containing BSA. Bands were visualized using 4-chloro-1-naphthol and hydrogen peroxide.

RESULTS

Adhesion assay

The adhesion assay used here is generally similar to that previously described (Ill *et al.*, 1984), but the use of microtitre modules facilitates handling procedures and requires much smaller quantities of reagents. Using the microtitre assay, there was a concentration-dependent increase in adherent platelets up to a platelet concentration of 5×10^7 /well, corresponding to 500000 platelets/ μl , using three different donors in triplicate determinations (Fig. 1a). The number of platelets adhering to the substrate was essentially independent of the laminin concentration above 6 μg /well for the three donors tested (Fig. 1b). The number of adherent platelets increased with time in all donors examined ($n = 4$) and reached a maximum at about 60 min, but with a reproducible lag phase in the first 5–10 min (Fig. 1c). The specificity of the adhesion reaction was shown by the fact that increasing concentrations of anti-laminin Fab fragments inhibited adhesion, with half-maximum inhibition at about 12 μg /well, whereas Fab fragments of non-immune IgG were without effect up to 100 μg /well (Fig. 1d). Anti-laminin Fab (30 μg /well) had no effect on platelet adhesion to fibronectin, fibrinogen or von Willebrand factor. As described in the Materials and methods section, the standard microtitre adhesion assay used 8 μg of laminin and 2.5×10^7 platelets per well and, for convenience, the incubation time was limited to 30 min. Under these conditions, about 12 % of the added platelets (or about 3×10^6 platelets per well) adhered to the laminin substrate, but the values obtained ranged from 6 % to 14 % among different donors, although similar platelet concentrations were used in each case.

Adhesion was strongly supported by Mg^{2+} at 0.5 mM (Fig. 2). On the other hand, Ca^{2+} ions alone supported adhesion only weakly (approx. 15 %) at 0.5 mM and even more weakly at higher concentrations. This inhibition by Ca^{2+} was confirmed using increasing concentrations of equimolar mixtures of Ca^{2+} and Mg^{2+} .

The observed inhibition by Ca^{2+} raised the question of whether platelet adherence to laminin would be observed in the presence of physiologically relevant concentrations of bivalent cations. To answer this, washed platelets were suspended in heparinized platelet-poor plasma so as to maintain levels of Ca^{2+} and plasma components that were at 80 % of the physiological level. Heparin itself, at the level present in the reconstituted plasma system (6.4 units/ml), caused about a 15 % decrease in the adherence of washed platelets to laminin (Table 1); this decrease was not unexpected, since laminin is known to bind heparin (Sakashita *et al.*, 1980). In the presence of heparinized platelet-poor plasma there was a further decrease of 10 %, so that the final adhesion was about 75 % of that in the heparinized control. Addition of EGTA (1 mM) to chelate free Ca^{2+} had little effect on adherence in this system. Adhesion in the washed platelet system containing 1.5 mM-EGTA was also compared with that of washed platelets which had been returned to plasma anticoagulated with EGTA (1.5 mM). Under these conditions, adhesion of 9.4 % was observed in the plasma-free system and similar adhesion (92 % of control) in the plasma-containing system.

Role of membrane glycoproteins

GPIb, the GPIIb/IIIa complex and GPIV have been identified as major adhesion receptors in platelets, but these glycoproteins did not appear to play a role in the interaction of platelets with laminin. Anti-glycocalicin Fab fragments against GPIb did not inhibit adhesion at concentrations as high as 40 μg /well, whereas platelets from two patients with Glanzmann's thrombasthenia, which lack GPIIb/IIIa, gave adhesion values that were respec-

Fig. 1. Characteristics of the adherence of platelets to laminin in the microtitre assay

Conditions were as for the standard assay procedure (see the Materials and methods section), except as noted. Values are given as means $\pm$ s.d., except in (d), where the averages of two determinations are shown. Where error bars are not visible they are within the area of the symbol. (a) Dependence of adherence on platelet number ($n = 3$). (b) Dependence of adherence on laminin content of the microtitre wells ($n = 3$). (c) Time course of the adhesion of platelets to laminin ($n = 4$). (d) Inhibition of adherence by Fab fragments of anti-(human laminin) antibody ($n = 2$). The Fab fragments were added to microtitre wells for 30 min before the addition of platelets. ●, Monospecific anti-laminin Fab; ○, non-immune IgG Fab.

Fig. 2. Dependence of platelet adhesion on bivalent cation concentration

The normalized control value was obtained as described for the standard microtitre assay with 2 mM-Mg²⁺. ○, Mg²⁺; ●, total concentration of equimolar amounts of Ca²⁺ and Mg²⁺; □, Ca²⁺.

Table 1. Adhesion of platelets to laminin in the presence of physiological concentrations of Ca²⁺ and plasma proteins

Values in parentheses are the actual percentages of adherent platelets in two experiments carried out in triplicate.

	Adhesion (% of control)
Washed platelets	100 (12.2, 12.5)
Washed platelets + heparin (6.4 units/ml)	84 (9.8, 11.0)
Washed platelets + heparin plasma	62 (7.4, 8.0)
Washed platelets + heparin plasma + EGTA (1.5 mM)	64 (7.2, 8.6)
Washed platelets + EGTA plasma	70 (8.3, 9.1)
Washed platelets + EGTA	76 (8.9, 9.9)

tively 95% and 90% of control values (means of triplicate determinations). Confirming this, the Arg-Gly-Asp-Ser peptide, which blocks the interaction of GPIIb/IIIa with adhesive proteins such as von Willebrand factor, fibrinogen and fibronectin, was without effect, and adhesion was 85% of control at an Arg-Gly-Asp-Ser concentration of 1 mM, as previously observed (Ill *et al.*, 1984).

GPIV (CD36) has recently been isolated from platelet membranes (Tandon *et al.*, 1989b) and shown to modulate the primary adhesion of platelets to collagen (Tandon *et al.*, 1989a), the attachment of *Plasmodium falciparum*-infected red blood cells to endothelium (Ockenhouse *et al.*, 1989) and the interaction of platelets with thrombospondin (Asch *et al.*, 1987). To determine whether GPIV might play a similar role in the adhesion of platelets to laminin, a monospecific polyclonal anti-GPIV Fab was incubated with platelets before the evaluation of adherence in the microtitre assay. Inhibition was not observed, but, at concentrations below 16 μg/well, the anti-GPIV Fab fragments increased adherence to laminin about 1.5-fold, while there was a return to control values at higher Fab concentrations (24 μg and 40 μg Fab/well) (results not shown).

Platelet modification

Although adherence to laminin does not in itself result in platelet activation (Ill *et al.*, 1984), it was not known whether activated but non-aggregated platelets would show differences in adherence from unactivated controls. The two agonists studied were ADP and thrombin, since these two agonists appear to mediate the activation of platelets by cultured tumour cells (Jamieson *et al.*, 1987; Grignani & Jamieson, 1988). Platelets activated with ADP (10 μM) showed no difference in adherence from unactivated controls (results not shown). However, incubation with relatively low concentrations of thrombin for 5 min caused a loss in the ability of platelets to attach to laminin. Adherence of 10.3% of platelets was found in controls, and this

Fig. 3. Elution pattern from laminin-Sepharose

Crude membranes from 1 unit of ¹²⁵I-labelled platelets were mixed with 20 units of unlabelled membranes, dissolved in Triton X-100 and applied to a column of laminin-Sepharose. The column was washed with application buffer (buffer B, see the Materials and methods section) until the absorbance (b) and radioactivity (a) in the eluate had fallen to approximately baseline values. Stepwise elution was then initiated with buffer B containing 1 M-NaCl (arrow 1), buffer B containing 5 mM-EGTA (arrow 2) and finally 0.2 M-glycine/HCl containing 0.1 % Triton X-100 (pH 2.85) (arrow 3). Fractions of 4 ml were collected and the pH was immediately adjusted to 7.6 with 75 μ l of Tris (1 M).

Fig. 4. Electrophoretic and immunological characterization of platelet laminin-binding proteins in the glycine/HCl eluate

Lanes 1 and 2 are silver stained. Lane 1, non-reducing conditions; lane 2, reducing conditions; lane 3, protein blot of glycine/HCl eluate against anti-(tumour cell laminin receptor) antibody.

Fig. 5. Cytofluorimetry

Indirect immunofluorescence analysis of the binding of rabbit anti-(human breast carcinoma) antibody (—) compared with that of normal IgG (.....).

was lowered to 5.7 % after treatment of platelets with 0.5 unit of thrombin/ml and to 4.8 % at 1.0 unit/ml.

Isolation of laminin-binding proteins

Membranes from ¹²⁵I-labelled platelets were solubilized in Triton X-100 and passed over laminin-Sepharose. As previously observed in other cells (Rao *et al.*, 1983; Malinoff & Wicha, 1983; Lesot *et al.*, 1983), the major portion of the radioactivity did not bind to laminin-Sepharose and radioactivity eluted with buffer B fell almost to baseline values (Fig. 3a). Addition of 1 M-NaCl to the buffer resulted in the elution of a peak of radioactivity which again fell to baseline values. Finally, a major peak of strongly adsorbed radioactivity was eluted with glycine/HCl containing Triton X-100. No further radioactivity was obtained when SDS was added to the elution buffer. No radioactivity was detected in glycine eluates from the laminin-Sepharose column using membranes from platelets that had been labelled in their surface glycoproteins by the periodate-borotritide procedure. Elution could be monitored, even in unlabelled platelets, by the negative u.v. absorption against Triton X-100 in the reference cuvette after application of glycine/HCl containing Triton X-100 (Fig. 3b), although there was little change in absorbance comparable with the increase in radioactivity with the other two eluting buffers.

SDS/PAGE of the glycine/HCl fraction after concentration by Amicon filtration showed the presence of a major component with a molecular mass of 67 kDa (reduced) or 53 kDa (un-reduced) (Fig. 4), identical with that previously reported for the laminin receptor of human carcinoma and murine fibrosarcoma cells (Malinoff & Wicha, 1983; Terranova *et al.*, 1983). The immunological identity of this protein with the laminin receptor of human carcinoma cells was confirmed by protein blotting using a polyclonal antibody prepared against human breast carcinoma tissue (Fig. 4, lane 3).

Flow cytometry

Flow cytofluorimetric analysis of platelets labelled with polyclonal antibody to the 67 kDa laminin receptor from human breast carcinoma tissue gave an intense fluorescence signal as compared with normal IgG and indicated that about 99 % of the platelets were labelled by fluorescein isothiocyanate-labelled secondary antibody (Fig. 5). The forward light scatter profile indicated that there was no detectable contamination of the sample by other cells.

Immunoinhibition of adhesion

We confirmed the functional status of the 67 kDa protein in platelets by showing that two monoclonal antibodies (3D1, 6G4)

Fig. 6. Immunoinhibition of platelet adhesion using the standard microtitre assay and culture supernatants containing IgM monoclonal antibodies 3D1 (○) and 6G4 (●) raised against the human tumour cell laminin receptor

The negative control (▲) is a culture supernatant containing anti-(human Factor IX) monoclonal antibody. The points shown are the averages of two determinations each carried out in triplicate.

Fig. 7. Adhesion of platelets to laminin-derived peptides

Peptides were dried on to microtitre wells in various amounts as indicated and adhesion of ^{51}Cr -labelled platelets was measured using the standard microtitre assay. Results are given as means $\pm$ S.D. for three separate experiments, each performed in triplicate. ○, Tyr-Ile-Gly-Ser-Arg; ●, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂; Δ, Gly-Ala-Arg-Gly (negative control). The bar shows the binding to laminin (8 $\mu\text{g}/\text{well}$) as a positive control.

Fig. 8. Characterization of platelet Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-Sepharose-binding proteins in the glycine/HCl eluate

SDS/PAGE was carried out under reducing conditions. Lane 1, silver stain; lane 2, protein blot against anti-(tumour cell 67 kDa laminin receptor) antibody.

raised against the LR-1 peptide sequence in the 67 kDa laminin receptor of malignant cells each inhibited the adhesion of platelets to laminin by 50–60 % in the microtitre assay system, whereas an irrelevant anti-(Factor IX) antibody was without effect (Fig. 6).

Peptide studies

The laminin-derived peptides Tyr-Ile-Gly-Ser-Arg and Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ strongly supported the adhesion of platelets, whereas an unrelated peptide (Gly-Ala-Arg-Gly) did not (Fig. 7). At 50 μg of peptide/well, 9 ± 1 % of the platelets in the well attached to the nonapeptide and about 6.0 ± 0.7 % to the pentapeptide; there was no further increase at Tyr-Ile-Gly-Ser-Arg levels as high as 150 $\mu\text{g}/\text{well}$. About 12 % of platelets attached to laminin (8 $\mu\text{g}/\text{well}$) as a positive control in the standard assay.

These peptides also inhibited the adhesion of platelets to laminin. In a typical adhesion assay, run in triplicate, platelet adhesion was 6.2 ± 0.3 % in the absence of added peptide and was decreased to 3.1 ± 0.5 % after a 30 min pre-incubation in the presence of 1 mM-Tyr-Ile-Gly-Ser-Arg. Adhesion was lowered to 2.7 ± 0.1 % in the presence of 1 mM-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂. These results show that the two peptides result in 51 and 57 % inhibition respectively in platelet adhesion at 1 mM concentrations.

Furthermore, pre-incubation of platelets with Fab fragments obtained from anti-Tyr-Ile-Gly-Ser-Arg antibody (50 $\mu\text{g}/\text{ml}$) for 30 min resulted in a decrease in platelet adhesion to laminin itself of about 50 %, from 6.3 ± 0.3 % to 3.5 ± 0.3 %. On the other hand, pre-incubation with the same concentration of anti-laminin Fab decreased the adhesion to 0.1 ± 0.01 %, whereas non-immune Fab fragments had no effect.

Finally, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-Sepharose was able to selectively bind the 67 kDa protein present in the Triton X-100-soluble platelet membrane lysate. The Triton X-100-soluble membrane fraction was passed over a 3 ml column of peptide-Sepharose as described in the Materials and methods section. The unbound material was removed by extensive washing with equilibration buffer followed by washing with 3 column vol. of 1 M-NaCl, and finally the tightly bound protein(s) was eluted with glycine/HCl, pH 2.85. Portions of the pooled washes and glycine/HCl-eluted fractions were concentrated for further analysis using an Amicon concentrator.

SDS/PAGE of the glycine/HCl eluates showed the presence of major component with a molecular mass of 67 kDa under reducing conditions (Fig. 8, lane 1), although occasionally two minor bands at 115 kDa and 45 kDa were observed upon over-staining of the gels by the silver method. Protein blotting utilizing a polyclonal antibody against the 67 kDa receptor of human breast carcinoma tissue confirmed immunological identity (Fig. 8, lane 2). The 67 kDa protein from platelets previously affinity purified on laminin-Sepharose also bound very tightly to Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-Sepharose and could be eluted with glycine/HCl (results not shown).

DISCUSSION

Adhesive proteins play a major role in platelet reactions. Platelets adhere to surfaces coated with von Willebrand factor or with collagen and undergo activation and spreading. Platelets also adhere to laminin-coated surfaces but with the unique difference that, in contradistinction to their interaction with von Willebrand factor and collagen, platelets that have adhered to laminin in static assay systems remain rounded up and do not spread or secrete 5-hydroxytryptamine (Ill *et al.*, 1984). The present studies have defined factors affecting the interaction between platelets and laminin in a microtitre assay system, and

have demonstrated the role in this interaction of the 67 kDa laminin receptor previously identified in a range of normal and malignant cells.

The microtitre system described here provides a simple and rapid assay for measuring platelet adhesion to laminin that is economical in the use of reagents and of platelets in pathological situations. Under the conditions of this assay system, about 12 % of the added washed or gel-filtered platelets in each well adhered to the laminin layer, but values ranged from 6 % to 14 % among different donors, similar to values previously reported in studies on the VLA-6-mediated adhesion (Sonnenberg *et al.*, 1988). Ill *et al.* (1984) obtained binding of approx. 17 % of applied gel-filtered platelets to laminin on 35 mm culture dishes and they demonstrated that platelets non-adherent to laminin on their first exposure showed normal adherence on subsequent exposures. This indicates that the percentage of platelets adhering is a function of the geometry of the system and is not due to the presence of populations of adherent and non-adherent platelets.

The adhesion process appears to be Mg^{2+} -dependent. The adhesion assay previously developed (Ill *et al.*, 1984) used Dulbecco's modified Eagle's medium which contains 1.8 mM- Ca^{2+} and 0.8 mM- Mg^{2+} . Using the present microtitre assay we found that equimolar concentrations of Ca^{2+} (final concentration 1 mM) caused a 50 % decrease in adhesion compared with buffer containing Mg^{2+} alone. The inhibitory effects of Ca^{2+} in the microtitre assay system raised the question of the physiological relevance of laminin adhesion. In heparinized plasma, where normal levels of Ca^{2+} , Mg^{2+} and plasma proteins are maintained, adhesion was at a level of about 75 % of that in the washed platelet system, indicating that adhesion is effective at physiological concentrations of plasma cations and other components. In confirmation of this, washed platelets resuspended in EGTA plasma to chelate Ca^{2+} specifically showed adhesion similar to that seen in a plasma-free system.

The absence of a role for GPIIb/IIIa in the adhesion of platelets to laminin was unequivocally demonstrated by the fact that platelets from two patients with Glanzmann's thrombasthenia, which lack GPIIb/IIIa, showed essentially undiminished adhesion in the microtitre assay. GPIb, which mediates adhesion to von Willebrand factor, and GPIV, which mediates adhesion to collagen, are also not involved in adhesion to laminin, based on the lack of effect of monospecific polyclonal antibodies.

Platelet activation induced by cultured tumour cell lines appears to be dependent on ADP and thrombin elaborated by the tumour cells themselves (Jamieson *et al.*, 1987; Grignani & Jamieson, 1988). Unlike the interaction of GPIIb/IIIa with adhesive proteins, the adhesion of platelets to laminin appeared to be unaffected by platelet activation in the case of ADP. The decrease in adhesion seen after treatment of platelets with thrombin was unexpected, and its cause is at present unknown. Since thrombin-induced proteolysis of the receptor was not detected, this effect could be due to conformational changes induced in the receptor by thrombin, similar to those recently noted in the conformation of GPIb, GPIX and the GPIIb/IIIa complex (Michelson & Barnard, 1987). This observation may be of relevance in the case of those tumour cells that induce platelet activation by the generation of thrombin.

The present studies indicate that platelet adhesion to laminin is mediated, at least in part, by the 67 kDa high-affinity receptor that has also been found in human breast cancer cells (Rao *et al.*, 1983), rodent striated muscle (Lesot *et al.*, 1983) and rodent macrophages (Wicha *et al.*, 1983), human monocytes (Huard *et al.*, 1984), and murine B16 melanoma (Iwamoto *et al.*, 1987) and fibrosarcoma (Varani *et al.*, 1983; Edgar *et al.*, 1984) cells, as described below.

First, the high-affinity 67 kDa protein was the major component identified by affinity chromatography on laminin-Sepharose of platelet membranes solubilized in Triton X-100. Its surface location was deduced from the fact that it could be labelled by lactoperoxidase-catalysed iodination, as has also been seen in other cell types (Edgar *et al.*, 1984) and by flow cytofluorimetry.

Secondly, monoclonal antibodies to the 67 kDa tumour cell receptor caused immunoinhibition of adhesion and established the 67 kDa protein in platelets as a true receptor, rather than solely as a laminin-binding protein. The fact that inhibition plateaued at about 50 % in the microtitre assay system may indicate that these antibodies are directed only partly against the laminin-binding domain of the receptor or that other receptors such as VLA-6 (GPIc/IIa) are also involved in the adhesive process. This would be consistent with the slight lag phase seen at 10 min in the time course of adhesion to laminin, since a similar lag phase may differentiate the contributions of GPIV and VLA-2 in platelet adhesion to collagen (Tandon *et al.*, 1989a). In that connection, it may be noted that integrin-mediated anchorage of platelets has been associated with another non-integrin receptor in other cases, as in their adhesion to von Willebrand factor mediated by GPIb and GPIIb/IIIa (Ruggeri, 1988), and their adhesion to collagen mediated by GPIV (Tandon *et al.*, 1989a) and GPIa/IIa (VLA-2) (Santoro *et al.*, 1988; Kunicki *et al.*, 1988).

Thirdly, we found significant adhesion to the peptides Tyr-Ile-Gly-Ser-Arg and Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂, which are thought to constitute a complementary domain for the 67 kDa receptor on the short arm of the B1 chain of laminin. Although a several-hundred-fold molar excess of these peptides was required to provide platelet adhesion comparable with that on laminin, similar results have been obtained in the attachment of HT-1080 cells to these peptides (Graf *et al.*, 1987). These results may indicate a lower affinity in the smaller peptides or that other laminin receptors are also participating in adhesive interactions with laminin. These peptides and the corresponding anti-Tyr-Ile-Gly-Ser-Arg Fab antibody fragments were also able to inhibit platelet adhesion to laminin. Finally, both the 67 kDa protein from crude platelet lysates and the purified platelet 67 kDa receptor obtained by affinity chromatography on laminin-Sepharose bound avidly to Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-Sepharose.

Further studies are required to elucidate the relative roles of the 67 kDa protein and the integrin GPIc/IIa in platelet adhesion to laminin. However, the present studies demonstrate that the 67 kDa laminin receptor and its Tyr-Ile-Gly-Ser-Arg domain play an important role in this interaction.

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