Tetrafibricin, a novel non-peptide fibrinogen receptor antagonist, induces conformational changes in glycoprotein IIb/IIIa

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Arg-Gly-Asp (RGD) is an amino acid sequence in fibrinogen recognized by platelet glycoprotein (GP) IIb/IIIa. Recently, it was found that RGD peptide binding to GPIIb/IIIa leads to conformational changes in the complex that are associated with the acquisition of high-affinity fibrinogen-binding function. In this study, we found that tetrafibricin, a novel non-peptidic GPIIb/IIIa antagonist, induced similar conformational changes in GPIIb/IIIa as did RGD peptides. Tetrafibricin increased the binding of purified inactive GPIIb/IIIa to immobilized pl-80, a monoclonal antibody that preferentially recognizes ligand-occupied GPIIb/IIIa. Exposure of the pl-80 epitope by tetrafibricin was also observed on resting human platelets by flow cytometry. On intact platelets, the conformational changes transformed GPIIb/IIIa into a high-affinity receptor for fibrinogen and triggered subsequent platelet aggregation. Tetrafibricin is the first non-peptidic GPIIb/IIIa antagonist reported that has the capacity to induce conformational changes in GPIIb/IIIa.

INTRODUCTION

Ligand binding to platelet-membrane GPIIb/IIIa is an obligatory step for the process of platelet aggregation. The complexed form of GPIIb/IIIa functions as a receptor for fibrinogen, von Willebrand factor (vWf) and vitronectin. For example, when platelets are stimulated with ADP, then soluble fibrinogen, vitronectin and vWf bind to the activated GPIIb/IIIa complexes [1,2]. The fact that only a few molecules of soluble fibrinogen bind to GPIIb/IIIa on resting platelets [3,4] indicates that the inactive conformer of GPIIb/IIIa is the predominant form on the surface of resting platelets. Cellular agonists such as thrombin or ADP are required to elicit the high-affinity fibrinogen-binding state of GPIIb/IIIa [5–8]. It was also postulated that certain monoclonal antibodies directed against GPIIb or GPIIIa could lead to conformational changes in GPIIb/IIIa resulting in high-affinity ligand binding [3,6,9,10].

Two amino acid sequences, Arg-Gly-Asp (RGD) existing in the α-chain and the dodecapeptide sequence His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (HHLGGAKQAGDV, residues 400–411) in the C-terminus of the γ-chain of fibrinogen, are considered as GPIIb/IIIa-binding domains within fibrinogen [11–14]. In contrast with macromolecular ligands, small synthetic ligands such as RGDS were demonstrated to bind to inactive GPIIb/IIIa in an agonist-independent manner [15]. Du et al. proposed that ligand-mimetic peptides provoke a change in GPIIb/IIIa, exposing neoepitopes (termed ligand-induced binding sites; LIBS) associated with the acquisition of the high-affinity fibrinogen-binding function [16]. Therefore, the RGD sequence may function as a partial agonist to trigger the high-affinity binding of a ligand rather than functioning as a simple competitive antagonist of GPIIb/IIIa. The precise mechanism that results in conformational changes within GPIIb/IIIa, rendering it competent to bind fibrinogen, remain to be elucidated.

In previous studies we demonstrated that tetrafibricin (Figure 1) was a competitive inhibitor of fibrinogen binding to GPIIb/IIIa [17–19]. We also proved that the anti-aggregatory effect of this compound could be attributed to its GPIIb/IIIa blockage rather than to its interference with other physiological pathways inside platelets. This conclusion was based on the inhibitory effects of this compound on: (1) the fibrinogen binding both to purified GPIIb/IIIa and to stimulated platelets; (2) the agonist-independent aggregation in chymotrypsin-treated platelets; and (3) the release reaction induced by ADP, but is not the same reaction induced by thrombin. Recently, several kinds of GPIIb/IIIa antagonist, including snake venoms containing the RGD sequence [20], monoclonal antibodies directed to GPIIb/IIIa [21] and synthetic peptidomimetic compounds [22,23] have been developed as platelet inhibitors. In spite of their potent antiplatelet action, some chemical modifications are required for these peptidic or peptidomimetic inhibitors in order to obtain (1) enough bioavailability, (2) long biological half-life and/or (3) sufficient specificity against other RGD-dependent integrins. Immunogenecity is also a matter of concern. Therefore, the elucidation of the action mechanism of tetrafibricin is of great interest for the rational design of non-peptidic GPIIb/IIIa inhibitors with widespread therapeutic potentials.

In an attempt to distinguish whether tetrafibricin interferes with the fibrinogen–GPIIb/IIIa binding by interacting with the receptor (GPIIb/IIIa) or with the ligand (fibrinogen), we examined whether or not tetrafibricin could induce conformational changes within inactive GPIIb/IIIa, previously reported to be caused by peptides containing the RGD sequence [16,24]. In the present study we found that tetrafibricin did expose neoepitopes on purified inactive GPIIb/IIIa and on resting platelets. Moreover, on intact platelets, tetrafibricin induced conformational changes in GPIIb/IIIa, transforming it into a high-affinity-binding state for fibrinogen (active conformer) and triggering the subsequent platelet aggregation.

These results suggest strongly that tetrafibricin interacts with GPIIb/IIIa. Tetrafibricin is the first non-peptide GPIIb/IIIa antagonist with the capacity to induce conformational changes in GPIIb/IIIa.

Abbreviations used: LIBS, ligand-induced binding sites; Aeg, aminoethyl-glycyl; PRP, platelet-rich plasma; GFP, gel-filtered platelets; GP, glycoprotein.

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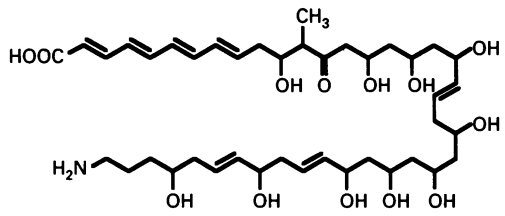

Figure 1 Structure of tetrafibricin

MATERIALS AND METHODS

Materials

The purification of tetrafibricin from the microbial broth of *Streptomyces neyagawaensis* NR0577 has been described previously [17]. The preparation proved to be more than 95% pure as assessed by the h.p.l.c. system. RGDS was purchased from Peptide Institute (Osaka, Japan), and dodecapeptide HHLGGAKQAGDV (residues 400–411) and RGES were from Peninsula Laboratories (Belmont, CA, U.S.A.). Human fibrinogen was from the IMCO Corporation (Stockholm, Sweden). Hexapeptide, KQAGDV (residues 406–411), was prepared by solid-phase peptide synthesis using a *p*-alkoxybenzyl alcohol polystyrene resin [25]. The purity of KQAGDV exceeded 98% as assessed by analytical h.p.l.c. and fast-atom-bombardment m.s. All other materials were of reagent grade and were purchased from Sigma (St. Louis, MO, U.S.A.) or Wako (Osaka, Japan) unless specified otherwise.

Inactive and active GPIIb/IIIa

The purification of GPIIb/IIIa was performed as described previously [26,27]. Briefly, the Triton X-100 lysate of human platelets from outdated blood was applied to a concanavalin A affinity chromatography column. Glycoproteins retained by concanavalin A were eluted and then applied to an aminoethyl-glycyl (Aeg)–RGDS–Sepharose column. The GPIIb/IIIa retained on the Aeg–RGDS column (active GPIIb/IIIa) was specifically eluted by RGDS (3 mM). RGDS was removed by dialysis against buffer A (0.1% Triton X-100, 150 mM NaCl, 20 mM Tris/HCl, 1 mM $MgCl_2$, 0.05% NaN_3, pH 7.0). GPIIb/IIIa in the flowthrough of the Aeg–RGDS affinity column (inactive GPIIb/IIIa) was further purified on a Sepharose S-300 gel-filtration column as described by Fitzgerald et al. [28]. The protein concentration of GPIIb/IIIa was determined by densitometry with the use of a t.l.c. scanner and the purity of each preparation (active and inactive GPIIb/IIIa) was determined to be $> 95\%$ and $> 85\%$ respectively, by electrophoretic evaluation.

Monoclonal and polyclonal antibodies

The monoclonal antibody, pl-80 and polyclonal antibody, Kan-2357, used in the present study were produced by standard techniques [29] using active GPIIb/IIIa as the immunogen. Kan-2357 is a polyclonal antibody directed against the GPIIb/IIIa complex. The Pl-80 clone was selected because of its capacity to recognize active GPIIb/IIIa immobilized on the plastic plate [30]. According to Western-blot analysis, the epitope of pl-80 was shown to be localized on GPIIb. As demonstrated in the cytofluorimetric analysis on resting platelets, pl-80 was identified as an anti-LIBS antibody on the basis of its preferential recognition of GPIIb/IIIa occupied by RGD peptide [30].

Exposure of LIBS on purified GPIIb/IIIa

The quantitative assay to examine the expression of the pl-80 epitope on GPIIb/IIIa was performed as described previously [27]. Briefly, the wells of microtitre plates were coated with 2 μg/ml of pl-80 (100 μl/well) overnight at 4 °C in buffer B (150 mM NaCl, 1 mM $MgCl_2$, 20 mM Tris/HCl, pH 7.4). After blocking with 3.5% (w/v) BSA, inactive GPIIb/IIIa (40 ng/ml) in buffer B containing 0.035% Triton X-100, 1% (w/v) BSA and various concentrations of the test compound was added and allowed to incubate overnight at room temperature. Bound GPIIb/IIIa was detected by using rabbit anti-GPIIb/IIIa antibody (Kan-2357) followed by anti-(rabbit IgG) (Amersham International, Amersham, Bucks., U.K.). The amount bound was finally quantified with the peroxide reaction by the use of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) as a substrate.

Preparation of platelets

Platelet-rich plasma (PRP) was prepared from whole blood anticoagulated with acid/citrate/dextrose. For the preparation of gel-filtered platelets (GFP), the platelets were isolated from PRP by gel filtration on a Sepharose 2B column (Pharmacia, Uppsala, Sweden) in modified Tyrode's buffer [3.5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 3 mM $NaHPO_4$, 2.5 μg/ml apyrase, 50 ng/ml prostaglandin I_2 (Funakoshi, Tokyo, Japan), 0.35% BSA, pH 7.2].

Exposure of neoepitopes on resting human platelets

In order to examine the exposure of the pl-80 epitope on intact platelets, we performed cytofluorimetric analysis according to the method described by Ginsberg et al. [31] with minor modifications. Before use in flow cytometry, pl-80 was conjugated with fluorescein isothiocyanate (FITC) according to the method described by Shattil et al. [32]. PRP (50 μl) was mixed with 20 μl of modified Tyrode's buffer containing 1 mM $CaCl_2$, various concentrations of an inhibitor and 2 μg/ml FITC–pl-80. Anti-(Fc receptor) antibody [14 μg/ml anti-(FcγRII IV.3) (Medarex, West Lebanon, NH, U.S.A.)] was also included to prevent Fc-receptor-mediated activation. The platelet suspension was incubated at room temperature for 15 min and then diluted 30-fold with modified Tyrode's buffer containing the appropriate inhibitor. The fluorescence intensity on the platelet surface was analysed on a FACScan flow cytometer (Becton Dickinson Immunocytochemistry System, Mountain View, CA, U.S.A.). The light scatter and fluorescence signal were acquired at logarithmic gain, and 10000 platelets in each sample were analysed.

Platelet aggregation

Platelet aggregation was observed by the use of a six-channel aggregometer (Hematracer 1, Niko Bioscience, Tokyo, Japan). When aggregation of fixed platelets was measured, GFPs were incubated with various concentrations of a test compound for 30 min at 37 °C in the presence of 1 mM $CaCl_2$. After fixation with 0.1% paraformaldehyde for 30 min, the platelet suspension was neutralized with an equal volume of 20 mM NH_4Cl solution

and washed once to remove the inhibitor. The aggregation of these fixed platelets was initiated by the addition of 1 mg of fibrinogen/ml. The extent of aggregation was estimated as the initial rate of the increase in the light transmission. When the reversibility of an antagonist was examined, GFPs were incubated with buffer alone, or with an inhibitor for 30 min at room temperature, and then were gel-filtered through Sepharose 2B. The aggregation was determined in the presence of 1 mg/ml fibrinogen after the addition of 20 μM ADP.

^{125}I-fibrinogen binding to stimulated platelets

Fibrinogen (500 μg) was radiolabelled with 1 mCi of $Na^{125}I$ (Amersham International) according to the modified chloramine T method as described by Parise and Phillips [33]. The GFPs [$(1.5–3) \times 10^8$/ml] were suspended in the presence of 0.07 μM ^{125}I-fibrinogen. After the addition of ADP (10 μM), ^{125}I-fibrinogen was incubated for 60 min and the bound radioactivity was separated from the free by spinning the platelets through an oil mixture (dioctylphthalate/dibutylphthalate, 2:2.5) as described by Mazoyer et al. [34]. The binding observed in the presence of 1 mM RGDS was considered as non-specific binding.

RESULTS

Tetrafibricin exposed neoepitopes on purified inactive GPIIb/IIIa

As described by Du et al. [16], RGD peptides induce conformational changes in GPIIb/IIIa, thereby exposing neoepitopes (LIBS). pl-80 was previously characterized as an anti-LIBS antibody, because it binds preferentially to GPIIb/IIIa occupied by RGDS [25]. By using inactive GPIIb/IIIa and immobilized pl-80, a conformational-specific antibody, the ability of tetrafibricin, RGDS and KQAGDV to expose the pl-80 epitope on GPIIb/IIIa was examined on a solid-phase system. As shown in Figure 2, tetrafibricin increased the binding of inactive GPIIb/IIIa to pl-80 immobilized on a plastic plate. According to dose-dependent curves, approx. 10 and 20 times more RGDS and KQAGDV (residues 406–411) than tetrafibricin were required to induce comparable binding of GPIIb/IIIa to pl-80 respectively. Exposure of the pl-80 epitope was specific, because an inactive peptide, RGES, exhibited no effect up to a concentration of 10 mM (results not shown). The concentrations

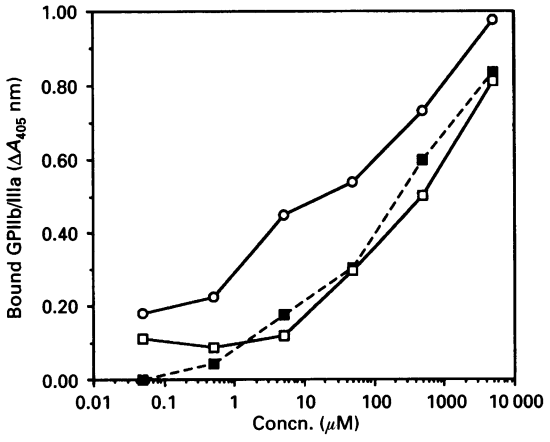

Figure 2 Exposure of the pl-80 epitope on purified inactive GPIIb/IIIa by various antagonists

Inactive GPIIb/IIIa (40 ng/ml) and various concentrations of a test compound (○, tetrafibricin; ■, RGDS; □, KQAGDV) were added to microtitre plates coated with pl-80 (2 μg/ml, 100 μl/well). After incubating overnight at room temperature, bound GPIIb/IIIa was detected by e.l.i.s.a. The absorbance in the absence of inhibitor (buffer alone, $A_{405} \approx 0.7$) was subtracted from each data point. The values are representative of three independent experiments.

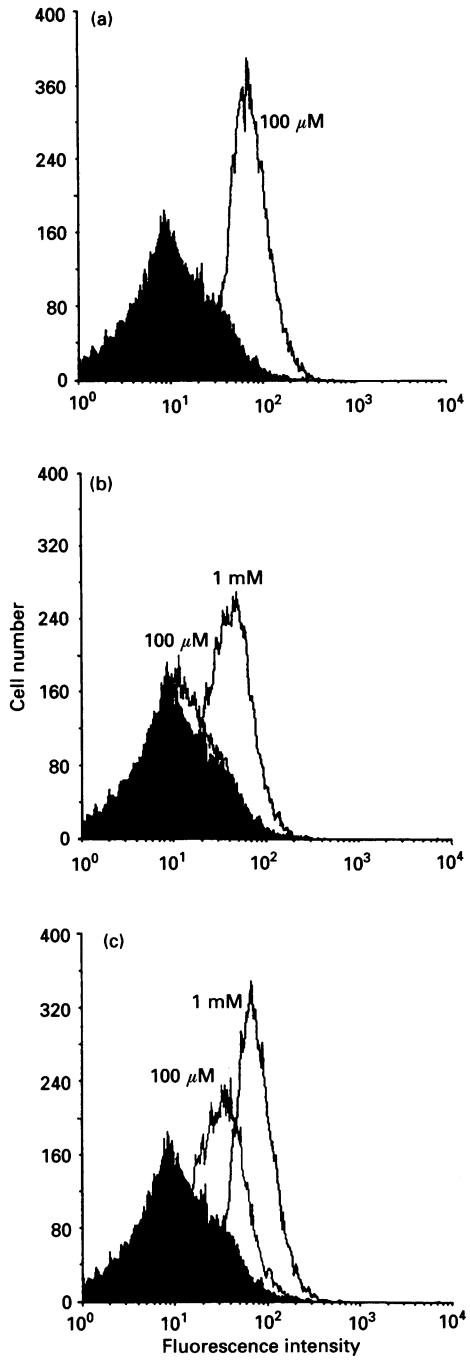

Figure 3 Exposure of the pl-80 epitope by GPIIb/IIIa antagonists in resting platelets

The binding of pl-80 to resting human platelets was examined by flow cytometry. FITC-conjugated pl-80 (2 μg/ml) and various concentrations of antagonists were incubated with 50 μl of PRP for 15 min. Samples were diluted 30-fold with modified Tyrode's buffer containing the appropriate antagonist. The solid areas denote pl-80 binding in the presence of the buffer, whereas the open areas denote that in the presence of tetrafibricin (**a**), KQAGDV (**b**) or RGDS (**c**).

Table 1 Effects of GPIIb/IIIa antagonists on ^{125}I-fibrinogen binding to stimulated platelets

Human platelets were purified by Sepharose 2B gel filtration. After preincubation with a test compound for 2 min, platelets [(1.5–3) × 10^8 cells/ml] were stimulated with 10 μM ADP in the presence of 0.07 μM ^{125}I-fibrinogen for 60 min at room temperature. Fibrinogen bound to platelets was determined in the platelet pellet after centrifugation in an oil mixture as described in the Materials and methods section. Non-specific binding was assessed in the presence of 1 mM RGDS. The indicated values represent the mean ± S.D. of the results from four to six experiments.

Compound	IC_{50} (μM)
Tetrafibricin	0.57 ± 0.1
RGDS	16.1 ± 0.2
KQAGDV	52.0 ± 4.5
RGES	> 1000

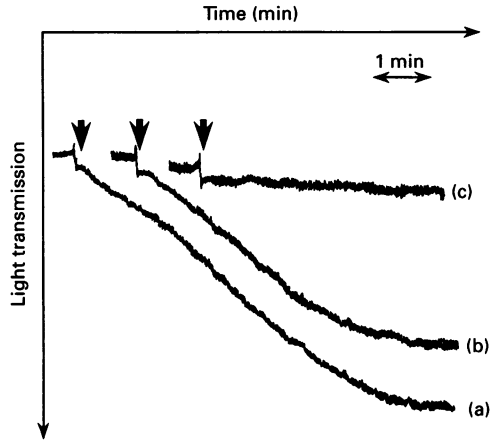

Figure 4 Aggregation of fixed platelets pretreated with GPIIb/IIIa antagonists

GFPs [(1–3) × 10^8 cells/ml] were incubated with 100 μM tetrafibricin (**a**), 100 μM RGDS (**b**) or 1 mM RGES (**c**) for 30 min at room temperature. After fixation with 0.1% paraformaldehyde and subsequent washing, platelet aggregation was initiated by the addition of 1 mg/ml fibrinogen (arrow).

of tetrafibricin and RGDS required to induce significant conformational changes in GPIIb/IIIa ($\geq$ 10 μM and $\geq$ 100 μM, for tetrafibricin and RGDS respectively) were approximately the IC_{50} values of these inhibitors for ADP-induced platelet aggregation in PRP which we reported previously (5.6 μM and 134 μM for tetrafibricin and RGDS respectively) [19]. Thus tetrafibricin is about 10-fold more potent than RGDS in inducing conformational changes in purified GPIIb/IIIa and about 20-fold more potent in inhibiting platelet aggregation.

Exposure of the pl-80 epitope on intact platelets by tetrafibricin

The above results indicated that tetrafibricin induces conformational changes in inactive GPIIb/IIIa, which was detected as the increase in pl-80 epitopes. In order to provide further evidence for the conformational changes, we next examined whether the same property was observed on the human platelet surface by the use of flow cytometry. As shown in Figure 3(a), human platelets incubated with 100 μM tetrafibricin stained brightly with pl-80.

Table 2 Aggregation of fixed platelets pretreated with tetrafibricin or RGDS

Platelet aggregation was induced in fixed platelets pretreated with various concentrations of tetrafibricin or RGDS as described in the Materials and methods section. The extent of aggregation was expressed as the increase in light transmission (%) per minute (LTU/min) after the addition of fibrinogen (1 mg/ml). Data are indicated as the mean ± S.D. of five or six experiments.

Concn. (μM)	Aggregation (LTU/min)	
	Tetrafibricin	RGDS
0	0.6 ± 0.6	0.9 ± 0.3
0.3	1.1 ± 0.9	0.6 ± 0.2
1	1.8 ± 1.4	0.1 ± 0.4
3	2.2 ± 1.6	0.8 ± 0.2
10	3.0 ± 1.3**	1.5 ± 0.3
30	5.2 ± 2.8*	2.5 ± 1.4
100	8.5 ± 2.9**	4.0 ± 1.1**
300	12.0 ± 1.5**	6.0 ± 2.0**
1000	13.0 ± 2.2**	8.4 ± 1.8**

* $P < 0.01$ or ** $P < 0.001$, significant difference from control aggregation, by paired Student's t test.

As compared with RGDS, tetrafibricin was at least 10 times more effective in increasing pl-80 binding to platelets, because about 1 mM of RGDS was required to induce comparable binding of pl-80 (Figures 3b and 3c). The rank of order among antagonists for increasing pl-80 binding to platelets was tetrafibricin > RGDS > KQAGDV, which was consistent with their potency in inducing the pl-80 epitope on purified GPIIb/IIIa as described above. RGES did not induce pl-80 binding even at a concentration of 1 mM (results not shown). These results were also consistent with the fact that tetrafibricin was approx. 30 and 90 times more potent than RGDS and KQAGDV respectively, on the inhibitory effect on ^{125}I-fibrinogen-binding to stimulated platelets (Table 1). Taken together, these results demonstrate that the conformational changes induced by tetrafibricin occurred not only in purified GPIIb/IIIa but also in GPIIb/IIIa present on resting platelets.

Tetrafibricin activates GPIIb/IIIa on intact platelets

The above data strongly indicated that tetrafibricin could induce conformational changes in GPIIb/IIIa resulting in the exposure of neoepitopes. As demonstrated previously [16,35], the interaction of RGDS with platelet results in an enhanced fibrinogen-binding capacity and in platelet aggregation. We next examined whether tetrafibricin can activate GPIIb/IIIa on intact human platelets. It was demonstrated that when GPIIb/IIIa was activated on intact platelets, it was capable of binding macromolecular ligands such as fibrinogen, this binding triggers subsequent platelet aggregation. In this respect, we employed a system in which platelets were preincubated with a test compound and then fixed with paraformaldehyde to freeze the conformation of GPIIb/IIIa. After fixation, platelets were washed to remove the compounds and fibrinogen was added to induce aggregation. As shown in Figure 4, platelets pretreated with tetrafibricin aggregated after the addition of fibrinogen without the need for agonist stimulation. This aggregation was specific, because platelets pretreated with RGDS, but not RGES, exhibited similar aggregation. Table 2 compares the extent of platelet aggregation following pretreatment with various concentrations of inhibitors. Tetrafibricin, when added at $\geq$ 10 μM, showed significant

Table 3 Effects of GPIIb/IIIa antagonists on aggregation of fixed platelets preactivated with tetrafibricin

GFPs [(1–3) × 10^8/ml] were treated with 100 μM tetrafibricin or buffer alone (pretreatment). After fixation with 0.1% paraformaldehyde and washing to remove the compound, platelet aggregation was induced by adding 1 mg/ml fibrinogen in the presence of a test compound or buffer alone. The extent of aggregation was assessed as the initial rate of the increase in the light transmission. Each value indicates the percentage of aggregation (mean ± S.D., $n = 3$) compared with the tetrafibricin-activated platelets.

Pretreatment	Test compound	Aggregation (% of control)
Tetrafibricin	Buffer	100
Buffer	Buffer	1 ± 0.7
Tetrafibricin	RGDS (1 mM)	2 ± 0.5
	HHLGGAKQAGDV (1 mM)	15 ± 2
	Tetrafibricin (100 μM)	3 ± 1
	EDTA (2 mM)	1 ± 0.5
	RGES (5 mM)	102 ± 15

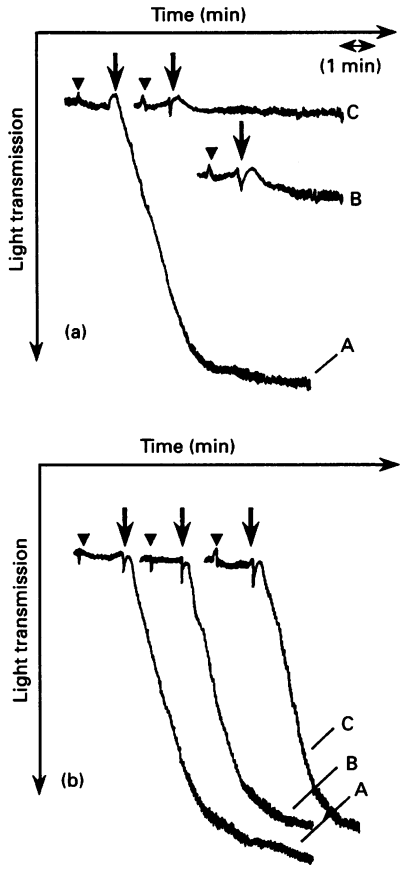

Figure 5 Reversible activation of GPIIb/IIIa on intact platelets by tetrafibricin or RGDS

GFPs [(1.5–3) × 10^8/ml] were incubated with buffer (A), 100 μM tetrafibricin (B) or 1 mM RGDS (C) for 2 min at 37 °C. After adding 1 mg/ml fibrinogen (triangle), platelets were stimulated with 20 μM ADP (arrow) (**a**). An aliquot of pretreated platelets were gel-filtered through Sepharose 2B to remove the inhibitor before platelet aggregation was induced (**b**).

activation of GPIIb/IIIa, whereas, at least 10 times more RGDS ($\geq$ 100 μM) was required to induce a comparable extent of aggregation. Similar to agonist-induced platelet aggregation, the aggregation of tetrafibricin-pretreated platelets was inhibited by agents such as RGDS, HHLGGAKQAGDV (residues 400–411), tetrafibricin or EDTA, indicating that fibrinogen binding to activated GPIIb/IIIa was responsible for this aggregation (Table 3). Direct activation of GPIIb/IIIa, not cellular activation, seems to be responsible for the effects of tetrafibricin, because agents that elevate the intracellular level of cyclic AMP (prostaglandins I_2 or E_1), when added simultaneously with tetrafibricin during the preincubation period, did not alter the effect of tetrafibricin (results not shown). Taken together, tetrafibricin can induce conformational changes on inactive GPIIb/IIIa, rendering it capable to bind fibrinogen and to support platelet aggregation after the addition of fibrinogen.

Tetrafibricin interacts with GPIIb/IIIa in a reversible manner

The foregoing data, by the use of a conformation-specific antibody and intact cells, indicated that tetrafibricin, after occupying GPIIb/IIIa, induced conformational changes in the receptor. Finally, we examined whether or not the interaction between tetrafibricin and GPIIb/IIIa, and the subsequent conformational changes, were reversible. As shown in Figure 5(a), when platelets were incubated with 100 μM tetrafibricin, a condition under which both significant conformational changes in GPIIb/IIIa and complete blockage of fibrinogen binding were observed, no aggregation occurred after the addition of fibrinogen (1 mg/ml) and ADP (20 μM). However, after the removal of the compound by gel filtration, these pretreated (unfixed) platelets had restored their ability to aggregate (Figure 5b). It should be noted that no aggregation was observed after the addition of fibrinogen alone (without agonist stimulation). The above observations suggest that on intact platelets, tetrafibricin interacts with the receptor in a reversible manner and that the conformational changes induced by this compound are also fully reversible.

DISCUSSION

Tetrafibricin is a competitive GPIIb/IIIa antagonist that has recently been discovered in the cultural broth of *Streptomyces neyagawaensis* NR0557. We have reported that the anti-aggregation activity of tetrafibricin was due to its capacity to block the binding of fibrinogen to GPIIb/IIIa [18,19]. Recently, there have been various studies reporting on GPIIb/IIIa inhibition by snake-venom proteins containing the RGD sequence [20], monoclonal antibodies directed to GPIIb/IIIa [21,36] and synthetic peptidomimetic compounds [22,23,37–39]. Considering that other GPIIb/IIIa antagonists are all derived from or designed based on RGD peptide, and are either peptides or peptidomimetics [36,40], the structure of tetrafibricin is unusual in having no RGD-mimicking regions.

The RGD sequence was initially identified as one of the domains in the fibrinogen molecule that is recognized by the GPIIb/IIIa receptor. Actually, competitive and complete inhibition of fibrinogen binding to GPIIb/IIIa could be observed with RGD peptides. Moreover, KYGRGDS has been shown to cross-link preferentially to amino acids 109–172 of GPIIIa [41]. Therefore, RGD-containing peptides or RGD mimetics are considered to bind to GPIIb/IIIa in a competitive manner with the ligand. Recently, Du et al. demonstrated that the binding of RGD peptides to GPIIb/IIIa induced conformational changes in GPIIb/IIIa complexes, transforming it into a high-affinity ligand-binding state [16]. It was suggested that certain RGD mimetics might function as partial agonists as well as competitive binding inhibitors for GPIIb/IIIa.

In this study, three lines of evidence clearly indicated that (1) tetrafibricin exerted its inhibitory effects on fibrinogen binding to GPIIb/IIIa by interacting with the GPIIb/IIIa receptor, and that (2) following receptor occupancy, tetrafibricin induced conformational changes in GPIIb/IIIa, transforming it into a high-affinity ligand-binding conformer. This is a novel finding as only RGD-containing peptides or peptidomimetic compounds have ever been reported to lead to similar conformational changes in GPIIb/IIIa. Our conclusions are based on the following observations. First, tetrafibricin, when incubated with purified inactive GPIIb/IIIa, significantly increased the binding of GPIIb/IIIa to pl-80, a monoclonal antibody that preferentially binds to RGD-occupied GPIIb/IIIa. Secondly, the exposure of the pl-80 epitope was also confirmed on resting platelets incubated with tetrafibricin. Thirdly, when platelets were pretreated with tetrafibricin and then fixed and washed to remove the compound, fibrinogen-dependent aggregation was observed without the addition of a cellular agonist. Tetrafibricin-induced aggregation was completely inhibited by GPIIb/IIIa antagonists, such as RGDS and HHLGGAKQAGDV, indicating that tetrafibricin is capable of activating the receptor *in situ* and that the fibrinogen binding to the activated receptor supports this aggregation. It is unlikely that tetrafibricin activates GPIIb/IIIa via other cellular activation processes, because there was little effect observed when a platelet-activation inhibitor, prostaglandin I_2 or prostaglandin E_1, was included during the preincubation period. The exposure of neoantigenic sites in GPIIb/IIIa was specific, because (1) other antagonists (e.g. RGDS or KQAGDV), but not the inactive peptide RGES, exhibited similar properties and (2) the order of potency (tetrafibricin > RGDS > KQAGDV ≫ RGES) for inducing conformational changes in GPIIb/IIIa was the same as for inhibiting platelet aggregation and ^{125}I-fibrinogen-binding to stimulated platelets. According to our previous observation, tetrafibricin inhibited the binding of fibrinogen, vWf and fibronectin to GPIIb/IIIa with similar IC_{50} values [18]. In contrast, the binding of vWf to GPIb/IX was not inhibited. These data, together with those from the present study, support the concept that tetrafibricin inhibits fibrinogen binding through interaction with the receptor and not with the ligand.

pl-80 is a useful tool to probe conformational changes in GPIIb/IIIa; however, there are different anti-LIBS antibodies with different epitopes on GPIIb/IIIa [24,31,42]. Moreover, ligand-induced conformational changes in the GPIIb/IIIa complex have been demonstrated from various approaches, such as susceptibility to proteolytic cleavage, decreases in intrinsic fluorescence and sedimentation coefficient, and increases in stroke radius [43,44]. We cannot, therefore, draw a simple conclusion that the conformational changes induced by tetrafibricin were exactly the same as those induced by RGDS. Although tetrafibricin does not contain an RGD sequence, it might involve a similar triggering feature. Different approaches, from both biochemical and immunological aspects, will be necessary to address this issue definitively. The IC_{50} values on LIBS exposure and on ^{125}I-fibrinogen binding for each inhibitor correlates well. These facts might indicate that tetrafibricin, after interacting with GPIIb/IIIa, exposes neoepitopes with similar efficiency to RGDS and γ-peptide. The fact that platelets, when preincubated with tetrafibricin before fixation (similar to RGDS-pretreated platelets), exhibited fibrinogen-dependent aggregation without the need for agonist stimulation suggests that this compound is able to convert inactive GPIIb/IIIa into its high-affinity ligand-binding stage. This finding has important functional implications. It suggests that tetrafibricin can function as a partial agonist for GPIIb/IIIa, as well as a competitive binding inhibitor.

Recently, exposure of LIBS was reported for the β_2 integrin [45]. Similar to GPIIb/IIIa (α_{IIb}/β_3), the initial interaction with the intact ligand, ICAM-1, causes exposure of neoepitopes in leucocyte function-associated antigen-1 (LFA-1). Intracellular adhesion molecule-1 (ICAM-1) itself can then bind with higher affinity to the altered LFA-1. It is not known at present whether or not non-peptide compounds are able to induce LIBS on other integrin molecules. Since tetrafibricin is the first non-peptide GPIIb/IIIa antagonist reported so far to induce LIBS, it would be interesting to explore the mechanisms of how tetrafibricin induces these conformational changes in GPIIb/IIIa. At present, we could not completely exclude the possibility that GPIIb/IIIa antagonists which induce LIBS lead to a thrombotic episode in physiological situations. However, we now speculate that such a possibility is not so likely, as no agonistic activity has ever been reported *in vivo* using GPIIb/IIIa antagonists that can induce conformational changes in the receptor [46]. Moreover, our results suggest that, in the suspension of GFPs, the activation of GPIIb/IIIa by tetrafibricin was fully reversible. Of course we admit that the number of *in vivo* studies is still limited and should be expanded before making final decisions on these issues. Further studies are also required to confirm the reversibility of GPIIb/IIIa activation in physiological situations. The ability of tetrafibricin to induce LIBS and to inhibit platelet aggregation may be a clue for the design of novel GPIIb/IIIa antagonists.

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