Fibroblasts Spread on Immobilized Fibrin Monomer by Mobilizing a β1-class Integrin, Together with a Vitronectin Receptor αvβ3 on Their Surface

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Human and murine fibroblasts were found to spread far more avidly on fibrin monomer monolayers than on immobilized fibrinogen, indicating that removal of fibrinopeptides by thrombin is a prerequisite for the fibrin-mediated augmentation of cell spreading. In fact, cell spreading was not efficiently augmented on monolayers of a thrombin-treated dysfibrinogen lacking the release of fibrinopeptide A due to an Aα Arg-16 → Cys substitution. Since a synthetic Arg-Gly-Asp (RGD)-containing peptide inhibited the fibrin-mediated cell spreading, subsequent dissociation of the carboxyl-terminal globular domain of the Aα-chains appears to render the RGD segments accessible to the cell-surface integrins. In support of this, fibrin-augmented cell spreading was inhibited by an antibody recognizing a 12-kDa peptide segment with γ Met-89 at its amino terminus, which is located in close association with the RGD segment at Aα 95–97 in the helical coiled-coil interdomainal connector. The fibrin-mediated augmentation of cell spreading was inhibited not only by an antibody against human vitronectin receptor (LM 609) but also by an antibody against the β1 subunit of integrin (mAb13), suggesting that the β1-class integrin together with a vitronectin receptor, αvβ3, is mobilized onto the surface of fibroblasts upon contact with the fibrin monomer monolayer.

Fibrinogen is a 340-kDa glycoprotein consisting of three pairs of polypeptide subunits, Aα, Bβ, and γ, linked together by multiple disulfide bonds. By structural studies including electron microscopic analysis together with biochemical data, there is now general agreement on the shape of the fibrinogen molecule (2–7). The fibrinogen molecule is composed of three major globular domains, i.e. one central E domain and two identical outer D domains connected by a single chain α-helical coiled-coil (4, 6). The distal part of the D domain is the carboxyl terminus of the γ-chain, while the proximal part is the carboxyl terminus of the Bβ-chain. The carboxyl-terminal two thirds of the Aα-chains fold back from the D domain and form two independently folded domains (αC domains) at their carboxyl-terminal parts. In the native fibrinogen molecule, the αC domains interact with each other and form an additional small globular (αC-αC) domain that is closely associated with the central E domain (4, 6, 7). Upon thrombin cleavage of fibrinopeptides A and B, the globular αC-αC domain is released from the E domain, and subsequently dissociated into individual αC domains (4, 6, 7). The fibrinogen molecule thus undergoes distinct conformational changes upon conversion to the fibrin monomer molecule (4), and thereby exposes several fibrin-specific regions that may participate in the functions of fibrin. The Arg-Gly-Asp (RGD) segments residing at Aα 95–97 and Aα 572–574, tentatively designated as RGD-1 and RGD-2, respectively, may also be categorized into this type of fibrin-specific segments. In this paper, we describe the binding of cultured human and murine fibroblasts to immobilized fibrin monomer, but not to immobilized fibrinogen, by focusing on the conformational changes induced in the fibrinogen molecule upon its conversion to fibrin.

EXPERIMENTAL PROCEDURES

Materials—All chemical reagents were of the highest analytical grade commercially available and were purchased from the sources shown below. Bovine serum albumin (BSA),1 soybean trypsin inhibitor type-1, trypsin, and sodium dodecyl sulfate (SDS) were from Sigma, and Microtiter 96- and 24-well flat-bottomed plates from Costar, Cambridge, MA. Rabbit antisera to the human vitronectin receptor and the human fibronectin receptor were purchased from Life Technologies, Inc., rabbit anti-human fibrinogen IgG and rabbit anti-human fibronectin from Dako Japan, Kyoto, Japan, and anti-human vitronectin antisera from The Binding Site Ltd., Birmingham, United Kingdom. The P1F6 antibody directed against the αvβ3 integrin receptor was from Telios/Life Technologies, Inc., and the KH33 antibody directed against the α5 chain of integrin receptor was from Seikagaku Corp, Tokyo, and others.

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Japan. Monoclonal antibody to the β subunit of the human fibrin fragment fibrinogen receptor, mAb13 (8), was a gift from Dr. Steven K. Akizawa (Howard University Cancer Center, Howard University College of Medicine, Washington, DC). Mouse monoclonal antibody LM609 was a gift from Dr. David A. Cheresh (Research Institute of Scripps Clinic, La Jolla, CA). This antibody was reported to be specific for the 99–117 complex and was previously shown to block the function of αβ (9).

Cells and Culture Media—3T3-fibroblast cells were purchased from Dainihon Pharma Co., Tokyo, Japan. Human fibroblasts (TIG-3) were a generous gift from Dr. Tadashi Shimo-Oka of Iwaki Glass Co., Chiba, Japan. Dulbecco's modified Eagle's essential medium (DME) with 10% fetal calf serum was obtained from Life Technologies, Inc. The cells were cultured in 10% (v/v) fetal calf serum-containing DME at 37 °C in a 6.0% CO₂ atmosphere.

Synthetic Peptides—Gly-Arg-Gly-Asp-Ser (GRGD) and Gly-Arg-Gly-Glu-Ser (GRGES) peptides were purchased from Iwaki Glass Co., Osaka, Japan, and a His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Gly-Glu-Ser (GRGES) peptides was from Sigma.

Immunoblotting—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting was carried out according to the method of Towbin et al. as described previously (10). Briefly, after separation of fibrinogen on 10% SDS-PAGE slab gels and electroblotting onto nitrocellulose filters, blots were soaked in 50 mM Tris-HCl, pH 7.4, containing 0.135 mM NaCl (TBS) and 3% BSA for 1 h at 37 °C, rinsed with TBS three times, and incubated with monoclonal antibodies (3 µg/ml) in TBS containing 0.1% BSA for 2 h at 22 °C. After washing three times with TBS, the filters were incubated for 1 h with peroxidase-conjugated rabbit anti-mouse IgG. After rinsing, blots were imersed in the substrate solution: 4-chloro-1-naphthol/H₂O₂.

Fibrinogen Purification and Plasmic Fragmentation—Fibrinogen was purified by repeated precipitation with 25% ammonium sulfate from human plasma harvested from blood freshly collected into 0.11 M trisodium citrate, 0.1 mM e-aminoacproic acid and 500 kallikrein inhibitor units (KIU)/ml aprotinin after removal of fibronectin and plasminogen by passing the plasma through columns of gelatin-Sepharose CL-6B and lysine-Sepharose CL-6B connected in tandem as described previously (11). The plasmic phase-3 digests of cross-linked fibrin (XDP) was prepared according to Francis et al. (12). Fragment D1 containing the amino-terminal 12-kDa peptide segment of the α-chain remnants of fragment D and D3 and E were separated by ion-exchange HPLC as described (12). Fragment D1 containing the α-chain remnants of fragment D1A, D1B, and D1C was prepared using 0.1 unit of plasmin/mg of fibrinogen in the presence of 10 mM CaCl₂ at 37 °C. One hundred microliters of 16 mg/ml human fibrinogen was diluted with 3 ml of TBS, and clotted with 0.5 unit/ml thrombin for 2 h at 37 °C, followed by another for 22 h at 4 °C to achieve gel formation as completely as possible. The fibrin gels formed were washed 5 times with 10 ml of TBS, dissolved with −0.5–1.0 ml of 2 M NaBr containing 1 mM GTP peptide as an antipolymerant (NaBr-GTPR) for 8 h at 20 °C, and further diluted with TBS containing 1 mM GTPR to appropriate concentrations required for immobilization on microtiter wells. When a small portion of a diluted fraction (500 µl, A₃₅₀ = 0.98) was subjected to gel filtration on a Sephacryl CL-6B column (1.0 × 80 cm), proteins were all eluted in a single peak (fractions 110–125) as fibrin monomer with a relative molecular mass of 3.4 × 10⁵. Though not shown here, no measurable fibrinogen-related peptides were identified in fractions eluted earlier than the peak fractions, indicating that the NaBr-solubilized fibrin was mostly constituted of monomeric fibrin molecules.

Structurally Elucidated Hereditary Dysfibrinogens—Two types of heterozygous dysfibrinogens were utilized, i.e. a dysfibrinogen with an Arg-263→Gly substitution and fibrinogen for 4 h at 22 °C, and the wells were washed with TBS followed by blocking with TBS containing 1% BSA. After washing three times with TBS, an anti-human fibrinogen polyclonal antibody (1:2000 dilution in TBS-Tween 80) was added to each well and the reaction was allowed to proceed for 3 h at 22 °C. The wells were rinsed three times with TBS and then incubated with 200 µl of a 1:1000 dilution of anti-rabbit IgG goat antiserum conjugated with horseradish peroxidase in TBS for 3 h at 22 °C. The wells were rinsed three times with TBS and finally incubated with a substrate for horseradish peroxidase at 37 °C. The reaction was analyzed by a kinetic ELISA. Data were expressed as the means of quadruplicate determinations. The concentrations of half-maximal saturation of fibrinogen and fibrin monomer to the surface are 0.04 µg/ml and 0.2 µg/ml, respectively (Fig. 2).

Cell Adhesion Assay—The cell adhesion, i.e. the initial attachment of cells to the substratum, and subsequent spreading thereon were measured by the method of Grinnell (21) with some modifications as described previously (22). Briefly, 2-cm² well (24-well Costar plates) polystyrene plates were coated with various concentrations of fibrinogen, fibrin monomer, fragment X, XDF, or heat-denatured BSA in TBS containing a synthetic antipolymerant GTP peptide at 1 mM.
blocking with 3% heat-denatured BSA in TBS for 1 h at 37 °C; plates were washed with TBS containing 0.2% BSA three times, 0.5-ml aliquots of suspensions of fibroblasts and HUVECs (2 × 10^4 cells/ml) were pipetted into the coated wells. Cells were allowed to adhere to the vitronectin- or BSA-coated surface for 60–90 min at 37 °C. Non-adherent cells were removed by washing with TBS, and the plates were examined by phase contrast microscopy and photographed. Spread cells were counted as described previously (22) and when necessary, they were analyzed using a computer image processing package to determine cell areas and diameters as described previously. The spread cells were defined essentially as described elsewhere (22), namely being polygonal in shape with a dark surface under phase-contrast microscopy and a larger surface area than round cells (+ 2 S.D. Vitronectin and fibronectin were used as control proteins for the cell adhesion assay.

Adhesion Inhibition Assay—After incubation of fibrin monomer, fibrinogen, fragments D, E, X, and XDP with DMEM and 0.2% BSA containing 0.5 mM GPRP peptide, cells were mixed with these solutions, and were added to the wells coated with fibrinogen or fibrin monomer. We applied the same assay conditions as those for the adhesion assay. All experiments were performed at least three times with two independent isolates.

RESULTS

Characterization of Fibrin Monomer-dependent Cell Adhesion—Human fibroblasts were found to spread on wells coated with fibrin monomer, but not on those coated with fibrinogen below the concentration of 10 μg/ml in DMEM-solution (Fig. 3). The cell spreading on the fibrin monomer substratum was apparently dependent on the concentration of fibrin monomer immobilized to the wells, as noted in different grades of morphological changes observed under microscopy. Since spontaneous aggregation of fibrin monomer was efficiently hindered by the addition of an antipolymerant GPRP peptide, the cell spreading on the fibrin monomer substratum is attributed most likely to specific changes induced in the molecule upon conversion to fibrin monomer rather than polymerized fibrin. Indeed, fibrinogen in solution added to cell suspensions was unable to block the cell spreading on the fibrin monomer substratum, but fibrin monomer inhibited it concentration-dependently (Fig. 4). However, both attachment and spreading of cells were inhibited by an RGD-containing peptide, GRGDSP, but not by a GRGESP peptide, indicating that the receptor for the RGD-containing peptide, i.e. an integrin, was involved in the adhesion and spreading of cells on the fibrin monomer substratum (Fig. 5). Thrombin-cleavage of fibrinopeptide A seemed to be mandatory for supporting the spreading of human fibroblasts on the fibrin monomer substratum, as shown by a study utilizing a hereditary dysfibrinogen with defective thrombin-catalyzed cleavage of fibrinopeptide A due to an AαR-16 to C substitution as compared with fibrinogen molecules manifesting normal thrombin-cleavage of fibrinopeptide A, i.e. normal fibrinogen and a dysfibrinogen with a γ R-275 to C substitution (Fig. 6). The newly exposed amino terminus of the fibrin α-chain appeared to be indifferent to the reactions because the synthetic GPRP peptide failed to inhibit both attachment and spreading of cultured fibroblasts (Fig. 5). The result thus suggested that a specific conformation newly induced in the fibrin monomer was responsible for the interaction with the cell-surface integrins.

There are two RGD-containing peptide segments assigned to the Aα 95–97, RGD-1, and the Aα 575–577, RGD-2, residues in each fibrinogen Aα-chain. To see whether or not either one or both of the two RGD segments, RGD-1 and RGD-2, are masked in native fibrinogen, and exposed on its conversion to fibrin

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monomer to support adhesion and spreading of cells, we utilized the JIF-25 antibody recognizing the \( g^{89-173} \) residue segment. Interestingly, by assignment of the amino acid residues of the \( \alpha \)-and \( g \)-chains in the helical coiled-coil regions based on the two ring-like disulfide bridges connecting the three polypeptides called disulfide swivels (1), RGD-1 is located close to the \( g^{73-75} \) residue segment, which is only 16 residues apart from the amino terminus of the 12-kDa \( g^{89} \) segment recognized by JIF-25. Thus, binding of JIF-25 to the 12-kDa \( g^{89} \) segment may affect the local conformation surrounding the RGD-1 segment. Indeed, when fibroblasts were seeded together with JIF-25, adhesion of fibroblasts to the fibrin monomer substratum was substantially inhibited (85%) (Fig. 7, A and B), indicating that RGD-1 was hidden in fibrinogen but exposed on immobilized fibrin monomer to modulate cell adhesion and spreading.

When adhesion and spreading was tested on immobilized fragment X lacking both RGD-2 segments, spreading of fibroblasts was less extensive than on fibrin monomer (data not shown). Thus, RGD-2 may also be involved in cell spreading.

Identification of the Cell-surface Receptor of Fibrin Monomer-dependent Cell Adhesion—To identify the cell-surface receptor responsible for cell adhesion to the fibrin monomer substratum, human fibroblasts were allowed to adhere to immobilized fibrin monomer in the presence of LM609, P1F6, or mAb13 recognizing \( \alpha_\beta_3 \), \( \alpha_\beta_5 \), and the \( \beta_1 \)-subclass integrin, respectively. Cell adhesion was inhibited by mAb13 (85.0%) and LM609 (42%) (Table I). The data indicate that both VNR and a \( \beta_1 \)-subclass integrin are involved in the fibrin monomer-dependent cell adhesion.

**DISCUSSION**

Adhesion and spreading of cells on immobilized thrombin-treated and non-treated fibrinogen have been studied extensively (24–34), but there still remain controversies on the mechanism of cell adhesion to the fibrinogen and fibrin substrata. Although experimental conditions are not necessarily comparable from one experiment to another, the controversies may largely arise from inconsistency regarding the configuration of individual fibrinogen or fibrin molecules immobilized onto tissue culture wells.
In this study, we established an assay system using NaBr-solubilized preformed fibrin, fibrin monomer, which had been immobilized onto tissue culture wells in the presence of a synthetic antipolymerant GPRP peptide. This system allowed us to achieve uniform immobilization of the fibrin monomer molecules on the wells as verified by homogeneously distributed adherent cells. We were thus able to count the adherent cells more accurately than by conventional assay systems.

Utilizing this assay system, we found that both human and murine fibroblasts were avidly adherent to immobilized fibrin monomer but not to immobilized fibrinogen (Figs. 3 and 4), and that the adhesion was dose-dependently inhibited by soluble fibrin monomer (Fig. 4) and a synthetic RGD peptide (Fig. 5). The results together indicate that the fibrin-specific regions are preserved satisfactorily after immobilization and that they are most likely two RGD segments, RGD-1 and RGD-2. Failure to inhibit the adhesion of fibroblasts to fibrin monomer by fragments X, D, and E may be explained at least partly by lack of one or both of the RGD segments in these plasmic fragments. Although fragment X still retains a pair of RGD-1 segments, both RGD-1 and RGD-2 may be required to cooperate with each other for full expression of cell-spreading supporting activity. Indeed, the involvement of separate sites in differentially mediating cell attachment and spreading has been observed in other adhesion molecules such as laminin (35) and thrombospondin (36). Thus, concerted action of the two RGD-segments may be required in order for fibroblasts to attach to the fibrin monomer substratum and avidly spread thereon. Since RGD-2 resides very close to a cluster of negatively charged residues (Aα 586–595), which may interact with a cluster of positively charged residues (Aα 601–608) in the carboxyl-terminal segment of the Aα-chain, RGD-2 may be inaccessible or hidden in the native fibrinogen molecule. When the (αc-αc) domain is fully dissociated upon conversion of fibrinogen to fibrin, RGD-2 may become available for supporting the cell adhesion. RGD-1 residing in the helical coiled-coil of the interdomainal connector may also be masked in the native fibrinogen molecule, and exposed upon fibrinogen to fibrin conversion. The adhesion of fibroblasts to fibrin monomer was inhibited by JIF-25, which recognized the 12-kDa γ 89 –segment (Fig. 7, A and B). The epitope for JIF-25 seems to be buried in native fibrinogen, because the JIF-25-conjugated Sepharose failed to adsorb fibrinogen in solution, although the γ-chain of SDS-denatured fibrinogen immobilized to the membrane was clearly visualized by JIF-25 on immunoblotting (14). Therefore, RGD-1 may also be buried in fibrinogen and exposed upon fibrinogen to fibrin conversion, and thereby serves as a functional site with the fibroblast in concert with RGD-2. Interestingly, RGD-1 has been found to be a receptor-induced binding site of fibrinogen with defective thrombin-cleavage of fibrinopeptide A failed to support the adhesion of fibroblasts (Fig. 6).

Of interest is the finding that the β1 subclass integrin is preferentially involved in the adhesion of fibroblasts to fibrin monomer together with αβ3, a vitronectin receptor, expressed on the fibroblast. The receptors for fibrinogen are shown to be α1β1/β3, which is specific for binding with the γ (400–411) residue segment of fibrinogen (26, 39–42) and αβ3, which interacts with vitronectin as well (9).

Dejana et al. (43) reported that cell spreading on the fibrinogen substratum was mediated by cellular fibrinogen synthesized by the cell itself. This possibility still remains to be resolved, but so far it seems to be unlikely, because a well characterized antibody, KH33, that inhibits the function of the α3 chain of integrins failed to block the fibrin monomer-dependent cell adhesion (data not shown). The mechanism of the involvement of the β1 subclass of integrin in the interaction with the RGD-segments of fibrin monomer is not clear at this stage of the investigation, but we speculate that the fibrin monomer molecule acquired affinity

| Spread cells | Fibrin-monomer | Fibrinogen |
|--------------|----------------|------------|
| Mouse IgG    | 100.0          | 100.0      |
| LM609 (α5β1) | 48.0 ± 4.5     | 110.0 ± 13.5 |
| P1F6 (αβ3)   | 109.5 ± 5.5    | 111.0 ± 14.0 |
| mAb13 (β1)   | 15.0 ± 1.0     | 18.3 ± 3.0  |

**TABLE I**

**Effects of monoclonal antibodies on human fibroblast spreading to immobilized fibrin-monomer and fibronectin**

Polyethylene wells were coated with fibrin-monomer (10 μg/ml) and fibronectin (10 μg/ml) at 4°C overnight. After blocking with 1% BSA, wells were washed with TBS and human fibroblasts were added with monoclonal antibodies against integrins or mouse IgG (50 μg/ml, respectively). Two hours later, spread cells were counted. This experiment was repeated four times, and results of one representative experiment were shown. Data are expressed as the means ± S.D. of three wells.
for the $\beta_1$-subclass integrin due to a newly induced conformational change upon contact with its authentic receptor $\alpha_{b2}$. Acquisition of such extraordinary affinity for non-authentic integrins has been shown in other adhesion molecules. For example, Neugenbauer et al. (23) showed that an integrin heterodimer in the $\beta_1$ family expressed on the HD11 chick myoblast cell line functions as a receptor for fibrinogen. They also showed that a mAb to the $\beta_1$-integrin enhanced attachment of HD11 cells to fibrinogen and inhibited attachment to vitronectin. Although the molecular mechanism still remains speculative, this report seems to be the first to describe acquisition by fibrin monomer of affinity for the $\beta_1$-subclass integrin.

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