Integrin $\alpha 2\beta 1$ -independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for $\alpha 2\beta 1$ -independent platelet reactivity

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The platelet reactivities of two simple collagen-like synthetic peptides, Gly-Lys-Hyp-(Gly-Pro-Hyp)₁₀-Gly-Lys-Hyp-Gly and Gly-Cys-Hyp-(Gly-Pro-Hyp)₁₀-Gly-Cys-Hyp-Gly, were investigated. Both peptides adopted a stable triple-helical conformation in solution. Following cross-linking, both peptides proved to be highly platelet-aggregatory, more active than collagen fibres, inducing aggregation at concentrations as low as 20 ng/ml. These peptides formed microaggregates in solution, and cross-linking was thought to stabilize these structures, allowing expression of their platelet reactivity at 37 °C. Like collagen fibres,

the peptides caused platelet secretion and release of arachidonate from platelet membrane lipids as well as activation of integrin $\alpha IIb\beta 3$ culminating in aggregation. Monoclonal antibodies directed against the integrin $\alpha 2\beta 1$ failed to prevent aggregation, release of arachidonate or platelet adhesion to the peptides. Our results indicate that collagen can activate platelets by a mechanism that is independent of integrin $\alpha 2\beta 1$ and for which collagen tertiary and quaternary structures are sufficient alone for activity without the involvement of highly specific cell-recognition sequences.

INTRODUCTION

The interaction between collagen fibres and blood platelets culminating in platelet aggregation is one of the basic underlying mechanisms responsible for the formation of the haemostatic plug, essential for the arrest of bleeding after injury. Collagen-induced platelet activation may also be an important cause of pathological platelet aggregation responsible for thrombosis [1].

The regulation of cell function by the extracellular matrix, as exemplified by collagen-platelet interaction, is mediated by specific cell-surface receptors, notably the integrins, which recognize discrete cell-reactive structures located within individual matrix constituents. Thus the amino acid sequence Arg-Gly-Asp-Xaa (where Xaa is one of several possible amino acids) serves as a cell-binding site in a number of matrix proteins, being recognized by a variety of integrins [2,3]. Cell adhesion to native (triple-helical) collagen type IV involving integrin $\alpha 1\beta 1$ is mediated by a single site encompassing an Asp residue at position 461 in the $\alpha 1(IV)$ chain and an Arg residue at the identical position in the $\alpha 2(IV)$ chain [4]. The integrin collagen receptor $\alpha 2\beta 1$ is thought to play an important role in the interaction of collagen with platelets [5–13]. Interaction between integrin $\alpha 2\beta 1$ and (rat) collagen type I is reported to be mediated by the sequence Asp-Gly-Glu-Ala occurring at residues 435–438 in the $\alpha 1(I)$ chain [14]. In contrast with these findings, we report here evidence, using simple, relatively short, collagen-like synthetic peptides, that all that is required for collagen to cause aggregation of platelets is collagen tertiary (triple-helical) and quaternary (polymeric) structures and that activation requires neither the mediation of integrin $\alpha 2\beta 1$ nor the presence of highly specific receptor-recognition sequences.

MATERIALS AND METHODS

Petri dishes (Falcon 1008; 35 mm) for use in adhesion assays were obtained from Becton–Dickinson, Oxford, U.K. Immulon 2 microtitre plates for the same purpose were from Dynatech Laboratories Ltd., Billingshurst, West Sussex, U.K. Na₂⁵¹CrO₄ and [³H]arachidonic acid (TRK 757) were from Amersham International, Amersham, Bucks., U.K. ATP Monitoring Reagent was supplied by Lab Systems (U.K.) Ltd., Basingstoke, Hants., U.K. Acetonitrile and water, both of h.p.l.c. grade, were from Fisons Scientific Equipment, Loughborough, Leics., U.K. Unless otherwise indicated, all other specialist reagents were from Sigma, Poole, Dorset, U.K.

Freshly collected citrated blood for platelet assays was received from the National Blood Transfusion Service, Cambridge, U.K.

Collagen type I was obtained from a limited pepsin digest of calf skin as described previously [15]. A suspension of native collagen (type I) fibres from bovine tendon, details of which have been reported previously [16], was from Ethicon, 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.

A solution, in 0.01 M Tris/0.15 M NaCl/0.05 % NaN₃, of affinity-purified monoclonal antibody (mAb) 6F1, a mouse mAb directed against human platelet integrin $\alpha 2\beta 1$ and recognizing the $\alpha 2$ -subunit [7], was a gift from Dr. B. S. Collier, School of Medicine, State University of New York, Stony Brook, NY, U.S.A. This antibody was dialysed against PBS before use. The murine mAb Gi9 recognizing human integrin $\alpha 2\beta 1$ was purchased from The Binding Site Ltd., Birmingham, U.K. Purified rat mAb 13 recognizing the human $\beta 1$ -integrin subunit [17] was donated

Abbreviations used: mAb, monoclonal antibody; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; TNBS, trinitrobenzenesulphonic acid; PGE₁, prostaglandin E₁.

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by Dr. M. J. Humphries, School of Biological Sciences, University of Manchester, Manchester, U.K. A mouse anti-(human platelet α Ib β 3) mAb (in ascites fluid), RFGP56 [18,19], was given by Dr. A. H. Goodall, Royal Free Hospital School of Medicine, Hampstead, London N.W.3, U.K.

Peptide synthesis

The peptides:

Gly-Lys-Hyp-(Gly-Pro-Hyp)₁₀-Gly-Lys-Hyp-Gly (Peptide 1)

and

Gly-Cys-Hyp-(Gly-Pro-Hyp)₁₀-Gly-Cys-Hyp-Gly (Peptide 2)

were synthesized on a Millipore 9050 Plus Pepsynthesizer using standard Fmoc [*N*-(9-fluorenylmethoxycarbonyl)] solid-phase chemistry [20]. Coupling cycles were of 30 min duration. At the completion of synthesis, peptides were cleaved from the resin with trifluoroacetic acid containing 5% water (h.p.l.c. grade), 5% phenol and 2% of tri-isopropylsilane. The crude peptide solutions were dried by rotary evaporation, the residue extracted with diethyl ether, dried, and then dissolved in 0.5 M acetic acid prior to freeze-drying. Peptides were then purified on a 1 cm $\times$ 70 cm column containing Vydac 218TPB1520 (Technicol, Stockport, Greater Manchester, U.K.) using a gradient of acetonitrile (h.p.l.c. grade) in 0.1% trifluoroacetic acid. Acetonitrile was removed from peptide-containing fractions by rotary evaporation; the peptide solution was then adjusted to 0.5 M acetic acid and freeze-dried.

Synthesis was also undertaken of a control peptide, designated 'Peptide 3', representing residues 463–501 of the α 1(III) chain of type III collagen (sequence GLQGLP*GTSGPP*GENGKP*GEP*GPKGEAGAP*GIP*GGKGDS; P* = Hyp), but including additionally Gly-Pro-Cys at the N- and C-terminus.

Homogeneity of the purified peptides was verified by h.p.l.c. The identity of peptides was confirmed by amino acid analysis.

Polarimetry

The stability of the triple-helical conformation of peptides 1, 2 and 3 was assessed by measurement of the change in optical rotation with increasing temperature using a polarimeter (Bellingham and Stanley, model A) equipped with a sodium lamp.

Cross-linking of peptides

Peptide 1 was cross-linked through reaction of lysine residues with glutaraldehyde, by dissolving at 0.3 mg/ml in cold phosphate buffer (*I* 0.4, pH 7.6) and, after some minutes, stirring on ice with glutaraldehyde, routinely 0.25%, for 1 h, as described previously [16]. After reaction, the peptide was dialysed against cold dilute acetic acid and freeze-dried.

Peptide 2 was cross-linked by allowing the spontaneous formation of disulphide bonds after the peptide was dissolved, at 5 mg/ml, in phosphate buffer (*I* 0.4) and left at 4 °C for at least 16 h. Alternatively, this peptide, and also Peptide 3, were polymerized by dissolving them at 5 mg/ml in ice-cold PBS and, after 1 h, adding 0.5 mg of the heterobifunctional cross-linking reagent *N*-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) in 0.4 ml of ethanol/ml of peptide solution [21,22]. The mixture was left on ice for 4 h, then left overnight at 4 °C before dialysis against cold dilute acetic acid.

Modification of lysine residues

Unchanged lysine residues remaining in peptide 1 after glutaraldehyde cross-linking were determined by reaction with trinitrobenzenesulphonic acid (TNBS) as described by Kakade and Liener [23]. For some experiments, these residues were blocked either with TNBS as described previously [24], using the method of Wilner et al. [25], or by acetylation [4], as follows. A 2 mg portion of the cross-linked peptide was suspended in 0.1 ml of water to which was added 0.9 ml of half-saturated sodium acetate and the mixture stirred on ice for 10 min. Six 2 μ l aliquots of acetic anhydride were added over 1 h while stirring and the mixture then dialysed against dilute acetic acid, followed by PBS. The completeness of modification in these experiments was checked by the procedure of Kakade and Liener [23].

Adhesion studies

Adhesion was measured using ⁵¹Cr-labelled gel-filtered platelets [15]. Where necessary, platelets were gel-filtered in the absence of the usual 2 mM Mg²⁺ and additions as required, 2 mM Mg²⁺ or 2 mM EDTA made to the platelet suspension after filtration. Dishes or wells were coated with collagen or peptide (10 μ g/ml) or collagen fibres (500 μ g/ml) in 0.01 M acetic acid for 2 h at room temperature. Adhesion was measured at room temperature over a period of 60 min. Assays were undertaken in triplicate, and results are expressed as means $\pm$ S.D. of the three determinations. Values are quoted to the nearest whole number. Percentage adhesion refers to bound radioactivity expressed as a percentage of the total added [15]. In some instances, to conserve reagents, adhesion was measured using microtitre plates [14]. Percentage adhesion was lower than with 35 mm-diameter dishes [14], probably due to an increased platelet suspension volume per unit surface area in the microtitre-plate assay. Adhesion to uncoated dishes or wells blocked with BSA was never more than 2% [13].

In inhibition studies, mAbs were included in the platelet suspension at least 5 min before adding to dishes or wells.

When required, prostaglandin E₁ (PGE₁) (100 ng/ml; freshly added at all stages) was included during platelet isolation and in the adhesion assay.

Measurement of platelet secretion and aggregation

Aggregation was measured turbidimetrically using human citrated platelet-rich plasma as described previously [24]. Gel-filtered platelets for aggregation studies were prepared as usual [15], except that gel filtration was performed in modified Tyrode's buffer (138 mM NaCl/2.7 mM KCl/2 mM MgCl₂/12 mM NaHCO₃/0.4 mM NaH₂PO₄/5.6 mM glucose/0.2% BSA/10 mM Hepes, pH 7.5). Inhibition of aggregation was assessed by preincubation of the platelet suspension with the test solution or antibody 2 min prior to the addition of agonist. The latter was added in an amount just sufficient on its own to cause a normal aggregatory response. The mAb 6F1 was dialysed against PBS prior to use (to effect the removal of Tris, which has been reported to inhibit collagen-induced platelet aggregation [26]). In some experiments, secretion was measured concomitantly with aggregation using a bioluminescence technique with the Chrono-log Aggro-Meter (model 550; Coulter Electronics Ltd.), using 0.45 ml of platelet-rich-plasma containing 50 μ l of ATP Monitoring Reagent.

Measurement of arachidonate release

Platelet-rich-plasma was centrifuged at 700 *g* for 15 min and the platelet pellet then resuspended in a buffered saline solution

(145 mM NaCl/5 mM KCl/10 mM glucose/1 mM MgSO₄/0.5 mM EGTA/10 mM Hepes, pH 7.4). The platelet number was determined in a haemocytometer and the suspension adjusted to 1×10^9 cells/ml. Ligand-stimulated arachidonic acid release was measured using a modification of the method of Joseph et al. [27]. Platelets were labelled for 1 h at 30 °C with [³H]arachidonate (1 µCi/ml). They were then diluted 3-fold with buffer and centrifuged at 700 *g* for 15 min to remove excess label. The platelet pellet was resuspended at a final concentration of 1×10^9 /ml. A 45 µl portion of this suspension was added to 5 µl of agonist, either Peptide 1, Peptide 2 or collagen fibres, vortex-mixed, and incubated at 30 °C for 6 min. The reaction was stopped by the addition of 50 µl of a 3.8 % formaldehyde/40 mM EDTA solution, and platelets were centrifuged at 10000 *g* for 1 min. A 75 µl portion of supernatant was counted for radioactivity by liquid scintillation to determine the amount of arachidonic acid released upon ligand stimulation. Assays were undertaken in triplicate and the results (corrected for release in the absence of ligand) expressed as the means of the three determinations.

Electron microscopy

Freeze-dried samples were dissolved in 0.05 M acetic acid at 4 °C at a concentration of 1 mg/ml. One drop of solution was then applied using a Pasteur pipette to the surface of a copper electron-microscope specimen grid coated with collodion. After approx. 30 s, negative staining was carried out by holding the grid at an angle of 45 ° and allowing three or four droplets of the stain solution to fall across the grid surface, mix with the sample and drain on to Velin tissue held below. Stains used were 1 % uranyl acetate, 1 % potassium phosphotungstate or 2 % ammonium molybdate. All stains were passed through a 2 µm-pore-size Millipore filter prior to use. Residual liquid was drained from the grid on to Velin tissue and the preparation was allowed to air-dry at room temperature.

Samples were examined in a Phillips 400 transmission electron microscope at 80 kV, and images were recorded on Kodak SO-163 film at magnifications of 100 000–170 000 ×.

RESULTS

Measurements of optical rotation indicated that Peptides 1 and 2 adopted in solution a highly stable triple-helical conformation with a 'melting' temperature (*T_m*) close to 60 °C in accord with published data for the peptide (Pro-Hyp-Gly)₁₀ [28]. In contrast, Peptide 3 was non-helical even at 1.5 °C, the lowest temperature tested.

Platelet activation by Peptides 1 and 2

Since both tertiary and quaternary structures of collagen are necessary for collagen-induced platelet aggregation [1], Peptides 1 and 2 were synthesized with either lysyl (Peptide 1) or cysteinyl (Peptide 2) residues present, with a view to obtaining a quaternary structure by cross-linking. Cross-linking of monomeric collagen with glutaraldehyde has been shown to yield a polymer possessing platelet-aggregatory activity [16,29], and we have utilized cross-linking in this way to ascertain the platelet-aggregatory activity of collagen CNBr-derived fragments [24,30]. Peptide 1 was cross-linked over a range of glutaraldehyde concentrations (0.1, 0.25 and 0.5 %; [29]). All cross-linked forms were highly platelet-aggregatory and consistently more active than our standard collagen-fibre preparation. As far as we are aware, the latter is the most platelet-reactive collagen available. Cross-linking at 0.25 % yielded the most active peptide species, able on occasion

Figure 1 Comparison of platelet aggregation by collagen (type I) fibres and cross-linked Peptide 1.

Aggregation was measured in platelet-rich plasma at 37 °C. The arrow indicates the point of addition of sample to the final concentration specified below. (a) Fibres; 0.25 µg/ml; (b) fibres; 0.13 µg/ml; (c) Peptide 1 cross-linked with 0.25 % glutaraldehyde; 0.2 µg/ml; (d) as (c); 0.02 µg/ml; (e) Peptide 1 cross-linked with 0.5 % glutaraldehyde; 0.17 µg/ml; (f) As (e), but heat-denatured; 1.7 µg/ml.

to cause platelet aggregation at concentrations of 20 ng/ml and above and at least ten times more active than collagen fibres tested at the same time (Figure 1). Cross-linking at 0.1 and 0.5 % glutaraldehyde yielded species active at best at about 150 ng/ml and above. Heat denaturation of cross-linked peptide (95 °C for 10 min) caused total loss of activity, testing up to ten times the concentration required for activity without denaturation (Figure 1).

Reaction with TNBS indicated that around 50 % of lysine residues in Peptide 1 were involved in cross-linking when using glutaraldehyde at 0.25 %. Complete modification of the remaining residues was effected either by trinitrophenylation or by acetylation. Modification had no effect whatsoever on the platelet reactivity (results not shown).

These results indicated that the aggregatory activity exhibited by Peptide 1 might be solely due to the tertiary and quaternary structures of the peptide. This was confirmed by synthesizing Peptide 2, where cysteine replaced the lysine in Peptide 1, to provide an alternative means of introducing quaternary structure.

Figure 2 Platelet secretion and aggregation induced by collagen fibres and cross-linked Peptides 1 and 2

Secretion and aggregation were measured concomitantly in the same sample, as described in the Materials and methods section. (a) Peptide 1 cross-linked with 0.25% glutaraldehyde. (b) Peptide 2 cross-linked with SPDP. (a) and (b) represent separate experiments. The arrow indicates the point of addition of sample at the specified final concentration.

Cross-linking of this peptide was then achieved either by spontaneous S-S-bond-formation or by the use of the cross-linking reagent SPDP. Both methods of polymerization yielded highly aggregatory species. Like Peptide 1, Peptide 2, cross-linked with SPDP, was more active than collagen fibres (Figure 2). The S-S-bonded Peptide 2 caused aggregation at around $3 \mu\text{g/ml}$ in comparison with fibres active at $0.5 \mu\text{g/ml}$ and above tested at the same time.

As with fibres, Peptides 1 and 2 both caused platelet secretion as well as aggregation (Figure 2). Both secretion and aggregation were totally inhibited with indomethacin at $10 \mu\text{M}$ (results not shown). In accord with our previous data [13], the anti-(integrin $\alpha\text{IIb}\beta 3$) mAb RFGP56 at $1 \mu\text{g/ml}$ completely blocked aggre-

Figure 3 Aggregation of gel-filtered platelets by cross-linked Peptide 1: lack of effect of mAb 6F1

Peptide 1 was cross-linked with 0.25% glutaraldehyde. Antibody in buffer, or buffer alone was added about 2 min before the addition of agonist (peptide or collagen fibres) at the specified final concentration.

Figure 4 Release of [^3H]arachidonic acid induced by Peptides 1 and 2 and collagen fibres

Assays were undertaken in triplicate, and the results are expressed as means $\pm$ S.E.M. ○, Peptide 1, cross-linked with 0.25% glutaraldehyde; ◆, Peptide 2, cross-linked by spontaneous disulphide bonding; □, collagen fibres.

gation by collagen fibres. It was found here to be equally effective in preventing aggregation by Peptides 1 and 2, whilst having no significant effect on secretion (results not shown).

Table 1 Platelet adhesion to Peptide 1

(a)–(e) Represent separate experiments. Adhesion was measured in the presence of 2 mM Mg^{2+} , except where EDTA (2 mM) is indicated. When included, mAb RFGP56 was tested at 10 $\mu g/ml$ [13], mAbs 6F1 and Gi9 at the concentrations indicated. Adhesion to monomeric collagen (type I) was 44% in (a) and 54% in (b). In (c) and (e), PGE_1 was included during platelet isolation and in the assay medium, as described in the Materials and methods section. In (d), adhesion was measured using Immulon 2 96-well plates.

(a)

Adhesion (%)

Control	mAb RFGP56	EDTA
43 $\pm$ 2	34 $\pm$ 3	32 $\pm$ 0

(b)

Adhesion (%)

Control	mAb 6F1 (5 $\mu g/ml$)	mAb RFGP56	Both
54 $\pm$ 1	58 $\pm$ 3	47 $\pm$ 1	48 $\pm$ 1

(c)

Adhesion (%)

mAb 6F1 ($\mu g/ml$) ...	0	1.25	2.5	5.0	25.0
Collagen (monomeric)	24 $\pm$ 1	0	0	2 $\pm$ 0	–
Peptide	25 $\pm$ 1	–	–	–	25 $\pm$ 1

(d)

Adhesion (%)

	Control	mAb Gi9 (20 $\mu g/ml$)
Collagen (monomeric)	14 $\pm$ 1	1 $\pm$ 0
Peptide	15 $\pm$ 1	16 $\pm$ 0

(e)

Adhesion (%)

	Control	EDTA
Peptide	53 $\pm$ 2	32 $\pm$ 1
Fibres	58 $\pm$ 2	39 $\pm$ 1

Table 2 Platelet adhesion to Peptide 2

(a)–(c) Represent separate experiments. Adhesion was measured in the presence of 2 mM Mg^{2+} , except where EDTA (2 mM) is indicated. Adhesion to monomeric collagen (type I) was 39 $\pm$ 0% in (a), reducing to 2 $\pm$ 0% in the presence of mAb 6F1 (2.5 $\mu g/ml$). In (b) and (c), adhesion was measured using Immulon 2 multi-well plates.

(a)

Adhesion (%)

Control	mAb 6F1 (2.5 $\mu g/ml$)	mAb RFGP56 (10 $\mu g/ml$)	EDTA
46 $\pm$ 2	45 $\pm$ 1	39 $\pm$ 0	37 $\pm$ 1

(b)

Adhesion (%)

	Control	mAb 6F1 (2.5 $\mu g/ml$)	mAb 6F1 (25 $\mu g/ml$)
Collagen (monomeric)	14 $\pm$ 1	1 $\pm$ 0	–
Peptide	17 $\pm$ 1	–	16 $\pm$ 0

(c)

Adhesion (%)

	Control	mAb Gi9 (20 $\mu g/ml$)	mAb 13 (10 $\mu g/ml$)
Collagen (monomeric)	12 $\pm$ 1	1 $\pm$ 0	1 $\pm$ 0
Peptide	11 $\pm$ 1	13 $\pm$ 1	12 $\pm$ 1

the loss of activity of cross-linked Peptides 1 and 2 on denaturation and with the failure of denatured collagen α -chains to exhibit aggregatory activity after cross-linking [30].

Both Peptides 1 and 2 were shown, like fibres, to be able to cause the aggregation of gel-filtered platelets. Aggregation by the peptides was totally unaffected by the anti-(integrin $\alpha 2$ -subunit) mAb, 6F1, tested up to 150 $\mu g/ml$ (Figure 3). This same antibody at 1 $\mu g/ml$ or less totally prevents the adhesion of gel-filtered platelets to monomeric collagen immobilized on plastic [7,13]. Aggregation by fibres at a concentration just sufficient for full response was partially inhibited by high concentrations of 6F1 (75 $\mu g/ml$), but inhibition was not observed on increasing the stimulus by doubling the fibre concentration, despite increasing the antibody level equivalently (Figure 3).

In accord with their higher aggregatory activity, Peptides 1 and 2 were found to be more active than fibres in causing arachidonate release from platelet membrane lipids. Even the disulphide-bonded Peptide 2, less aggregatory than fibres, was more active than fibres in the release assay (Figure 4). Release of arachidonate was totally unaffected by mAb 6F1 tested at 5 $\mu g/ml$ (results not shown).

Adhesion studies

Platelet adhesion to Peptides 1 and 2 immobilized on plastic was as good as that to monomeric collagen or collagen fibres (Tables 1 and 2). In contrast with the adhesion of platelets to monomeric

Peptides 1 and 2 failed to aggregate platelets in the absence of cross-linking, even when tested up to concentrations of 2 mg/ml, although they were able to inhibit aggregation by fibres (50% inhibition at around 100 $\mu g/ml$; results not shown).

Cross-linking of the non-helical Peptide 3 yielded a species totally devoid of aggregatory activity, testing up to 4 mg/ml, confirming that cross-linking in the absence of a triple-helical conformation is insufficient for activity. This is in agreement with

Figure 5 Microaggregates of Peptide 2 observed by electron microscopy

A solution of Peptide 2 in dilute acetic acid was negatively stained with ammonium molybdate to reveal the presence of numerous microaggregates. The bar represents 100 nm.

collagen, which is totally Mg^{2+} -dependent [31,15], and fully mediated by integrin $\alpha 2\beta 1$ [7,8,13], adhesion to the peptides was largely bivalent-cation-independent (Tables 1a and 2a) and fully independent of integrin $\alpha 2\beta 1$; mAb 6F1 tested up to $25 \mu g/ml$ was totally without effect, although fully preventing adhesion to monomeric collagen at $1.25 \mu g/ml$ in accord with our previously obtained data, indicating full inhibition at $1 \mu g/ml$ or less [13] (Tables 1b, 1c, 2a and 2b). The relatively small stimulation of adhesion to the peptides by Mg^{2+} appeared to be attributable to a small amount of adhesion mediated by the integrin $\alpha IIb\beta 3$ (Tables 1a, 1b and 2a). These characteristics of platelet adhesion to Peptides 1 and 2, bivalent-cation-independent apart from a small amount of adhesion mediated by integrin $\alpha IIb\beta 3$ and no involvement of integrin $\alpha 2\beta 1$, are features also to a large extent of adhesion to collagen fibres [13,15] (see Table 1e). We have reported previously that mAb 6F1 causes only a slight inhibition of adhesion to fibres [13]; in the present study, adhesion was reduced by 20% at a mAb concentration of $2.5 \mu g/ml$, and there was no further inhibition on increasing the concentration to $25 \mu g/ml$. Interestingly, when the platelet-activation inhibitor PGE_1 was included throughout platelet isolation and in the assay medium, adhesion to Peptides 1 and 2, as to collagen fibres, occurred as well as in its absence. In accord with our previous data [13], 'activation-dependent' adhesion to both synthetic peptides and to collagen, mediated by integrin $\alpha IIb\beta 3$, was not prevented by the presence of PGE_1 as a platelet-activation inhibitor (Table 1e).

The total lack of involvement of integrin $\alpha 2\beta 1$ in the platelet recognition of Peptides 1 and 2 was confirmed with the mAb Gi9. This antibody completely inhibited platelet adhesion to monomeric collagen at concentrations above $2.5 \mu g/ml$ (the lowest tested), but had no effect on adhesion to Peptides 1 and 2 tested up to $20 \mu g/ml$ (Tables 1d and 2c). MAb 13 was also without effect (Table 2c), whilst fully inhibiting adhesion to monomeric collagen, in accord with our previous data [13].

Electron microscopy

Examination of solutions of Peptides 1 and 2 under the electron microscope revealed the presence of large numbers of microaggregates (Figure 5), reminiscent of microcrystalline structures described for the peptide $(Pro-Hyp-Gly)_{10}$, which are formed by a regular lateral association of molecules [32].

DISCUSSION

Our previous studies [24,30,33] indicated that platelet adhesion to collagen and subsequent platelet activation could be regarded as distinct events involving different reactive sites in the collagen molecule and, perhaps, then, different receptors. Others [34,35] have also proposed a two-step mechanism of collagen-platelet interaction involving initially adhesion, where integrin $\alpha 2\beta 1$ recognizes highly specific binding sites in collagen such as the

sequence Asp-Gly-Glu-Ala (residues 435–438 in the (rat) collagen $\alpha 1(I)$ chain [14]), and then subsequent recognition of specific activation sequences leads to platelet aggregation. Several studies have indicated that lysine residues in collagen are important for the expression of collagen platelet-aggregatory activity [24,25,34,36,37]. In a previous study we postulated that the sequence Gly-Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys-Gly-Glu-Ala [residues 478–489 in the $\alpha 1(III)$ collagen chain] might be a platelet-activation sequence in collagen type III [24]. Chemical modification of lysine residues indicated their involvement in the aggregatory activity of the type III-derived fragment, $\alpha 1(III)CB4$, containing this sequence. Others independently concluded that this region of the type III collagen molecule contained a platelet-activation site [38]; the peptide Gly-Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys (residues 478–486) was shown to inhibit collagen-induced platelet aggregation [38,39]. However, we considered that the inhibition was indirect, arising as a result of inhibition of collagen fibrillogenesis (this leading to an absence of fibres necessary for aggregation) [40]. Nevertheless, when the sequence concerned was synthesized within a triple-helical structure and added to platelets in a polymeric (cross-linked) form, it was able to cause their aggregation [40]. This seemed to confirm the concept of specific platelet-activation sequences in collagen involving lysine. However, this view is refuted by the present study. Simple collagen-like peptides consisting essentially of a repeating Gly-Pro-Hyp sequence proved to be highly platelet-reactive. The collagen triple-helical conformation was necessary for activity. Cross-linking was also required. These peptides formed microaggregates in solution, and we consider that cross-linking was necessary to stabilize these structures to allow the expression of their aggregatory activity at 37 °C. The activity of these peptides can, in our opinion, be attributed solely to their triple-helical and polymeric structures. The dimensions of the microaggregates may be such as to offer a much larger surface area relative to mass than that presented by the collagen fibre, and this could account for their higher platelet reactivity in comparison with fibres. Cross-linking (of Peptide 1) with 0.25 % glutaraldehyde yielded the most reactive species. Conceivably cross-linking at 0.1 % failed to fully stabilize the microaggregates, whilst cross-linking at 0.5 % yielded larger polymeric assemblies, in effect reducing the surface area relative to mass. We found no evidence that the lysine residues in Peptide 1 not involved in cross-linking were required for the platelet-aggregatory activity of this peptide. This conclusion was confirmed by the ability of Peptide 2 with cysteine in place of lysine to cause platelet aggregation. Side-chain-modification studies have shown that negative charges in collagen (aspartic acid and glutamic acid carboxy groups) inhibit collagen interaction with platelets [36,37]. It is possible that positive charges (lysine and arginine residues) will counteract this effect, and this could explain why modification of lysine residues causes a decrease of collagen platelet reactivity. Positive charges may conceivably increase the affinity of the collagen triple-helix for platelets, whilst negative charges may have the opposite effect.

Given our evidence here that collagen tertiary and quaternary structures are sufficient alone for platelet activation, it might be supposed that any collagen, collagen-derived fragment or collagen-like peptide should exhibit aggregatory activity provided that it possesses triple-helical and polymeric form. However, we have shown that not all collagen CNBr-derived fragments are aggregatory [24,30]. This might be due to incorrect molecular alignment, triple-helical instability or inability of our cross-linking procedure to produce an adequate polymeric form. This seems unlikely, though, in the case of the inactive fragment $\alpha 1(I)CB3$ from collagen type I, which is structurally similar to

the highly reactive fragment $\alpha 1(III)CB4$ from collagen type III [24,30]. We believe the inactivity of $\alpha 1(I)CB3$ might be due to an unfavourable balance between positive and negative charges. Whilst in $\alpha 1(III)CB4$ there is an equal number of positively and negatively charged residues, in $\alpha 1(I)CB3$ the number of negatively charged residues (14) is appreciably higher than the number of residues of the opposite charge (10) [41].

Platelet aggregation by Peptides 1 and 2, as by collagen fibres, involved genuine platelet activation, as evidenced by the occurrence of platelet secretion and the release of arachidonic acid from platelet membrane lipids as a result of the activation of phospholipase A_2 . Platelet-platelet interaction (aggregation) was mediated by integrin $\alpha IIb\beta 3$; it was not simply agglutination by the peptides. Both peptides caused platelet secretion independently of platelet aggregation and can be regarded therefore, like collagen fibres, as 'strong' platelet agonists.

Several studies have focussed on the involvement of integrin $\alpha 2\beta 1$ in collagen-platelet interaction [5–14]. There is no doubt that this integrin contributes to the adhesion of platelets to immobilized monomeric collagen, either in static adhesion assays [7,8,13] or under flow conditions [10,12]. Patients have been described with a deficiency of the platelet integrin $\alpha 2$ subunit or with autoantibodies directed against this protein. These patients present with defective platelet adhesion to collagen and impaired collagen-induced platelet aggregation [5,6,9], testifying to the involvement of $\alpha 2\beta 1$ in collagen-platelet interaction *in vivo*. However, we noted previously that platelet adhesion to collagen fibres under static conditions is largely independent of integrin $\alpha 2\beta 1$ [13] and, as shown in the present study, this integrin is not involved at all in platelet adhesion to Peptides 1 and 2. In accord with this evidence of $\alpha 2\beta 1$ -independent adhesion, we found that neither the release of arachidonate in platelets nor the aggregation of gel-filtered platelets caused by Peptides 1 and 2 involved integrin $\alpha 2\beta 1$. Collagen fibres can also seemingly cause platelet aggregation without the involvement of integrin $\alpha 2\beta 1$, as judged by the relative ineffectiveness of 6F1 to block the aggregation of gel-filtered platelets. Others have noted previously that mAb 6F1 does not prevent collagen-induced platelet aggregation in platelet-rich plasma [7,11]. It is reported to delay the onset to aggregation, but this could be an effect of the antibody on fibrillogenesis. However, Coller et al. [7] have described inhibition by relatively low concentrations (about 3 $\mu\text{g}/\text{ml}$) of mAb 6F1, of the aggregation of gel-filtered platelets by collagen fibres, in contrast with our findings here, where partial inhibition only was noted, and only at very much higher mAb concentrations. We do not know the reason for this difference in findings. Our data indicating $\alpha 2\beta 1$ -independent aggregation *in vitro* by Peptides 1 and 2 and collagen fibres are consistent with the occurrence of $\alpha 2\beta 1$ -independent adhesion and with the absence of any obvious specific $\alpha 2\beta 1$ -binding sequences in our peptides. We believe that integrin $\alpha 2\beta 1$ may be more important *in vivo*, where interaction has to occur under flow conditions, possibly serving to increase the affinity between platelets and the fibre surface under flow and thereby allowing subsequent interaction between the collagen helix and a second receptor involved more directly in the activation process. The latter receptor may, perhaps, have sufficient affinity for the collagen triple helix under *in vitro* conditions for activation to be able to occur without any involvement of integrin $\alpha 2\beta 1$. Alternatively, our data may indicate the existence, *in vivo*, of separate mechanisms of collagen-platelet interaction acting in concert: one initiated by cell-signalling via integrin $\alpha 2\beta 1$ [42–44] following recognition of $\alpha 2\beta 1$ -binding sites in collagen and the other involving signalling following the recognition of the simple collagen helix by a receptor other than integrin $\alpha 2\beta 1$ and yet to be identified. There

are numerous reports of platelet proteins other than integrin $\alpha 2\beta 1$ that can bind to collagen or are required for collagen-platelet interaction and may function as collagen receptors, including integrin $\alpha \text{IIb}\beta 3$, CD36 (glycoprotein IV) and glycoprotein VI [10,45–56]. The evidence of Beer et al. [57], indicating that clinical bleeding associated with impaired collagen-platelet interaction only arises when there is more than one defect, supports the proposal advanced here that there may be more than one mechanism of platelet activation by collagen.

On the basis of the relatively simple structure of Peptides 1 and 2, comprising essentially of just a repeat Gly-Pro-Hyp sequence, we have concluded that collagen tertiary and quaternary structures may be sufficient alone for collagen to induce platelet aggregation without the need for the recognition within the primary structure of highly specific platelet-reactive sequences. We cannot, however, exclude the possibility that the sequence Gly-Pro-Hyp may itself serve as such a platelet-recognition sequence.

Note added in proof (received 20 December 1994)

Since completion of this manuscript, Fields and colleagues [58] have reported platelet aggregation by a relatively complex collagen-like synthetic peptide containing the type IV collagen sequence $\alpha 1(\text{IV})1263$ –1277. The concentration needed for activity was 100-fold or more higher than concentrations required of the simple collagen-like peptides described here. The involvement of integrin $\alpha 2\beta 1$ was not examined nor the specific need for the sequence $\alpha 1(\text{IV})1263$ –1277.

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