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Distinct determinants on collagen support $\alpha_2\beta_1$ integrin-mediated platelet adhesion and platelet activation

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Recent studies have revealed that the sequence of amino acids asp-gly-glu-ala represents an essential determinant of the site within the α1(I)-CB3 fragment of collagen recognized by the $\alpha_2\beta_1$ integrin cell surface collagen receptor (Staatz *et al.*, 1991). Studies employing chemical modifications of collagen amino acid side chains confirm both the essential nature of the acidic side chains of aspartic acid and glutamic acid residues and the nonessentiality of lysine ϵ-amino groups in supporting adhesion mediated by the $\alpha_2\beta_1$ integrin. The approach also indicates the presence of a distinct determinant on collagen separate from the $\alpha_2\beta_1$ recognition site that contains essential lysine side chains and that is necessary for subsequent interactions with the platelet surface that give rise to collagen-induced platelet activation and secretion. The two-step, two-site model for cellular signaling involving both an integrin and a signal-transducing coreceptor suggested by these data may be common to other integrin-mediated processes.

Introduction

Fibrillar collagen is the most thrombogenic macromolecular constituent of the vascular subendothelium (Baumgartner, 1977). It serves not only as an effective substrate for platelet adhesion but is a potent initiator of platelet activation. The $\alpha_2\beta_1$ integrin (very late activation antigen 2, extracellular matrix receptor II, glycoprotein Ia–IIa) serves as a cell surface collagen receptor on platelets and fibroblastic cells (Wayner and Carter, 1987; Kunicki *et al.*, 1988; Santoro *et al.*, 1988; Takada *et al.*, 1988; Coller *et al.*, 1989; Elices and Hemler, 1989; Languino *et al.*, 1989; Staatz *et al.*, 1989; Kirchofer *et al.*, 1990) and as both a collagen and a laminin receptor on endothelial cells and some epithelial cells (Elices and Hemler, 1989; Languino *et al.*, 1989; Kirchofer *et al.*, 1990). Virtually all of the Mg^{2+}-dependent adhesion of platelets to collagen is mediated by the $\alpha_2\beta_1$ integrin (Kunicki *et al.*, 1988; Coller *et al.*, 1989; Staatz *et al.*, 1989). In an earlier study, we established that platelets that adhere to fibrillar collagen substrates via the $\alpha_2\beta_1$-mediated, Mg^{2+}-dependent mechanism subsequently undergo activation and secretion of granule contents (Santoro, 1986). Under some, but not all, conditions, monoclonal antibodies directed against the $\alpha_2\beta_1$ integrin specifically inhibit collagen-induced platelet aggregation (Coller *et al.*, 1989). Platelets deficient in the $\alpha_2\beta_1$ integrin or from patients who have acquired antibodies directed against the $\alpha_2\beta_1$ integrin exhibit not only impaired adhesion to collagen but also markedly impaired collagen-induced platelet aggregation (Nieuwenhuis *et al.*, 1985; Deckmyn *et al.*, 1990). Collectively, these observations suggest that adhesion to collagen mediated by the $\alpha_2\beta_1$ integrin is an important step along the pathway of collagen-induced platelet activation.

We recently established that a binding site for the $\alpha_2\beta_1$ integrin is located within the α1(I)-CB3 fragment of collagen (Staatz *et al.*, 1990) and that the amino acid sequence asp-gly-glu-ala (DGEA) is an important structural determinant of the $\alpha_2\beta_1$ recognition site (Staatz *et al.*, 1991). We now describe the use of chemical modification of collagen amino acid side chains to confirm that the acidic side chains of aspartic acid and glutamic acid residues, but not the ϵ-amino groups of lysine side chains, are critical determinants for Mg^{2+}-dependent adhesion of platelets to collagen. This approach has also enabled us to delineate a distinct determinant, separate from the $\alpha_2\beta_1$ recognition site on collagen, that contains essential lysine side chains and that is necessary for subsequent interactions with the platelet surface that give rise to collagen-induced platelet activation.

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Results

To examine the role of lysine residues of collagen in mediating Mg^{2+}-dependent platelet adhesion, the amino groups of collagen substrates were modified by acetylation, succinylation, reductive methylation, or guanidination. These reactions are illustrated schematically in Figure 1A. The modifications preserve centers of positive charge (reductive methylation, guanidination), convert positively charged side chains to neutral side chains (acetylation), or convert positively charged side chains to negatively charged side chains (succinylation). After modification by acetylation, succinylation, or reductive methylation, collagen substrates supported platelet adhesion determined in either Mg^{2+}- or EDTA-containing media to extents that did not substantially differ from the levels observed on untreated or reagent-control sub-

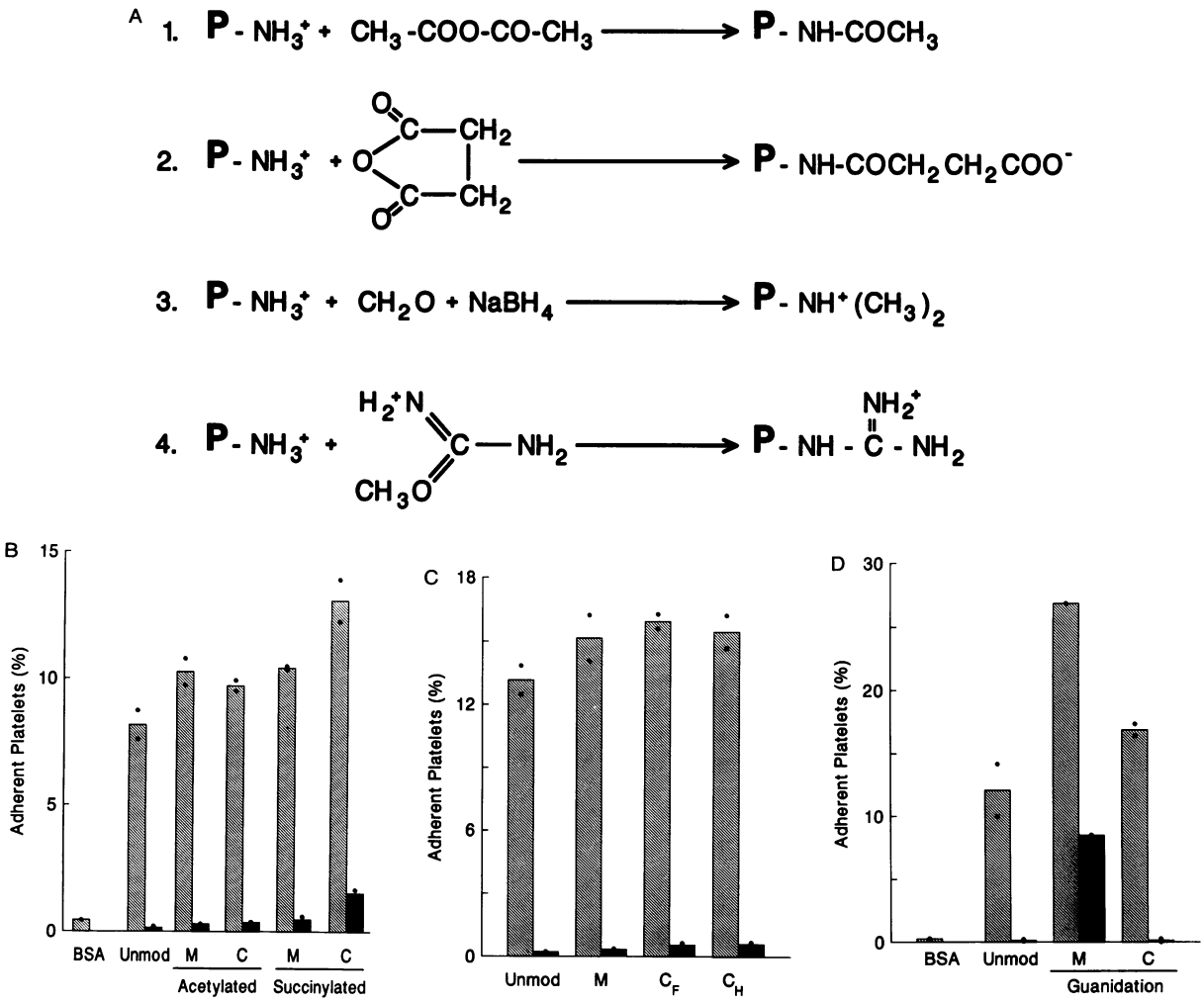

Figure 1. **Adhesion of platelets to lysine-modified collagen substrates.** (A) Summary of modifications showing the protein with an ϵ-amino group ($P\text{-}NH_3^+$), the modifying reagent, and the resultant modified amino group: 1. acetylation with acetic anhydride, 2. succinylation with succinic anhydride, 3. reductive methylation with formaldehyde and sodium borohydride, 4. guanidination with o-methylisourea to yield homoarginine residues. (B) Platelet adhesion to collagen substrates with lysine side chains modified (M) by either acetylation or succinylation. Controls included unmodified collagen substrates (unmod) or reagent controls (C) from which the modifying agent was omitted. (C) Platelet adhesion to collagen substrates modified by reductive methylation (M). Controls included unmodified collagen substrates (unmod) and reagent controls in which either formaldehyde (C_F) or sodium borohydride (C_H) was omitted. (D) Platelet adhesion to collagen substrates in which lysine residues were converted to homoarginine residues by guanidination with o-methylisourea (M). Control substrates consisted of unmodified collagen (unmod) and a reagent control (C) from which o-methylisourea was omitted from the reaction mixture. Adhesion to BSA was also routinely measured. Hatched bars represent adhesion in the presence of 2 mM Mg^{2+}. Solid bars represent adhesion in the presence of 2 mM EDTA. Data are presented as the mean of duplicate determinations.

A

1. $P\text{-}COO^- + H_2N\text{-}CH_2\text{-}CH_2\text{-}NH_3^+ \xrightarrow{CMC} P\text{-}CONH_2\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$

2. $P\text{-}COO^- + H_2N\text{-}CH_2\text{-}COOCH_3 \xrightarrow{CMC} P\text{-}CONH_2\text{-}CH_2\text{-}COOCH_3$

3. $P\text{-}COO^- + H_2N\text{-}CH_2\text{-}CH_2\text{-}SO_3^- \xrightarrow{CMC} P\text{-}CONH_2\text{-}CH_2\text{-}CH_2\text{-}SO_3^-$

4. $P\text{-}COO^- + CH_3OH \xrightarrow{HCl} P\text{-}COOCH_3$

B

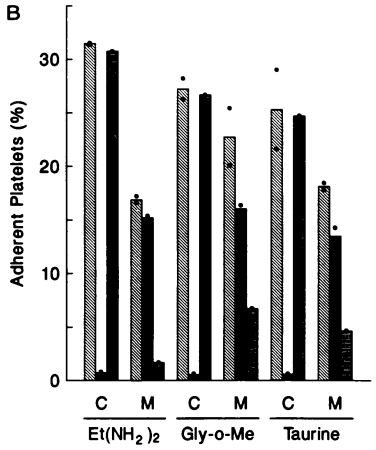

Figure 2. **Adhesion of platelets to collagen substrates with chemically modified aspartic acid and glutamic acid residues.** (A) Summary of the reactions showing the starting protein ($P\text{-}COO^-$), the modifying reagent, and the resultant modifications: 1. addition of ethylene diamine, 2. addition of glycine methyl ester, 3. addition of taurine, 4. esterification in anhydrous acidic methanol. Reactions 1–3 were mediated by carbodiimide (CMC). (B) Platelet adhesion to collagen substrates modified with ethylene diamine ($Et(NH_2)_2$), glycine methyl ester (Gly-o-Me), or taurine. Controls included substrates incubated in the presence of $Et(NH_2)_2$, Gly-o-Me, or taurine, respectively, but in the absence of CMC. Results shown are the mean of duplicate determinations. Cross-hatched bars are total adhesion measured in the presence of 2 mM Mg^{2+}; solid bars are divalent cation-independent adhesion in the presence of 2 mM EDTA; dotted bars are Mg^{2+}-dependent adhesion determined as the difference between total adhesion and adhesion measured in the presence of EDTA.

strates (Figure 1,B and C). After guanidination of the collagen substrates with o-methylisourea to convert lysine residues to the more basic homoarginine residues, adhesion measured in EDTA increased from essentially undetectable levels to 8.5% (Figure 1D). Total adhesion measured in Mg^{2+}-containing buffer increased comparably from 17 to 26%. The Mg^{2+}-dependent component of adhesion onto guanidinated collagen substrates was not altered (Figure 1D). Adhesion to both native collagen substrates and substrates subjected to amino group modification by acetylation was inhibited by the P1H5 monoclonal antibody directed against the $\alpha_2\beta_1$ integrin.

Virtually identical results were obtained when collagen was first modified in solution and then used to prepare substrates for use in adhesion assays (data not shown). Under the conditions employed for the studies described above, 90% of collagen amino groups was modified by acetylation, 75% by succinylation, 90% by reductive methylation, and 60% by guanidination.

The carboxyl groups of aspartic acid and glutamic acid side chains were modified by carbodiimide-mediated conjugation with ethylene diamine, glycine methyl ester, or taurine. As illustrated in Figure 2A, these reactions resulted in the addition of positively charged, uncharged, or negatively charged groups, respectively, to the initially negatively charged carboxyl side chains.

As shown in Figure 2B, modification of the carboxyl groups of collagen substrates with ethylene diamine reduced total adhesion, measured in the presence of 2 mM Mg^{2+}, to ~50% of control levels. The divalent cation-independent component of adhesion, measured in EDTA-containing buffer, increased ninefold over control levels after modification with ethylene diamine. In contrast, the Mg^{2+}-dependent component of adhesion was diminished by 95% (from 30 to 1.6%) after reaction of collagen carboxyl groups with ethylene diamine.

The results of the glycine methyl ester and taurine modifications are also shown in Figure 2B. In general, the effects were comparable with those described above for ethylene diamine: slightly decreased total adhesion, increased divalent cation-independent adhesion, and marked inhibition of the Mg^{2+}-dependent component of adhesion. The effects of modification

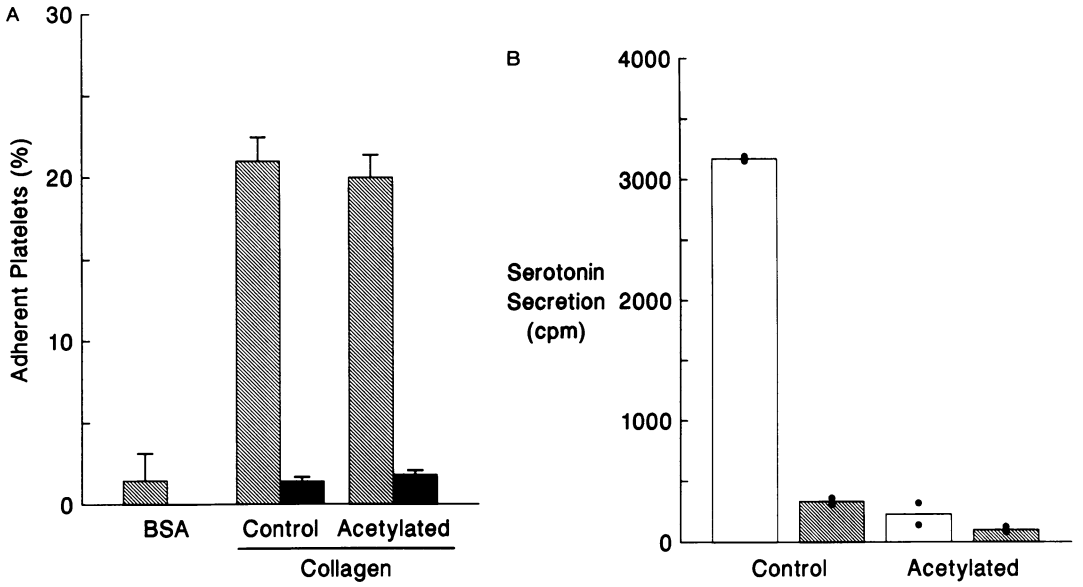

Figure 3. **Effect of acetylation of fibrillar collagen substrates on platelet adhesion and activation.** (A) Adhesion was measured to substrates of BSA, fibrillar collagen, or acetylated fibrillar collagen in the presence of 2 mM Mg^{2+} (▨) or 2 mM EDTA (■). Data shown are the means ± SD of quadruplicate determinations. (B) Lack of [^{14}C]serotonin secretion by platelets adherent to acetylated fibrillar collagen substrates. Serotonin secretion from platelets adherent to control or acetylated fibrillar collagen substrates was determined in medium containing 2 mM $MgCl_2$ (□) or 2 mM EDTA (▨). Data are the mean of duplicate determinations.

with glycine methyl ester or taurine on Mg^{2+}-dependent adhesion were somewhat less marked (80% and 85% inhibition, respectively) than the effect of modification with ethylene diamine. As the $\alpha_2\beta_1$ integrin is dependent on divalent cations for ligand-binding activity (Staatz *et al.,* 1989), it is unlikely that the platelet adhesion onto collagen substrates containing modified carboxyl groups, which is mostly independent of divalent cations, is mediated by the $\alpha_2\beta_1$ integrin (Figure 2B). The inability of the P1H5 monoclonal antibody directed against the $\alpha_2\beta_1$ integrin to inhibit effectively adhesion to substrates in which collagen carboxyl groups were modified with taurine (Walsh and Santoro, unpublished observations) is consistent with this interpretation.

In a manner consistent with the above results, modification of collagen carboxyl groups by esterification with methanol also markedly inhibited Mg^{2+}-dependent platelet adhesion to collagen (data not shown). Comparable results were obtained when collagen was modified in solution and the modified collagen used to prepare substrates, rather than modifying preformed substrates as in the experiments described above. The results of these chemical-modification experiments demonstrating the essential contribution of collagen carboxyl groups to Mg^{2+}-dependent platelet adhesion are entirely consistent with our recent determination, using synthetic peptides, that the DGEA sequence plays a critical role in the recognition of collagen by the $\alpha_2\beta_1$ integrin (Staatz *et al.,* 1991).

We have previously demonstrated that platelets that adhere to fibrillar collagen substrates via the $\alpha_2\beta_1$ integrin-mediated, Mg^{2+}-dependent adhesive mechanism become activated and secrete the contents of their dense storage granules (Santoro, 1986), a process readily monitored by quantitating the secretion of [^{14}C]-serotonin. In contrast to the lack of effect of amino group modification on $\alpha_2\beta_1$ integrin-mediated adhesion we have observed in this study (Figure 1), earlier studies revealed that collagen-induced platelet aggregation was markedly inhibited by modifications that diminished the positive charge of collagen amino group side chains (Wilner *et al.,* 1968, 1971; Balleisen *et al.,* 1976; Chesney, 1983). This suggested to us that distinct determinants on collagen might be involved in the two processes.

This issue was addressed by examining the effect of acetylation of the amino groups of fibrillar collagen substrates on their ability to support both adhesion and activation as judged by the secretion of [^{14}C]serotonin. As shown in Figure 3A, Mg^{2+}-dependent adhesion of platelets to the fibrillar collagen substrate was not

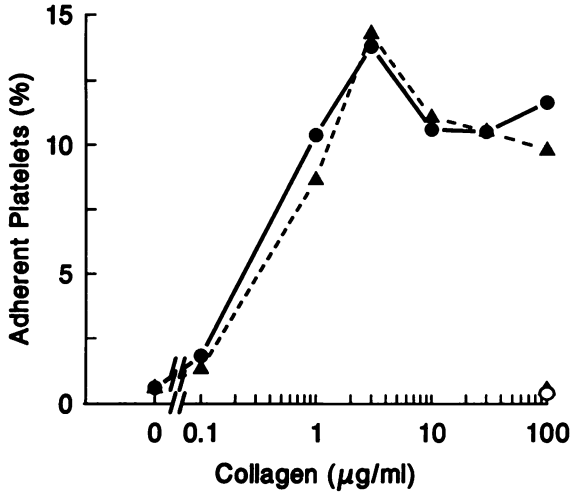

Figure 4. **Concentration-dependence of platelet adhesion to acetylated and unmodified collagen substrates.** Substrates were prepared with the indicated concentrations of monomeric collagen and were then either treated with acetic anhydride or with reagent control. Platelet adhesion to the unmodified (●, ○) and acetylated substrates (▲, △) was then determined in the presence of either 2 mM $MgCl_2$ (●, ▲) or 2 mM EDTA (○, △). Values shown are the mean of duplicate determinations.

altered by acetylation of the substrate. This observation is in agreement with the data shown in Figure 1 for substrates composed of monomeric collagen. As expected from our earlier study and shown in Figure 3B, platelets adherent to the fibrillar collagen substrate in the presence of Mg^{2+} were activated and secreted [^{14}C]serotonin from the dense granule pool into the assay medium. Under the assay conditions employed, serotonin in the medium is derived entirely from the adherent population with no contribution from nonadherent platelets (Santoro, 1986). The secreted serotonin corresponds to ~30% of the initial [^{14}C]serotonin content of the adherent population. As also shown in Figure 3B, in the presence of 10 μM prostaglandin E_1 (PGE_1), an inhibitor of platelet activation that has no effect on Mg^{2+}-dependent adhesion to either fibrillar or monomeric collagen substrates (Santoro, 1986), secretion was inhibited by 90%. Despite unimpaired adhesion to substrates of acetylated fibrillar collagen (Figure 3A), there was virtually no secretion of serotonin from platelets adherent to the acetylated substrate (Figure 3B). The low level of serotonin detected in the medium was not different from that observed in the presence of PGE_1 (Figure 3B) or the combination of PGE_1 and EDTA (data not shown).

It is unlikely that the disparity between the ability of acetylated collagen to support adhesion and the ability to induce secretion is due to a decreased affinity of the $\alpha_2\beta_1$ integrin for acetylated collagen such that adhesion still occurs, but activation and secretion do not. As shown in Figure 4, substrates composed of unmodified or acetylated collagens supported comparable extents of adhesion even at suboptimal concentrations where any differences due to impaired affinity should have been most apparent. The rates of platelet adhesion to the two substrates were also comparable (Figure 5).

Discussion

The experiments described in this report establish two points relevant to the process of collagen-induced platelet activation. First, chemical modification of carboxyl and amino group side chains revealed that carboxyl side chains of collagen, but not amino group side chains, are essential for the Mg^{2+}-dependent platelet adhesion to collagen mediated by the $\alpha_2\beta_1$ integrin. The essential nature of the acidic side chains is in complete agreement with the results of our recent studies using synthetic peptides that delineated the sequence DGEA as a critical recognition site on collagen for the $\alpha_2\beta_1$ integrin (Staatz *et al.*, 1991). Side chain length, as well as negative charge, appears to be important in the recognition because modification with taurine, which preserves negative charge but extends side chain length, also resulted in loss of the ability to support Mg^{2+}-dependent adhesion.

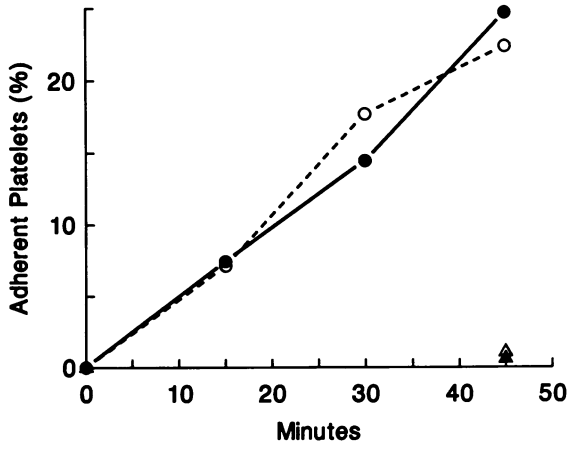

Figure 5. **Rate of adhesion to acetylated and unmodified collagen substrates.** Substrates were prepared by coating plates with monomeric collagen (10 μg/ml) and then treated with either acetic anhydride (○, △) or reagent control (●, ▲). Adhesion at the indicated times was measured in the presence of 2 mM $MgCl_2$ (●, ○) or 2 mM EDTA (▲, △). Values shown are the mean of duplicate determinations.

Studies of other integrin recognition sites have yielded similar conclusions. The very conservative substitution of glutamic acid for aspartic acid has been shown to markedly impair the ability of arg-gly-asp peptides to inhibit ligand binding to the $\alpha_5\beta_1$ integrin fibronectin receptor (Pierschbacher and Ruoslahti, 1984) and the platelet IIb–IIIa complex (Ginsberg *et al.,* 1985; Haverstick *et al.,* 1985; Plow *et al.,* 1985).

The second major finding of this study is that chemically distinct determinants on collagen support $\alpha_2\beta_1$-mediated platelet adhesion and subsequent platelet activation. The DGEA sequence plays a critical role in the former, whereas the latter determinant includes the positive charge of one or more amino group side chains. The receptor that recognizes the amino group-containing determinant remains to be identified. A corollary to this finding is that interaction of the DGEA sequence of collagen with the $\alpha_2\beta_1$ integrin cell surface collagen receptor alone is insufficient to induce platelet activation.

These observations and other previously published observations discussed below have been incorporated into a model of the platelet-collagen interaction shown in Figure 6. Platelet-collagen adhesive reactions have been classified as direct (primary) or indirect (secondary) interactions depending on whether adhesion to collagen is mediated by a plasma membrane protein that binds directly to collagen or by an intermediary bridging molecule such as von Willebrand factor or fibronectin (Santoro, 1988). The initial direct or primary interaction of platelets with collagen occurs via the divalent cation-dependent recognition of the DGEA sequence of collagen by the $\alpha_2\beta_1$ integrin on the platelet surface. This high-affinity adhesive interaction brings and holds in close proximity a lower-affinity receptor that binds the determinant containing the critical amino group(s). It is the occupancy of this second receptor site, likely mediated through multiple, simultaneous, linked interactions as proposed earlier (Santoro and Cunningham, 1977), that leads to activation, secretion, and subsequent aggregation. Once activation has occurred, other indirect interactions such as those mediated by fibronectin and von Willebrand that can bind to both the activated IIb–IIIa complex and collagen would further strengthen adhesion to collagen (Santoro, 1986).

Several issues regarding each of the steps in the model merit discussion. Obviously, other plasma proteins, such as von Willebrand factor and fibronectin that mediate indirect adhesive reactions of platelets with collagen, contribute to the early adhesive interactions (Baumgartner *et al.,* 1980; Houdijk *et al.,* 1985). For example, under the conditions present in citrate-anticoagulated plasma, undefined plasma proteins and platelet receptors can substitute, at least partially, for the $\alpha_2\beta_1$ integrin in facilitating initial adhesive interactions (Coller *et al.,* 1989). Under other conditions, interaction with collagen through the $\alpha_2\beta_1$ integrin alone is adequate to support initial adhesion leading to activation and aggregation (Santoro, 1986; Coller *et al.,* 1989). Study of a patient deficient in surface expression of the $\alpha_2\beta_1$ integrin indicated that under more physiological flow conditions, neither von Willebrand factor nor other plasma adhesive proteins could substitute for the direct interaction of the $\alpha_2\beta_1$ integrin with collagen to promote platelet deposition onto vascular subendothelium (Nieuwenhuis *et al.,* 1986). Platelets from a patient with $\alpha_2\beta_1$ integrin deficiency exhibited not only impaired adhesion but also impaired collagen-induced aggregation (Nieuwenhuis *et al.,* 1985), as predicted by the model shown in Figure 6.

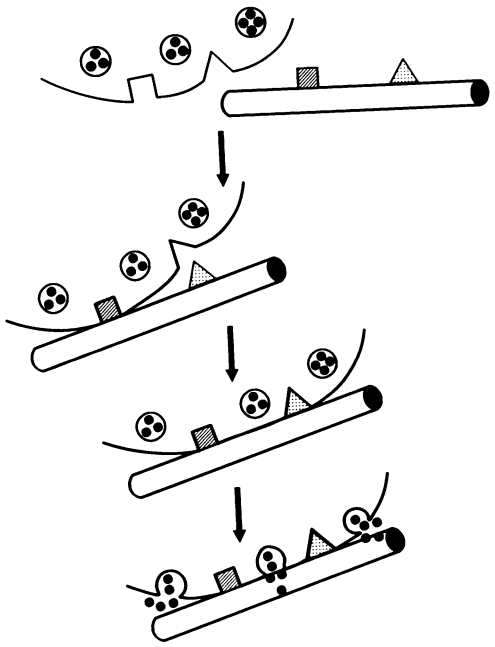

Figure 6. **Model for adhesion of platelets to collagen and subsequent platelet activation.** Platelets initially adhere to collagen fibers in a divalent cation-dependent manner through a mechanism mediated by binding of the $\alpha_2\beta_1$ integrin to the DGEA sequence (▨) of collagen. This high-affinity interaction facilitates the binding of a distinct determinant on collagen containing a critical amino group(s) (△) to a second lower-affinity receptor in a divalent cation-independent manner. Occupancy of the second receptor site results in platelet activation and secretion of granule contents.

As established earlier, the adhesive mechanism mediated by the $\alpha_2\beta_1$ integrin supports much greater rates and extents of adhesion than does the operationally distinct divalent cation-independent mechanism (Santoro, 1986). In contrast to the divalent cation-dependent adhesive mechanism mediated by the $\alpha_2\beta_1$ integrin, the lower-affinity divalent cation-independent mechanism is sensitive to amino group modifications such as acetylation and succinylation that diminish centers of positive charge (Wilner *et al.,* 1968; Balleisen *et al.,* 1975; Santoro, unpublished observations). The essential role for collagen amino groups in the second step of the model shown in Figure 6 is consistent with the several earlier reports documenting the essential nature of collagen amino groups for induction of secretion and aggregation (Wilner *et al.,* 1968, 1971; Balleisen *et al.,* 1976; Chesney *et al.,* 1983).

Several platelet membrane components (reviewed in Santoro, 1988), including a 65-kDa polypeptide (Chiang and Kang, 1982), a 61-kDa polypeptide (Kotite and Cunningham, 1986; Moroi *et al.,* 1989), and glycoprotein IV (Tandon *et al.,* 1989), have been proposed as mediators of divalent cation-independent platelet interactions with collagen. Interestingly, antibodies against the 65-kDa polypeptide (Chiang *et al.,* 1987) as well as a 62-kDa polypeptide (Sugiyama *et al.,* 1987) have been shown to inhibit collagen-induced platelet aggregation. Antibodies against glycoprotein IV have been reported to inhibit collagen-induced as well as ADP- and epinephrine-induced aggregation (Tandon *et al.,* 1989). Although these are expected properties of the lower-affinity divalent cation-independent signal-transducing receptor, the identity of the receptor that actually transmits the signal giving rise to collagen-induced platelet activation remains to be established.

Although the model depicted in Figure 6 shows the critical amino group interacting with a receptor-binding site distinct from the $\alpha_2\beta_1$ integrin, at present the available data do not exclude the possibility that the critical collagen amino group(s) is part of an extended $\alpha_2\beta_1$ binding site composed of both the amino group and the DGEA sequence. Independent evidence that suggests that adhesion- and aggregation-inducing sites are located on spatially distinct determinants has been presented (Morton *et al.,* 1989).

There is at present considerable interest in the mechanisms by which integrin adhesive receptors transmit signals from the extracellular matrix through the cell membrane to the cell interior despite their generally extremely short cytoplasmic domains (Ruoslahti, 1991). The findings of this investigation concerning the mechanisms of platelet activation and the role of the $\alpha_2\beta_1$ integrin cell surface collagen receptor in the process may well be relevant to the more general issue of integrin signaling. The model depicted in Figure 6 and the data on which it is based suggest that inhibition of a cell-signaling process by an inhibitory anti-integrin antibody cannot alone be considered adequate evidence that the signaling event is mediated directly through the integrin receptor. Other molecules, as delineated in this report, may also be involved.

Materials and methods

Materials

Bovine acid-soluble type I calf skin collagen (C3511), bovine serum albumin (BSA), glycine methyl ester, ethylenediamine, and taurine were obtained from Sigma Chemical (St. Louis, MO). 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-p-toluene sulfonate (CMC) was obtained from Pierce Chemical (Rockford, IL). Acetic anhydride, succinic anhydride, sodium borohydride, and formaldehyde were purchased from Fisher Scientific (St. Louis, MO). O-methyl isourea was from Aldrich Chemical (Milwaukee, WI). $Na^{51}CrO_4$ was obtained from ICN, Biomedicals, Inc. (Costa Mesa, CA). [^{14}C]serotonin was purchased from New England Nuclear Research Products (Boston, MA). The P1H5 monoclonal antibody was provided by Dr. William G. Carter, Fred Hutchinson Cancer Research Center, Seattle, WA.

Platelet preparations

Platelets were isolated from freshly drawn, citrate-anticoagulated whole blood by centrifugation and washing as previously described (Haverstick *et al.,* 1985). Platelets were radiolabeled with either [^{14}C]serotonin or $Na^{51}CrO_4$ as previously described in detail (Santoro, 1983, 1986; Haverstick *et al.,* 1985). For use in adhesion or secretion assays, washed platelets were resuspended in 0.15 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris) HCl (pH 7.4), 5 mM glucose, 0.5% BSA at $1–2 \times 10^8$ platelets/ml. $MgCl_2$ or EDTA was added as indicated for individual experiments.

Adhesion and secretion assays

Adhesive substrates were prepared as previously described in detail (Santoro, 1986). Polystyrene dishes were coated with BSA (5 mg/ml) and monomeric type I collagen (20 μg/ml) dissolved in 0.5% acetic acid or reconstituted collagen fibers (100 μg/ml) prepared by dialysis of monomeric collagen against 0.02 M Na_2HPO_4 as described (Bruns and Gross, 1974). The adhesion of ^{51}Cr-labeled platelets to collagen-coated or BSA-coated substrates in 30-mm polystyrene dishes was determined in media containing either 2 mM $MgCl_2$ or 2 mM EDTA exactly as described earlier (Santoro, 1986). The secretion of [^{14}C]serotonin into the medium by adherent platelets also was determined as previously described in detail (Santoro, 1986).

Chemical modifications

Monomeric and fibrillar collagen substrates were subjected to chemical modification as described below after coating onto plastic plates. The ϵ-amino groups of lysine were acetylated with acetic anhydride in sodium acetate buffer under the conditions described by Fraenkel-Conrat (1957). Amino groups were modified with succinic anhydride in carbonate buffer as described by Chu *et al.* (1969). Reductive methylation with formaldehyde and sodium borohydride was carried out as described (Means and Feeney, 1968). Guanidination was carried out with O-methylisourea as described by Klee and Richards (1957). Controls included reagent blanks that were identical to the reaction conditions above except for omission of the reactive species, as well as untreated collagen substrates. At the conclusion of the reactions, substrates were washed extensively with water to remove soluble reactants and reaction products and then with 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4) containing 0.5% BSA. The extent of amino group modification was determined by measurement of residual unmodified amino groups with trinitrobenzene sulfonic acid under quantitative conditions (Habeeb, 1966).

Carboxyl groups of aspartate and glutamate residues were modified by conjugation with ethylene diamine, glycine methyl ester, and taurine using the carbodiimide coupling method (Hoare and Koshland, 1967). Reactions were carried out for 22 h at 4°C in the presence of 7.5 M urea and 0.4 M CMC as described (Hoare and Koshland, 1967). Plates were then washed extensively and blocked with BSA as described above before use as substrates for platelet adhesion. Controls included reactions with ethylene diamine, glycine methyl ester, or taurine in the absence of CMC, as well as untreated substrates.

Carboxyl groups of collagen substrates were also esterified in anhydrous methanol adjusted to pH 4.5 with HCl (Fraenkel-Conrat and Olcott, 1945). Reactions were carried out for 72 h at 23°C, then stopped by the addition of water and thorough rinsing. Control reactions included samples from which either the methanol or the HCl was omitted and the use of 50% aqueous methanol instead of anhydrous methanol.

For some experiments, modifications of collagen were carried out in solution. In these cases the washing steps described above were replaced by extensive dialysis against 3% acetic acid, followed by lyophilization. Control and modified collagens were then dissolved in 0.05% acetic acid and used to prepare adhesive substrates.

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