Adhesion of Platelets to Laminin in the Absence of Activation

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ABSTRACT The binding of platelets to components in the subendothelial matrix is an initial event in hemostasis and thrombosis. The glycoprotein components of the matrix are considered important in this interaction. Of these, collagen binds and activates platelets and induces their aggregation. In this study we demonstrate that substrate-bound laminin causes time- and concentration-dependent adherence of human platelets to the substrate. The binding of platelets to laminin was found to be similar in some respects, but different in others, to their binding to surfaces coated with fibronectin or collagen. The binding of platelets to laminin or fibronectin was not associated with their activation under conditions in which type I collagen activates the platelets as measured by [^{14}C]serotonin secretion. Platelets bound to laminin and fibronectin differed in their appearance; they remained rounded on laminin whereas they flattened completely on fibronectin. Binding of platelets to fibronectin, but not laminin, is inhibited by a recently described peptide (Pierschbacher, M., and E. Ruoslahti, 1984, *Nature (Lond.)*, 309:30–33) containing the cell-attachment tetrapeptide sequence of fibronectin, which suggests that separate receptors exist for laminin and fibronectin. These studies establish laminin as a platelet-binding protein and suggest that laminin can contribute to the adhesiveness of exposed tissue matrices to platelets. Since laminin and fibronectin do not activate platelets, whereas collagen does, and laminin differs from fibronectin in that it does not induce spreading of the attached platelets, all three proteins appear to confer different signals to the platelets. Some of these may be related to platelet functions other than those necessary for the formation of a hemostatic plug.

The vascular endothelium in vivo is a nonthrombogenic entity. This property stems from inability of the endothelial cell apical surface to bind platelets. In vitro cultures of vascular endothelium are also nonthrombogenic if they are maintained under conditions that support their normal contact-inhibited, nonoverlapping monolayer configuration (1–3). If, however, the cell layer is removed, the platelets can adhere to the underlying extracellular matrix which resembles a basement membrane (4). Exposure of the subendothelial matrix in vivo also leads to platelet attachment (5) and this phenomenon has been suggested to play a role in the etiology of atherosclerosis (6).

Extracellular matrices contain proteins known to be adhesive to platelets. For example, the binding of platelets to various types of collagen (7–9) and to fibronectin (10) has been reported. Endothelial and other basement membranes also contain laminin, which like fibronectin is a large glycoprotein adhesive to various types of nucleated cells (11–13). Whether insolubilized laminin also interacts with platelets has not been studied previously. The potential importance of such an interaction as a component of the platelet-extracellular matrix interactions has led us to study the adherence of platelets to surfaces coated with purified laminin.

We show here that platelets bind to laminin-coated surfaces. Comparison of this binding to the binding of platelets to fibronectin- and type I collagen-coated surfaces indicates that beyond binding, each of these matrix proteins has different effects on platelets with regard to spreading, aggregation, and activation of the platelets.

MATERIALS AND METHODS

Isolation and Labeling of Platelets: Human blood (60 ml) was collected on the day of the experiment in polypropylene tubes containing 5 ml of anticoagulant solution (2.5% sodium citrate, 1.4% citric acid, and 2% dextrose, pH 4.6). Donors were females who had not ingested aspirin for at least 10 d prior to collection. Platelets were isolated by differential centrifugation and gel filtration on Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) (14). The isolated platelets were labeled with either [^{14}C]serotonin (2 μCi/ml of

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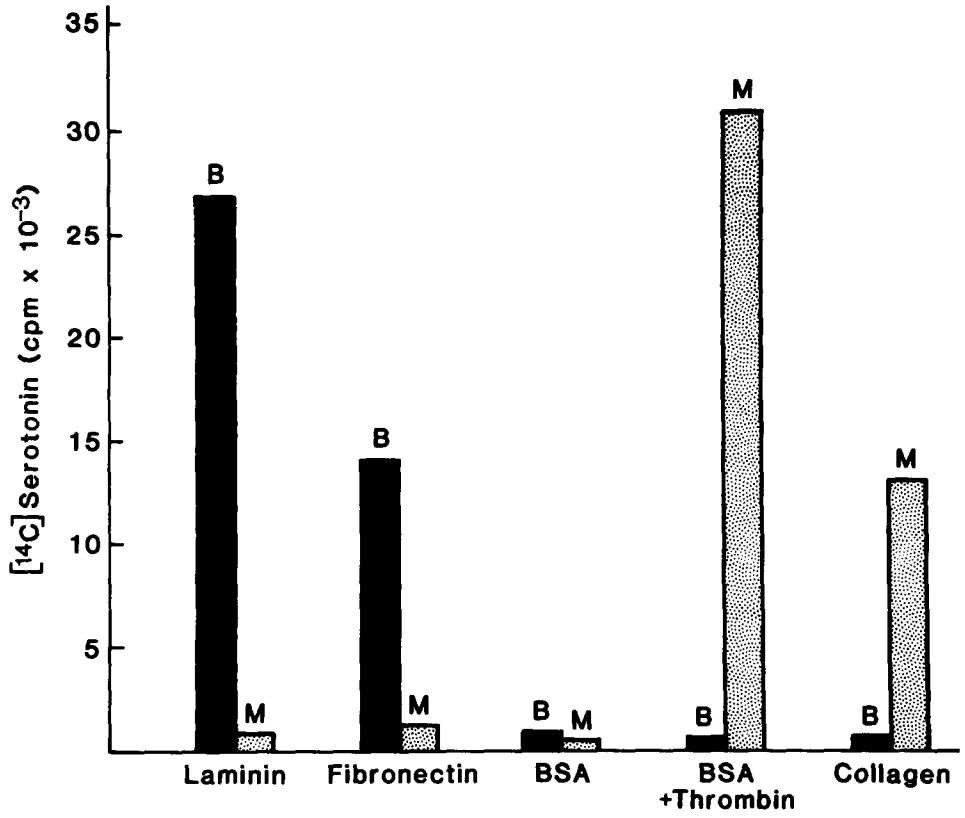

FIGURE 1 Binding and activation of human platelets on surfaces coated with extracellular matrix components. Petri dishes were coated with rat laminin (30 μg/ml), fibronectin (10 μg/ml), or type I collagen (50 μg/ml) as described in Materials and Methods. Dishes coated with BSA (5 mg/ml) were used as controls. Human platelets labeled with [^{14}C]serotonin (1×10^8 platelets, 1.5×10^5 cpm) in 1 ml of DME supplemented with 0.5% BSA were added to these dishes and incubated for 90 min at 37°C. Thrombin was added to some dishes at a final concentration of 1 U/ml. The media with nonbound platelets were collected and centrifuged. The dishes with bound platelets were washed extensively with PBS that contained 0.5% BSA. [^{14}C]-Serotonin radioactivity was then measured and values were expressed as [^{14}C]serotonin in the bound (*B*) or cell-free media (*M*) fractions. The radioactivity not present in these two fractions was recovered in the nonbound platelet fraction which is not shown.

50.7 mCi/mmol) or 5 μCi/ml of $Na_2[^{51}Cr]O_4$ in sterile saline (New England Nuclear, Boston, MA), by incubation in a 37°C water bath for 1 h before gel filtration. The average yield of platelets from 60 ml of blood was approximately 5×10^9, and [^{14}C]serotonin or $Na_2[^{51}Cr]O_4$ labeling averaged 10^3 cpm/10^6 platelets.

Proteins and Coating Procedures: Laminin was prepared as described from rat (15) or mouse (16) tumors. Large fragments of human laminin were isolated from pepsin extracts of placenta using affinity chromatography on monoclonal antibody (17). Fibronectin was prepared from human plasma (18). Type I collagen was from the Collagen Corp. (Palo Alto, CA), and BSA and thrombin were from Sigma Chemical Co. (St. Louis, MO). Synthetic peptides were from Peninsula Laboratories, Inc. (Belmont, CA). Protein concentrations were determined by Coomassie Blue binding assay (19) using BSA as standard, except for collagen which was determined by the method of Lowry et al. (20) using type I collagen as a standard. Coating of 35-mm petri dishes (Falcon, LabWare, Div. of Becton Dickinson & Co., Oxnard, CA) with the various proteins was carried out overnight at 4°C or for 1–2 h at 37°C in 1 ml of PBS at the concentrations indicated in the figure legends. The coating was followed by washing and further incubation of the dishes with PBS containing 0.5% BSA to prevent any nonspecific platelet adherence to the plastic.

Binding/Secretion Assays: In a typical binding assay, $0.5–2 \times 10^8$ platelets labeled with [^{14}C]serotonin or $Na_2[^{51}Cr]O_4$ in 1 ml of Dulbecco's modified Eagle's medium (DME)[1] containing 0.5% BSA were plated onto the various protein-coated petri dishes. Incubations at 37°C in a humidified incubator were varied between 30 min and 4 h. After incubation, the media with nonbound platelets were collected. For $Na_2[^{51}Cr]O_4$-labeled platelets, radioactivity in the platelets bound to the dishes was determined after extensive washing. For [^{14}C]serotonin-labeled platelets, the radioactivity of three fractions was determined; i.e., radioactivity associated with the bound platelets after washing, that found in the supernatant after removal of the nonbound platelets by centrifugation, and that associated with the nonbound platelets.

Antibodies/Inhibition Assay: Goat anti-rat laminin antibodies were affinity purified on laminin-Sepharose as described (17). Affinity-purified goat antibodies to human alpha-fetoprotein (21) were used as a control. The affinity-purified antibodies were incubated on coated dishes for 2 h at 37°C before the addition of platelets.

Scanning Electron Microscopy: 12-mm circular glass coverslips were coated with laminin or fibronectin as described above. Platelets (2×10^8) were incubated on these coverslips in Dulbecco's modified Eagle's medium containing 0.5% BSA. Unbound platelets were washed away and bound platelets were fixed in 2.5% glutaraldehyde, postfixed in 2% OsO_4, and dried by Freon to liquid CO_2 substitution in a Polaron 3000 critical-point dryer (Polaron Instruments Inc., Hatfield, PA). Coverslips were coated using a gold-palladium (80:20) source in an E5100 Polaron sputter coater and viewed with an Hitachi H-600 electron microscope.

[1] *Abbreviation used in this paper:* DME, Dulbecco's modified Eagle's medium.

RESULTS

Binding and Activation of Platelets on Surfaces Coated with Extracellular Matrix Components

Binding of platelets to dishes coated with laminin was observed visually and by quantitation of [^{14}C]serotonin in the bound platelets (Fig. 1). No significant release of [^{14}C]serotonin into the media, which would have been indicative of activation, occurred on laminin. Binding of platelets also occurred on dishes coated with fibronectin as has been observed earlier (10, 22). However, twice as many platelets bound to laminin-coated than to fibronectin-coated dishes, reflecting the fact that the platelets on laminin remained rounded, whereas those on fibronectin were completely flattened (see Fig. 3). Controls showed that no binding or activation of the platelets took place on BSA-coated substrates, i.e., all the [^{14}C]serotonin was found in the nonbound platelets. When thrombin was added, all of the radioactivity was released into the media. On dishes coated with collagen, the platelets bound to the substrate, but their [^{14}C]serotonin was released into the media, indicating activation. Only the platelets that had bound to the coated surface were activated, however, as nonbound platelets retained their [^{14}C]serotonin (not shown).

The degree of platelet binding to laminin and fibronectin was dependent on the concentration of the coating protein. Increased concentrations of laminin or fibronectin coated

onto petri dishes caused increased platelet binding as measured by binding of radioactivity from platelet suspensions labeled with [^{14}C]serotonin or $Na_2[^{51}Cr]O_4$ (Fig. 2). Similar results were obtained with rat and mouse laminin and with the peptic fragments of human laminin.

Since the binding of platelets to laminin or fibronectin was always fractional, it was necessary to address the question of whether different subpopulations of platelets were binding to the two proteins. This was studied by the transfer experiment shown in Table I. After platelets were incubated on laminin-coated dishes, the platelets that remained nonbound were transferred to a second set of laminin-coated dishes. A similar percentage of platelets bound to the first and second series of dishes, suggesting that no depletion of a putative laminin-binding subpopulation had occurred in the first incubation. When the platelets that remained nonbound through two cycles of attachment to laminin were transferred to fibronectin-coated dishes, the percentage that attached was similar to that from the original platelet suspension, indicating no significant enrichment of a fibronectin-binding population. A reciprocal experiment in which the first two incubations were on fibronectin also showed no evidence of separation of the platelets into subpopulations. These results suggest that the capacity of platelets to bind to fibronectin or to laminin is not a characteristic of specific subpopulations of platelets.

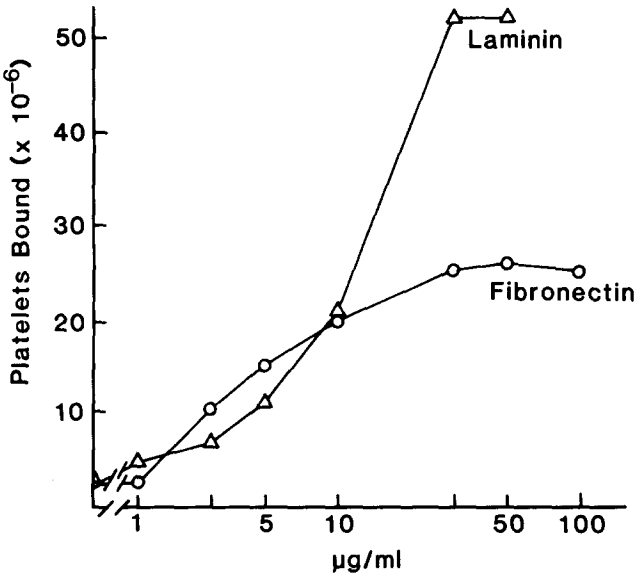

FIGURE 2 Platelet binding to substrates coated with different concentrations of laminin or fibronectin. Petri dishes were coated with increasing concentrations (1.0–100 μg/ml) of either human laminin or human fibronectin. Human platelets (2×10^8) labeled with $Na_2[^{51}Cr]O_4$ in 1 ml of DME supplemented with 0.5% BSA were incubated for 45 min at 37°C. The media with nonbound platelets were removed and any nonbound platelets remaining in the dish were washed away with PBS that contained 0.5% BSA.

Morphology of Platelets Attached to Different Surfaces

Scanning electron microscopy showed that platelets remained rounded when attached to laminin whereas attachment to fibronectin was associated with complete flattening (Fig. 3, *b* vs. *a*). No evidence of lysis or aggregation of the bound platelets was seen on laminin or fibronectin, in agreement with the lack of activation demonstrated by the [^{14}C]-serotonin release measurements.

Lack of Platelet Activation upon Prolonged Contact with Laminin

To determine whether activation as measured by [^{14}C]-serotonin secretion might occur over a prolonged incubation of platelets on laminin, we carried out time-course experiments. No [^{14}C]serotonin was released into the media over a 3-h incubation when platelets were incubated on laminin (Fig. 4) or fibronectin-coated (data not shown) substrates. In contrast, on type I collagen-coated substrate, [^{14}C]serotonin was continually secreted and was completely released by 3 h (Fig. 4).

To investigate whether activation was a function of platelet density, we incubated increasing concentrations of platelets on laminin-coated substrates. There was no secretion of [^{14}C]serotonin by platelets on laminin substrates at any platelet density. On collagen-coated substrates, the release of [^{14}C]serotonin into the media increased in proportion to the number of platelets present (data not shown). These results suggest that binding of platelets to laminin (or fibronectin) is not associated with secretion of [^{14}C]serotonin either temporally or as a function of platelet density.

Differential Inhibition of Binding of Platelets to Laminin and Fibronectin

Recent studies from this laboratory have demonstrated that fibroblasts adhere to fibronectin through the amino acid sequence arginine-glycine-aspartic acid-serine contained in the cell attachment domain of fibronectin and that the attachment of cells to fibronectin-coated substrates can be inhibited by micromolar quantities of synthetic peptides containing this sequence (23). Since a number of other proteins that interact with cell surfaces share this sequence (23), the peptides offered a useful probe for the analysis of the mechanism of platelet adherence to various substrates. When equimolar amounts (5×10^{-8} M) of laminin and fibronectin were used to coat petri dishes, and platelets were incubated in media with a peptide

TABLE I

Binding of Platelets upon Repeated Incubation on Laminin- or Fibronectin-coated Dishes

	First incubation	Second incubation	After 2 incubations on fibronectin	After 2 incubations on laminin
On laminin	17 ± 0.2	17.5 ± 0.1	17.9 ± 2.2	—
On fibronectin	7.5 ± 0.7	6.5 ± 0.4	—	8.0 ± 0.3

Platelets were isolated and labeled with [^{14}C]serotonin to 10,000 cpm/1.8×10^7 platelets. Petri dishes were coated with laminin or fibronectin (30 μg/ml) as described in Materials and Methods, and 2×10^8 platelets were added to them in the initial incubation. Each incubation was for 45 min in 2 ml of DME supplemented with 0.5% BSA at 37°C. The unbound platelets were then transferred to another dish as indicated. Each transfer was done in triplicate and data are expressed as percentage of attached cells (mean ± SE).

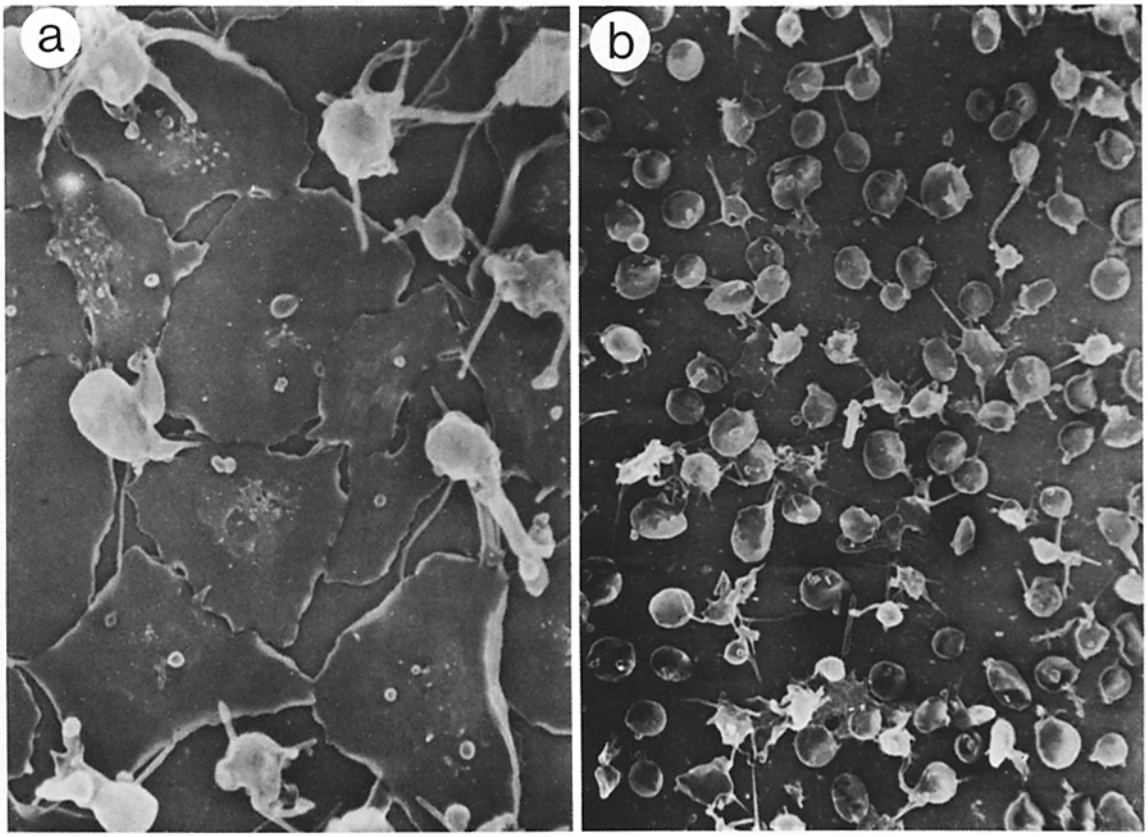

FIGURE 3 Scanning electron microscopy of platelets bound to laminin or fibronectin. Laminin and fibronectin (50 μg/ml) were coated onto glass coverslips as described in Materials and Methods. The platelets (2×10^8) were incubated on the coated surfaces for 1 h at 37°C in 1 ml of DME supplemented with 0.5% BSA. At the end of this incubation, the coverslips were fixed and examined by scanning electron microscopy. Platelets bound to fibronectin (*a*) (× 5,000) or to laminin (*b*) (× 2,000).

containing the arg-gly-asp-ser sequence, the binding of platelets to fibronectin was inhibited in a dose-dependent fashion, whereas their attachment to laminin was unaffected (Fig. 5). A control peptide, gly-asp-ser-pro, previously shown to be inactive in the adhesion of fibroblasts, had no inhibitory effect on platelet binding to either fibronectin or laminin. Similar results were obtained with another inactive peptide, arg-val-asp-ser-pro-ala-cys (results not shown). Neither of the peptides at the concentrations indicated caused platelet activation as measured by secretion of [^{14}C]serotonin into the media. That the binding of platelets to laminin could be specifically inhibited with an appropriate reagent was shown using affinity-purified laminin antibodies, which inhibited the binding of platelets to dishes coated with laminin (Table II). Attempts to show reciprocal inhibition with fibronectin antibodies were not successful because fibronectin-coated dishes treated with antifibronectin tended to activate the platelets (not shown).

DISCUSSION

The studies presented here identify laminin as a new adhesive protein for platelets. The specificity of the interaction of laminin with platelets is supported by several observations. First, the binding was dependent on the concentration of laminin and reached a level 20 times higher than the background binding observed on dishes coated with albumin. Second, this effect was obtained with laminin preparations purified from three different species. These laminins were purified using different methods, affinity chromatography on monoclonal antibodies was used for human laminin, and salt fractionations and heparin-Sepharose affinity chromatography were used for the rodent laminins. Thus, the effect on platelets is likely to be inherent to the laminin molecule and not caused by a contaminant. Furthermore, the adhesion of platelets to laminin showed characteristics fundamentally different from those observed on fibronectin or collagen.

Adhesion of platelets to laminin was distinct in that it was not associated with the spreading observed on fibronectin or with the activation that took place on type I collagen. In addition, antilaminin antibodies inhibited binding of platelets to laminin, while a soluble cell attachment peptide from fibronectin inhibited the binding of platelets to fibronectin but not to laminin. These observations suggest the existence of different cellular receptors for fibronectin and laminin on platelets, and that adhesion of platelets through these receptors results in different responses. That laminin and fibronectin would differ with regard to the cellular response they elicit is not without precedent. Neuronal cells form neurites in response to adherence to laminin, but they respond much less to fibronectin (24, 25).

Our data demonstrate that the binding of platelets to laminin is not accompanied by their activation as shown by the lack of release of serotonin from the platelets and their intact appearance in scanning electron microscopy after binding to laminin-coated surface. These data similarly confirm that platelets attach and spread on fibronectin (10, 22), but they also show that activation as measured by [^{14}C]serotonin release does not occur.

In contrast to laminin and fibronectin, type IV collagen of basement membranes, like type I collagen, has been shown

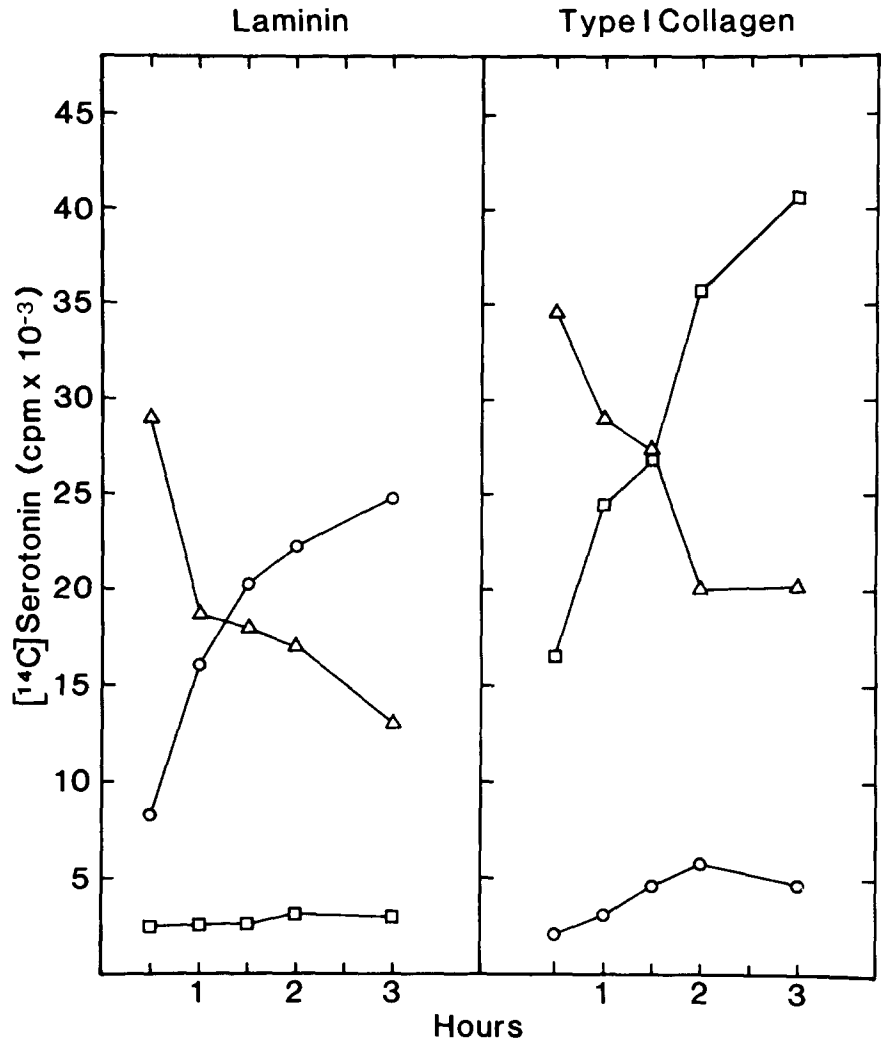

FIGURE 4 Time course of platelet binding and secretion on laminin- or Type I collagen-coated substrates. Petri dishes were coated with either 30 μg/ml laminin or 50 μg/ml type I collagen. Human platelets (1×10^8) labeled with [^{14}C]-serotonin were added to these dishes in 1 ml of DME supplemented with 0.5% BSA. Duplicate dishes at the indicated times were collected and processed as described in Materials and Methods. Data are presented as [^{14}C]serotonin in the bound (○), media (□), or nonbound (△) fractions.

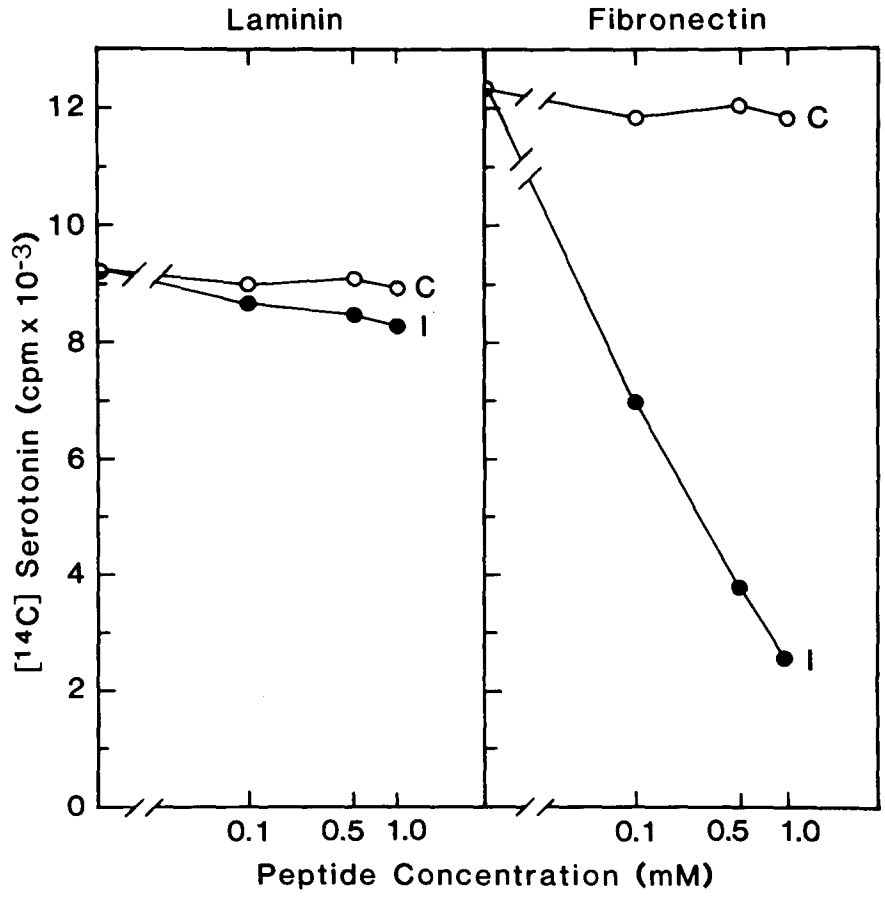

FIGURE 5 Inhibition of platelet binding to fibronectin and laminin by synthetic peptides. Petri dishes were coated with 40 μg/ml of laminin or 20 μg/ml of fibronectin. Human platelets (1×10^8) labeled with [^{14}C]serotonin were added to these dishes with and without increasing concentrations of either gly-arg-gly-asp-ser-pro (*I*) or gly-asp-ser-pro (C) in 1 ml of DME supplemented with 0.5% BSA. The dishes were then incubated 1 h at 37°C. Data represent radioactivity associated with the bound platelet fraction.

TABLE II

Inhibition of Platelet Binding to Laminin by Antibodies

Substrate	Condition	Bound platelets
		%
Laminin	Control	100 ± 9.6
Laminin	+ Antilaminin	8.4 ± 0.3
Laminin	+ Control antibody	84 ± 0.7

Petri dishes were coated with 10 μg/ml of rat laminin. After incubation with antibodies (10 μg/ml), the dishes were washed and 1×10^8 [^{14}C]serotonin-labeled human platelets were added in 1 ml of DME supplemented with 0.5% BSA. Incubation time was 1 h at 37°C. Bound platelets and media [^{14}C]serotonin were determined as described in Materials and Methods. [^{14}C]Serotonin secreted into the media by platelets on laminin-coated dishes was <7% of the total counts added, indicating a lack of activation. No further secretion was measured on antibody-coated laminin substrates. Triplicate determinations were made and data are expressed as the mean ± SE.

to be capable of activating platelets (7). Basement membrane-like endothelial cell matrices formed in vitro also bind platelets but appear not to activate them (2, 26, 27). Since laminin and fibronectin, unlike collagen, do not activate platelets, they may be the molecules to which platelets attach in a basement membrane in this situation. It is also possible that these glycoproteins modify the response of platelets to collagen in an extracellular matrix. It has been reported that binding of fibronectin to collagen suppresses the platelet-aggregating activity of collagen (28). Laminin might play a similar role in basement membranes. However, further data will be needed to determine whether the platelet-laminin interaction could occur in vivo. For instance, whether plasma proteins could affect the interaction remains to be studied. Our binding assays were carried out by using platelets separated from the plasma because divalent cations, which have previously been reported to be essential for the binding of fibronectin to platelets (29), had been removed from the plasma by the anticoagulant.

The lack of activation of the thrombogenic responses by platelets attaching to noncollagenous adhesive proteins and basement membranes suggests that such binding may serve purposes other than hemostasis. Under some circumstances alpha-granule proteins such as platelet-derived growth factor might be released without the release of serotonin from the dense granules (30). It will be interesting to know whether binding of platelets to laminin and fibronectin might be a sufficient stimulus to enable platelets to play a role in endothelial cell proliferation over an area of exposed basement membrane.

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