Conformation-dependent platelet adhesion to collagen involving integrin $\alpha 2\beta 1$ -mediated and other mechanisms: multiple $\alpha 2\beta 1$ -recognition sites in collagen type I

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Platelet adhesion has been measured to type-I monomeric collagen, collagen fibres, $\alpha 1(I)$ and $\alpha 2(I)$ chains and the chain fragments $\alpha 1(I)CB3$, $\alpha 1(I)CB6$, $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$, and $\alpha 2(I)CB3,5$ and $\alpha 2(I)CB4$. Little if any adhesion occurred to any denatured species at 37 °C, demonstrating the importance of the collagen helix. However, on coating at 4 °C to promote helix formation, and assaying at room temperature to avoid denaturation, adhesion was observed to both α -chain types and all fragments, the exact level of which depended on the identity of the species in question. Adhesion was strongly Mg^{2+} -dependent. Antibodies against the integrin $\alpha 2\beta 1$ partially inhibited adhesion to α -chains and all fragments except $\alpha 1(I)CB6$, indicating a wide distribution of $\alpha 2\beta 1$ -binding sites in the collagen molecule. 'Activation-dependent' adhesion to monomeric collagen, totally

secondary to $\alpha 2\beta 1$ -mediated adhesion, involved at least two mechanisms, one mediated by integrin $\alpha IIb\beta 3$ and insensitive to prostaglandin E_1 , the other inhibitable by prostaglandin E_1 but independent of integrin $\alpha IIb\beta 3$. $\alpha IIb\beta 3$ -mediated adhesion to fragments was, at least in part, independent of the $\alpha 2\beta 1$ -mediated adhesion. Adhesion to fibres was largely bivalent-cation-independent with only minor involvement of integrin $\alpha 2\beta 1$. Some $\alpha IIb\beta 3$ -mediated adhesion occurred but was independent of any $\alpha 2\beta 1$ -initiated adhesion. Total 'activation-dependent' adhesion to fibres was less than to monomeric collagen. Affinity chromatography revealed bivalent-cation-independent binding to fibres of three main platelet surface proteins, 90, 150 and 190 kDa in size.

INTRODUCTION

The adhesion of platelets to collagen leads to platelet aggregation, important in haemostasis but also a possible cause of thrombosis [1]. Adhesion is mediated by the Mg^{2+} -dependent integrin $\alpha 2\beta 1$ (platelet $gpIa/IIa$) [2], although other mechanisms may exist [3,4]. Recognition of integrin $\alpha 2\beta 1$ by collagen type I has been reported to involve the sequence Asp-Gly-Glu-Ala, residues 435–438 of the collagen $\alpha 1(I)$ chain [5]. In the present study, we define three different adhesion processes: (1) conformation-dependent adhesion to collagen involving a number of $\alpha 2\beta 1$ -recognition sites widely distributed in the collagen molecule; (2) secondary 'activation-dependent' adhesion, comprising adhesion mediated by integrin $\alpha IIb\beta 3$ (platelet $gpIIb/IIIa$) but insensitive to prostaglandin E_1 (PGE_1) and adhesion inhibitable by PGE_1 but $\alpha IIb\beta 3$ -independent; (3) primary adhesion to collagen fibres that involves neither $\alpha 2\beta 1$ nor $\alpha IIb\beta 3$ and may be integrin-independent.

A preliminary account of this work has already been published [6].

MATERIALS AND METHODS

Petri dishes (Falcon 1008; 35 mm) for use in adhesion assays were obtained from Becton Dickinson UK Ltd., Oxford, U.K. $Na_2^{51}CrO_4$ was from Amersham International, Amersham, Bucks., U.K., and $Na^{125}I$ was from ICN Biomedicals, High

Wycombe, Bucks., U.K. Other specialist reagents were from Sigma, Poole, Dorset, U.K.

Collagen and collagen-derived fragments

Collagen type I was purified from a limited pepsin digest of calf skin as before [3]. Type-I collagen $\alpha 1$ - and $\alpha 2$ -chains were obtained from the parent collagen by CM-cellulose chromatography and further purified by gel filtration [7]. Type-I collagen was fragmented with CNBr, and the resultant peptides isolated in pure form as described earlier [8]. A suspension of native collagen (type I) fibres from bovine tendon, details of which have been reported previously [7], was from Ethicon Inc., Somerville, NJ, U.S.A. Before use, the suspension was dialysed against 0.01 M acetic acid and diluted with the same solution to the required concentration. Collagen, isolated α -chains and CNBr-derived peptides were denatured by heating in solution for 30 min at 60 °C. Renaturation was achieved by gradual stepwise cooling [7,8]. Fibres were denatured by heating at 90 °C for 30 min.

Antibodies

The following monoclonal antibodies (mAbs) were employed in this study: an affinity-purified mouse mAb, 6F1, directed against human platelet integrin $\alpha 2\beta 1$ and recognizing the $\alpha 2$ -subunit [9], which was a gift from Dr. B. S. Collier, School of Medicine, State University of New York, Stony Brook, NY, U.S.A.; purified rat mAb 13 recognizing the human $\beta 1$ -integrin subunit [10]; and a mouse anti-(human platelet $\alpha IIb\beta 3$) mAb (in ascites fluid),

Abbreviations used: PGE_1 , prostaglandin E_1 ; mAb, monoclonal antibody; TBS, Tris-buffered saline (0.05 M Tris/HCl, pH 7.4, containing 0.14 M NaCl and 5 mM glucose); PRP, platelet-rich plasma; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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RFGP56 [11,12]. mAbs were used routinely at the following concentrations: 6F1, 1 $\mu\text{g/ml}$; 13 and RFGP56, 10 $\mu\text{g/ml}$.

Platelet adhesion

Adhesion was measured using ^{51}Cr -labelled gel-filtered human platelets [3]. Dishes were coated with a solution of collagen (10 $\mu\text{g/ml}$) or a suspension of collagen fibres (500 $\mu\text{g/ml}$), in 0.01 M acetic acid, for 2 h at room temperature, or with denatured collagen, collagen α -chains or collagen-derived peptides (10 $\mu\text{g/ml}$ in 0.01 M acetic acid), normally at 4 °C overnight, occasionally, as required, for 2 h at 37 °C. Collagen α -chains and CNBr-cleaved peptides, unless specifically renatured beforehand as described above, were routinely heat-denatured immediately before coating in order to dissociate any partially helical species that might be present. We have shown previously that a collagen coating concentration of 10 $\mu\text{g/ml}$ is well in excess of the minimum required for optimum adhesion [3]. Using iodinated species at this concentration we have also found that collagen and collagen fragments bind to plastic in comparable amounts (all within the range 0.2–0.4 $\mu\text{g/cm}^2$). After coating, dishes were routinely blocked with BSA, as before [3]. Unless indicated otherwise, adhesion was measured in Tris-buffered saline (TBS) (plus 0.5 % BSA) containing 2 mM MgCl_2 [3] for 60 min at room temperature or 37 °C as defined later. Assays were undertaken in triplicate. Individual values generally did not differ from one another by more than 10 %. Results are expressed as the mean of three determinations. Percentage adhesion refers to bound radioactivity expressed as a percentage of the total added [3]. Where a range of values is quoted, this is normally based on at least six separate experiments. As reported previously [3], there was negligible adhesion to uncoated dishes blocked with BSA which were used as controls.

In some experiments, PGE_1 (100 ng/ml; freshly added at all stages) was included during platelet isolation and in the adhesion assay. In other instances, as required, PGE_1 (200 ng/ml) was included only during adhesion.

Affinity chromatography

Platelet-rich plasma (PRP; 27 ml) prepared as before [3] was diluted with 0.1 vol. of acid/citrate/dextrose, and the platelets were pelleted by centrifugation at 1300 g for 10 min. The pellet was washed in TBS and then suspended in 1 ml of 0.05 M Tris/HCl, pH 7.4, containing 0.14 M NaCl, 10 mM glucose, 0.3 % BSA, lactoperoxidase (20 $\mu\text{g/ml}$), glucose oxidase (0.1 unit/ml) and 1 mCi of Na^{125}I . The suspension was incubated at room temperature for 30 min. PGE_1 (100 ng/ml) was freshly added at each stage. After 30 min, platelets were pelleted at 10000 g for 5 min, washed once with TBS containing 1 mM NaI, then four times with cold (4 °C) TBS. The platelet pellet was lysed for 2 h at room temperature in 1 ml of 0.05 M Tris/HCl, pH 7.4, containing 0.14 M NaCl (where indicated), 100 mM octyl β -D-glucopyranoside, 1 mM MnCl_2 , 2 mM MgCl_2 and the protease inhibitors pepstatin (10 $\mu\text{g/ml}$), 3,4-dichloroisocoumarin (1 mM) and E64 (10 μM). Insoluble material was removed by centrifugation.

Coupling of ligand to CNBr-activated Sepharose 4B was carried out according to the manufacturer's instructions, using 30 mg of ligand per g of dry Sepharose. Collagen and renatured collagen fragments were coupled overnight at 4 °C, all subsequent operations then being carried out at room temperature or 15 °C in the case of fragments. Denatured collagen was coupled for 2 h at 37 °C, and all subsequent operations were undertaken at this

temperature. Fibres were coupled at 4 °C exactly as described by Brass et al. [13], and subsequent operations carried out at room temperature. After coupling, the affinity matrices were equilibrated in a chromatography column with 0.05 M Tris/HCl, pH 7.4, containing 0.14 M NaCl (when present in lysis buffer), 25 mM octyl β -D-glucopyranoside, 1 mM MnCl_2 and 2 mM MgCl_2 (column buffer). Up to 1 ml of the lysate of ^{125}I -labelled platelets was applied to the column and recirculated for 2 h (at the appropriate temperature). Unbound material was washed from the column with column buffer, and the column then sequentially eluted with column buffer adjusted to 0.5 M NaCl, 20 mM EDTA in 0.05 M Tris/HCl, pH 7.4, 25 mM octyl β -D-glucopyranoside and finally 6 M urea containing 2 % (w/v) SDS. Fractions (1 ml) were collected and counted on a γ -counter. Appropriate fractions were pooled, dialysed against deionized water and freeze-dried. SDS/PAGE of these fractions was performed using 6 % (w/v) polyacrylamide gels and the dried gels exposed to autoradiography.

RESULTS

Conformation-dependent adhesion to collagen and collagen fragments mediated by Integrin $\alpha 2\beta 1$

Adhesion was measured to collagen, collagen α -chains, the four fragments $\alpha 1(\text{I})\text{CB}3$, $\alpha 1(\text{I})\text{CB}6$, $\alpha 1(\text{I})\text{CB}7$ and $\alpha 1(\text{I})\text{CB}8$, representing 90 % of the total $\alpha 1(\text{I})$ chain length and to the two fragments $\alpha 2(\text{I})\text{CB}3,5$ and $\alpha 2(\text{I})\text{CB}4$, constituting together 95 % of the total $\alpha 2(\text{I})$ chain. When dishes were coated at 37 °C (to maintain the non-helical form), little if any adhesion occurred either to denatured collagen, $\alpha 1$ - or $\alpha 2$ -chains or any fragments when measured at 37 °C, establishing the importance of the collagen triple-helical structure for adhesion (Figures 1 and 2). Substantial adhesion, however, did occur at room temperature, presumably as a result of renaturation to a helical form with the fall in temperature. Results obtained with denatured collagen and collagen α -chains illustrating this point are shown in Figure 1. With the conformational requirement in mind, dishes were routinely coated at 4 °C overnight to promote helix formation. Adhesion was then measured at room temperature to avoid

Figure 1 Platelet adhesion to collagen type I and its constituent α -chains: effect of conformation

Adhesion was measured at 90 min in the presence of 2 mM Mg^{2+} at room temperature (RT) or 37 °C as indicated. Samples, except native collagen, were first heat-denatured and then coating was undertaken at the temperature indicated, 37 °C for 2 h or 4 °C overnight.

Figure 2 Platelet adhesion to collagen type-I-derived fragments $\alpha 1(I)CB3$ (a) and $\alpha 1(I)CB7$ (b): lack of adhesion to non-helical forms

Adhesion was measured at the times indicated in the presence of 2 mM Mg^{2+} . Samples, first heat-denatured, were coated at 37 °C (2 h) and adhesion then recorded at this temperature ($\square$) or coated at 4 °C (16 h) and adhesion measured at room temperature ($\blacksquare$). (a) and (b) were separate experiments.

denaturation, as at 37 °C adhesion (to α -chains and fragments) was appreciably lower (Figure 1), presumably because of loss of helical conformation at the higher temperature. Under these standard conditions, adhesion occurred to collagen α -chains and all collagen fragments tested. Generally speaking, adhesion was similar to that obtained when coating with the same sample optimally renatured beforehand by slow stepwise cooling. Over the course of several experiments, adhesion to $\alpha 1(I)CB3$ was always close to that observed for the parent collagen, and adhesion to $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$ and to $\alpha 2(I)CB3,5$ and $\alpha 2(I)CB4$ was generally somewhat lower, but occasionally adhesion to $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$ was as good as to collagen. Adhesion to $\alpha 1(I)CB6$ ranged from intermediate to poor. Typical data are shown in Figures 1–3 and Tables 1–3. Adhesion was in all cases Mg^{2+} -dependent (Table 1).

Adhesion to native monomeric collagen was totally inhibited by the anti-($\alpha 2$ -subunit) mAb 6F1, as described by others [9], or the anti-($\beta 1$ -subunit) mAb 13 (Figures 3a and 3c). These two

Figure 3 Platelet adhesion to collagen: inhibition by anti-integrin mAbs

(a) Inhibition of adhesion to native type-I collagen by the anti-(integrin $\alpha 2$ -subunit) antibody, mAb 6F1; (b) partial inhibition of adhesion to the collagen type-I-derived fragment, $\alpha 1(I)CB7$, by mAb 6F1; (c) inhibition of adhesion to native type-I collagen by the anti-(integrin $\beta 1$ -subunit) antibody, mAb 13; (d) partial inhibition of adhesion to native type-I collagen by the anti-(integrin $\alpha 1Ib\beta 3$) antibody, mAb RFGP56. Adhesion was measured at 60 min at room temperature in the presence of 2 mM Mg^{2+} and mAb at the concentrations indicated. Dishes were coated with collagen (10 µg/ml) for 2 h at room temperature or with $\alpha 1(I)CB7$ (10 µg/ml), first denatured, at 4 °C overnight.

Table 1 Mg^{2+} -dependent platelet adhesion to collagen and collagen-derived fragments

Dishes (35 mm) were coated with monomeric type-I collagen (at room temperature), collagen $\alpha 2(I)$ chains or CNBr peptides (at 4 °C) as described in the Materials and methods section. Adhesion at 60 min was measured at room temperature [23 °C in (a), 24 °C in (b)] in the presence of 2 mM $MgCl_2$ or 2 mM EDTA as indicated. (a) and (b) are separate experiments.

	Adhesion (%)			Adhesion (%)	
	Mg	EDTA		Mg	EDTA
(a)			(b)		
Collagen	51	2	Collagen	49	0
$\alpha 1(I)CB3$	49	1	$\alpha 2(I)$ chain	48	3
$\alpha 1(I)CB6$	3	1	$\alpha 2(I)CB3,5$	38	1
$\alpha 1(I)CB7$	34	1	$\alpha 2(I)CB4$	42	2
$\alpha 1(I)CB8$	28	1			

Table 2 Platelet adhesion to collagen $\alpha 1(I)$ -derived CNBr fragments: effect of mAbs 6F1 and RFGP56

Dishes (35 mm) were coated with heat-denatured peptide solution (10 μ g/ml) at 4 °C overnight. Adhesion at 60 min was measured at room temperature (25 °C) in the presence of 2 mM $MgCl_2$. MAb 6F1 was tested at 1 μ g/ml and RFGP56 at 10 μ g/ml. Adhesion to collagen (monomeric type I) was 48%.

Fragment	Adhesion (%)			
	No antibody	6F1	RFGP56	Both
$\alpha 1(I)CB3$	49	17	34	9
$\alpha 1(I)CB6$	38	36	15	14
$\alpha 1(I)CB7$	48	33	26	15
$\alpha 1(I)CB8$	47	32	22	13

Table 3 Platelet adhesion to collagen, collagen α -chains, and the collagen-derived CNBr fragments $\alpha 1(I)CB3$, $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$ and $\alpha 2(I)CB3,5$ and $\alpha 2(I)CB4$: effect of renaturation (by stepwise cooling)

Dishes (35 mm) were coated with peptide solution (10 μ g/ml) at 4 °C overnight. Peptides were either heat-denatured before coating or, where indicated (by the letter R in parentheses), renatured by stepwise cooling. Adhesion at 60 min was measured in the presence of 2 mM $MgCl_2$ at room temperature (20 °C) in either the presence or absence, as indicated, of mAb 13 (10 μ g/ml). (a) and (b) represent separate experiments. $\beta 1$ -mediated adhesion is equated with $\alpha 2\beta 1$ -mediated adhesion as mAbs 6F1 and 13 were indistinguishable in terms of their ability to inhibit adhesion.

Species	Adhesion (%)		
	(1) — mAb 13	(2) + mAb 13	(3) $\alpha 2\beta 1$ - mediated (1) — (2)
(a) Collagen	31	1	30
$\alpha 1(I)CB3(R)$	35	6	29
$\alpha 1(I)CB7(R)$	20	4	16
$\alpha 1(I)CB8(R)$	29	2	27
$\alpha 1(I)CB3$	33	13	20
$\alpha 1(I)CB7$	20	7	13
$\alpha 1(I)CB8$	24	4	20
(b) Collagen	34	1	33
$\alpha 1(I)$ chain(R)	35	12	23
$\alpha 2(I)$ chain(R)	34	12	22
$\alpha 2(I)CB3, 5(R)$	22	10	12
$\alpha 2(I)CB4(R)$	30	10	20
$\alpha 1(I)$ chain	28	4	24
$\alpha 2(I)$ chain	22	7	15
$\alpha 2(I)CB3, 5$	23	19	4
$\alpha 2(I)CB4$	25	16	9

mAbs also caused partial inhibition of adhesion to all fragments except $\alpha 1(I)CB6$ (see Figure 3b and Tables 2 and 3). The two mAbs, when used alongside each other, invariably caused very similar inhibition, indicating that $\beta 1$ -mediated adhesion was entirely due to the integrin $\alpha 2\beta 1$. $\alpha 2\beta 1$ -mediated adhesion to $\alpha 1(I)CB3$ was usually highest, sometimes as high as that to the parent collagen, that to $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$ generally around one-third lower, and that to $\alpha 2(I)CB3,5$ and $\alpha 2(I)CB4$ lower still. Negligible $\alpha 2\beta 1$ -mediated adhesion occurred to $\alpha 1(I)CB6$. $\alpha 2\beta 1$ -mediated adhesion, expressed as a proportion of the total, was substantially increased when fragments were renatured by

Figure 4 Affinity chromatography of platelet proteins on collagen fragments bound to Sepharose

Experimental details, identical for each sample, are described in the Materials and methods section. CNBr peptides were first renatured before coupling to Sepharose. Affinity chromatography was undertaken at 15 °C to avoid denaturation. Columns (5 mm $\times$ 40 mm), loaded with 0.25 ml of ^{125}I -labelled platelet lysate (containing 0.14 M NaCl), were eluted in turn with salt (0.5 M), EDTA and SDS/urea as described in the Materials and methods section. Eluted material was separated by SDS/PAGE and examined by autoradiography. Lane 1, salt wash; 2, EDTA eluate; 3, SDS/urea eluate. Sizes as indicated (kDa) are based on molecular-mass markers run at the same time.

gradual stepwise cooling beforehand rather than allowing renaturation to occur during coating (Table 3).

Affinity chromatography was undertaken to confirm the presence of $\alpha 2\beta 1$ -recognition sites in fragments $\alpha 1(I)CB3$, $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$. In accord with other reports [2,14], EDTA was found to cause the elution from a collagen-Sepharose column of two proteins, 155 and 120 kDa in size (Figure 4), increasing to 165 and 140 kDa respectively after reduction. Their identity as integrin subunits $\alpha 2$ and $\beta 1$ was confirmed by immunoprecipitation (not shown). In accord with the lack of platelet adhesion to denatured collagen, there was little if any binding of platelet membrane proteins to denatured collagen immobilized on Sepharose (not shown). As illustrated in Figure 4, bivalent-cation-dependent binding of integrin- $\alpha 2\beta 1$ was detected to $\alpha 1(I)CB3$, $\alpha 1(I)CB7$ and $\alpha 1(I)CB8$ fragments immobilized on Sepharose. However, EDTA failed to elute any detectable integrin $\alpha 2\beta 1$ from $\alpha 1(I)CB6$ -Sepharose (not shown). The EDTA eluate from the $\alpha 1(I)CB7$ -bound column revealed a 90 kDa component. This band was only obvious in the SDS/urea eluate from the other columns.

'Activation-dependent' adhesion to collagen and its fragments

Anti- $\alpha IIb\beta 3$ mAbs block platelet aggregation by preventing the binding of fibrinogen to $\alpha IIb\beta 3$ on activated platelets. As with other anti- $\alpha IIb\beta 3$ mAbs [15,16], mAb RFGP56 strongly inhibited platelet aggregation by collagen fibres, causing total loss of aggregation at 1 μ g/ml (not shown). It invariably also caused a partial inhibition of adhesion to collagen, of the order of 25–50% (Figure 3d), suggesting the occurrence of appreciable activation-dependent adhesion involving integrin $\alpha IIb\beta 3$. When PGE_1 was included as an inhibitor of platelet activation, either added

Table 4 Platelet adhesion to native intact collagen (type I) fibres: effect of mAbs 6F1, 13 and RFGP56

Dishes (35 mm) were coated with fibres (500 $\mu\text{g}/\text{ml}$) for 2 h at room temperature. Adhesion was measured for 60 min at room temperature (25 °C) in the presence of 2 mM MgCl_2 or 2 mM EDTA, as indicated. Antibodies were tested at a concentration, as stated, sufficient to produce the maximum response.

Addition	Adhesion (%)	
	Mg^{2+}	EDTA
None	53	36
mAb 6F1 (1 $\mu\text{g}/\text{ml}$)	47	35
mAb 13 (10 $\mu\text{g}/\text{ml}$)	51	34
mAb RFGP56 (10 $\mu\text{g}/\text{ml}$)	38	34
mAbs 6F1 + RFGP56	24	34
mAbs 13 + RFGP56	27	36

throughout platelet isolation and adhesion or simply in the adhesion medium, adhesion to collagen, in either case, was reduced, on average by one-third. However, even in the presence of PGE_1 , RFGP56 still caused inhibition of adhesion to proportionately the same extent as in the absence of PGE_1 . In a typical experiment, adhesion in the presence of PGE_1 was 30 %, reducing to 20 % on addition of mAb RFGP56 to the adhesion medium. To investigate further the relationship between platelet activation and $\alpha\text{IIb}\beta 3$ -mediated adhesion, we examined the effects of PGE_1 and RFGP56 alone and in combination. The two combined always produced more inhibition than either alone. In one experiment, for example, adhesion of 44 % was reduced to 27 % by PGE_1 , to 25 % by RFGP56 and to 13 % when both were present. These data indicate that 'activation-dependent' adhesion involves, in roughly equal amounts, PGE_1 -sensitive adhesion unrelated to $\alpha\text{IIb}\beta 3$ and adhesion mediated by $\alpha\text{IIb}\beta 3$ but unaffected by PGE_1 . $\alpha\text{IIb}\beta 3$ -mediated adhesion, inhibitable by PGE_1 , is only a minor component.

Adhesion to fragments was also partially inhibited by RFGP56. Results for $\alpha 1(\text{I})$ chain fragments are shown in Table 2. In the case of fragments CB6, CB7 and CB8, inhibition was greater than that caused by 6F1. In the case of CB3, CB7 and CB8, which are able to support $\alpha 2\beta 1$ - as well as $\alpha\text{IIb}\beta 3$ -mediated adhesion, inhibition by 6F1 and RFGP56 in combination was greater than either alone.

Adhesion to collagen fibres

In contrast with the total lack of adhesion to monomeric collagen in the presence of EDTA, adhesion to fibres in the presence of the chelating agent was relatively high, around three-quarters of that observed in the presence of Mg^{2+} , indicating the ability of fibres to support substantial bivalent-cation-independent adhesion (Table 4). No adhesion occurred to heat-denatured collagen fibres at 37 °C after coating at this temperature. Adhesion to fibres in the presence of EDTA was unaffected by mAbs 6F1 (or 13) and RFGP56, singly or in combination. In the presence of Mg^{2+} , adhesion was only slightly reduced by anti-($\alpha 2\beta 1$ -integrin) antibodies, more so by RFGP56. For reasons unknown, combination of mAbs 6F1 and RFGP56 produced more inhibition than the sum of the two individually. Nevertheless, substantial adhesion still occurred in the presence of these antibodies, confirming the existence of considerable bivalent-cation-independent binding to fibres even in the presence of Mg^{2+} . Unexpectedly, 'activation-dependent' adhesion, measured as the

Figure 5 Affinity chromatography of platelet proteins on a collagen fibre-Sepharose column

Chromatography conditions are detailed in the Materials and methods section. Platelet lysate (1 ml) was applied to the column (10 mm $\times$ 45 mm) which was then successively eluted, as indicated by the arrows, with salt, EDTA and SDS/urea. Inset: autoradiograph of material eluted from a collagen fibre-Sepharose column by salt (lane 1) and SDS/urea (lane 2) and separated by SDS/PAGE. In this case, the column was loaded with radiolabelled platelet lysate in the presence of 20 mM EDTA which was also included in elution solvents. Sizes (kDa) as shown were derived by comparison with standard markers.

inhibition produced by PGE_1 and RFGP56 together, was less to fibres than to monomeric collagen. In one experiment, for example, adhesion to monomeric collagen was reduced from 48 to 17 % in the combined presence of PGE_1 and RFGP56, much in accord with data already presented. However, adhesion to fibres measured at the same time fell from 47 to 38 %. Equivalent figures in another experiment were 50 and 33 % and 47 and 37 % respectively.

Affinity chromatography indicated little if any bivalent-cation-dependent ligand binding to fibres (Figure 5). In the presence of EDTA, binding of three major species, 90, 150 and 190 kDa in size, was detected (Figure 5).

DISCUSSION

Conformation-dependent adhesion involving integrin $\alpha 2\beta 1$

Previous studies by us and others have reported adhesion, at room temperature, to denatured collagen [3,17,18] and to unfolded α -chains and fragment $\alpha 1(\text{I})\text{CB3}$ [18]. Our data here, however, clearly demonstrate that adhesion at 37 °C is strongly dependent on conformation. Previously noted adhesion to non-helical species at room temperature may have been due to their renaturation when dishes coated with the denatured species at 37 °C were then brought to room temperature for assay of adhesion. In support of this, we have observed that adhesion at room temperature to denatured collagen (coated at 37 °C) is Mg^{2+} -dependent and totally inhibitable by mAb 6F1. Others have found poor adhesion to denatured collagen [4,9] and commercial gelatin [18] even at room temperature. Possibly, denaturation conditions in these cases were so severe as to prevent renaturation.

Platelet adhesion to monomeric collagen mediated by integrin $\alpha 2\beta 1$ has been attributed to a recognition site in the fragment $\alpha 1(\text{I})\text{CB3}$; little adhesion was observed to other $\alpha 1$ -chain fragments [5,18]. However, our studies demonstrate $\alpha 2\beta 1$ -mediated

adhesion to fragments CB7 and CB8 as well as CB3 and to the $\alpha 2(I)$ chain fragments $\alpha 2(I)CB3,5$ and $\alpha 2(I)CB4$, implying a wide distribution of $\alpha 2\beta 1$ -binding sites. $\alpha 2\beta 1$ -mediated adhesion to CB7 and CB8 as well as CB3 has also been detected under flow [19]. Although we consider that adhesion to collagen chains and fragments is due to the adoption of a helical conformation during coating, we cannot exclude the possibility that some adhesion at room temperature is independent of conformation. This could explain why adhesion to fragments may be the same whether coating is under standard conditions or with fragments optimally renatured beforehand, and yet $\alpha 2\beta 1$ -mediated adhesion is greater with the previously renatured material. The latter observation suggests that conformation is especially critical for $\alpha 2\beta 1$ -dependent adhesion. It is possible that differences between fragments, particularly as regards $\alpha 2\beta 1$ -mediated adhesion, are really due to differences in the degree of correct triple-helical alignment achieved during renaturation. This could explain why adhesion to the parent monomeric collagen is totally $\alpha 2\beta 1$ -dependent but adhesion to fragments is only partially so.

In contrast with our findings with human platelets, rat fibroblasts exhibit $\alpha 2\beta 1$ -mediated adhesion to $\alpha 1(I)CB3$ but not CB8 [20]. Conceivably, this may reflect conformational differences between integrin $\alpha 2\beta 1$ in platelets and fibroblasts [21].

Inhibition studies have indicated that platelet adhesion via integrin $\alpha 2\beta 1$ is mediated by Asp-Gly-Glu-Ala in collagen [5]. However, we have been unable to inhibit adhesion with peptides containing this sequence (L. F. Morton and M. J. Barnes, unpublished work) or $\alpha 2\beta 1$ -dependent adhesion of chondrosarcoma cells to collagen type II (M. J. Humphries and D. S. Tuckwell, unpublished work). In any event, $\alpha 2\beta 1$ -mediated platelet adhesion to $\alpha 2(I)CB4$ must involve a different type of sequence, as Asp-Gly-Glu-Ala or similar sequences are absent from this fragment [22]. The same holds true for $\alpha 2\beta 1$ -mediated adhesion to the cell-binding domain of type-IV collagen [23].

'Activation-dependent' adhesion to monomeric collagen and collagen fragments

At least two types of 'activation-dependent' adhesion have been detected in this study, occurring to approx. equal extent, one that is insensitive to PGE_1 and mediated by $\alpha IIb\beta 3$, the other that is inhibitable by PGE_1 but does not involve $\alpha IIb\beta 3$. As mAb 6F1 (or 13) totally blocked adhesion to collagen, this activation-dependent element must be entirely secondary to $\alpha 2\beta 1$ -mediated adhesion. Some $\alpha IIb\beta 3$ -mediated adhesion to collagen has been detected previously [9,24], but other studies noted little effect of PGE_1 on adhesion [25]. As phase-contrast microscopy indicated adhesion basically only of single platelets, with little if any platelet-platelet interaction [17], we consider that $\alpha IIb\beta 3$ -dependent adhesion must involve platelet contact with collagen, possibly mediated by the adhesive proteins, fibronectin and/or von Willebrand factor. Both proteins are known to bind to integrin $\alpha IIb\beta 3$ after their release from activated platelets [26] and to be able to bind to collagen [27,28]. Others have speculated on the existence of interactions of this nature in collagen-induced platelet aggregation in PRP [9]. The failure of PGE_1 to prevent $\alpha IIb\beta 3$ -dependent adhesion agrees with the observation that collagen, unlike other agonists, can activate platelets in the presence of elevated levels of cyclic AMP [29].

We do not know the mechanism responsible for PGE_1 -sensitive adhesion independent of integrin $\alpha IIb\beta 3$. ADP activation of platelets in PRP leads to increased $\alpha 2\beta 1$ -mediated adhesion to collagen, an effect prevented by blocking activation with prostaglandin I_2 . However, this phenomenon was not seen with washed platelets [30].

$\alpha IIb\beta 3$ -mediated adhesion occurred to all fragments tested. mAbs 6F1 and RFGP56 in combination caused more inhibition of adhesion to CB3, CB7 and CB8 than either alone, and it follows that $\alpha IIb\beta 3$ -mediated adhesion must at least in part be independent of $\alpha 2\beta 1$ -mediated adhesion. In the case of CB6 where there was no $\alpha 2\beta 1$ -mediated adhesion, $\alpha IIb\beta 3$ -mediated adhesion cannot be dependent on prior $\alpha 2\beta 1$ -mediated adhesion. We assume that primary adhesion to fragments by other mechanisms must support some secondary $\alpha IIb\beta 3$ -mediated adhesion.

Adhesion to fibres

Adhesion to monomeric collagen requires Mg^{2+} and is totally dependent on integrin $\alpha 2\beta 1$. Partial inhibition by RFGP56 indicates some $\alpha IIb\beta 3$ -mediated adhesion secondary to primary $\alpha 2\beta 1$ -supported adhesion. In contrast, adhesion to fibres is mostly bivalent-cation-independent with relatively minor involvement of $\alpha 2\beta 1$. In accord with the results of others [31], some $\alpha IIb\beta 3$ -mediated adhesion occurs but this appears to be totally independent of any $\alpha 2\beta 1$ -mediated adhesion, as 6F1 and RFGP56 together actually produce more inhibition than the sum of either alone. $\alpha IIb\beta 3$ -mediated adhesion to fibres may, conceivably, involve direct interaction between the integrin and collagen independent of platelet activation.

It is accepted that to cause platelet aggregation (activation) collagen must be polymeric (fibrous) [1]. However, we have found in this study evidence of substantial 'activation', in the presence of Mg^{2+} , by (immobilized) monomeric collagen, greater in fact than that observed with fibres. There is no doubt of the potent aggregatory activity of these same fibres in PRP, presumably in this case as a result of the presence of essential Ca^{2+} . The precise role of integrin $\alpha 2\beta 1$ in collagen fibre interaction with platelets is uncertain. We have demonstrated here $\alpha 2\beta 1$ -independent adhesion to fibres, and others have noted that the aggregation in PRP is not prevented by mAb 6F1 [9].

The identity of the three bands, 90, 150 and 190 kDa in size, that bind to collagen fibres in the absence of bivalent cations is under investigation. A 90 kDa protein was also detected that bound to monomeric collagen and collagen fragments. There are several reports of a membrane protein of this size involved in collagen-platelet interaction, including CD36 [4,24,32-35]. However, we cannot exclude the possibility that bivalent-cation-independent adhesion to fibres involves a non-collagenous constituent such as dermatan sulphate proteoglycan.

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