CROSS-LINKING OF FIBRIN BY ACTIVATED FACTOR XIII STIMULATES ATTACHMENT, MORPHOLOGICAL CHANGES AND PROLIFERATION OF FIBROBLASTS

SHUNJI KASAI¹, TAKEHIKO KUNIMOTO² and KAZUO NITTA²

¹Department of System Engineering for Materials, Faculty of Technology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, and ²Chemotherapy Division, National Cancer Center Research Institute, Tsukiji, Chuoku, Tokyo 104, Japan

ABSTRACT

The response of fibroblasts to the conversion of fibrinogen into fibrin was investigated to clarify the relationship between the conversion and wound healing. The formation of fibrin by thrombin little affected fibroblast attachment and morphology. In contrast cells rapidly attached and subsequently spread on fibrin cross-linked by activated Factor XIII. The introduction of cross-linking also stimulated cell proliferation. However neither enzyme had much effect on cellular behavior. These results indicate that the introduction of cross-linking by activated Factor XIII directly promotes the cellular responses, and suggest that the formation of fibrin stabilized by cross-linking plays a significant role not only in stoppage of blood flow but also in subsequent migration and proliferation of fibroblasts.

The conversion of fibrinogen into fibrin is the terminal step in blood clotting. In this process, thrombin cleaves fibrinogen to release sequentially fibrinopeptide A and fibrinopeptide B. This trimming results in the spontaneous polymerization of the molecules. Although the first stage of fibrin polymerization by thrombin does not involve covalent bonds, the polymer is subsequently stabilized by the introduction of intermolecular γ -glutamyl- ϵ -lysine peptide bonds. The formation of these covalent bonds between side chains is catalyzed by an enzyme called activated Factor XIII or fibrin-stabilizing factor (FSF). Factor XIII deficiency, first described by Duckert *et al.* (1, 5), is characterized by recurrent bleeding, hematoma formation after trauma, prolonged wound healing, and extensive scarring. The stimulation of fibroblast proliferation and collagen formation

are required in wound healing (8). Thus the delay of wound healing in Factor XIII-deficiency patients may be due to the lack of stimulation of fibroblast proliferation.

To clarify the relationship between the conversion of fibrinogen into fibrin and wound healing, we investigated the responses of fibroblasts to the conversion. In this study serum-free defined medium was used to eliminate effects of hormones, growth factors and cold-insoluble globulin present in serum.

MATERIALS AND METHODS

Purification of Fibrinogen

Fibrinogen free of blood coagulation Factor XIII and plasminogen was prepared by treating bovine fibrinogen (Daiichi Pure Chemicals Co.; 97% clottable) with 3.3 M urea (17) and lysine-Sepharose affinity chromatography (19). The solution containing fibrinogen was dialyzed against 0.14 M sodium chloride buffered at pH 7.4 with 0.01 M Tris-HCl (NaCl/Tris-HCl) and stored in portions at -20°C .

¹Present address: Biotechnology Center, The Green Cross Corporation Research Division, Hirakata, Osaka 573, Japan

Conversion of Fibrinogen into Fibrin

Non-cross-linked and cross-linked fibrin were prepared in the following manner (13, 14, 17). An aliquot of fibrinogen solution was placed in a 35-mm culture dish. Fibrin formation was initiated by adding a solution containing thrombin (Sigma Chem. Co.) and CaCl_2 . The final concentrations were 2 mg/ml fibrinogen; 20 mM CaCl_2 ; 2 units/ml thrombin. When exogenous Factor XIII (The Green Cross Co.; specific activity 10 units/mg protein) was to be included in the clotting mixture, it was added to the fibrinogen solution before the addition of the thrombin- CaCl_2 mixture. In this case the final concentration of Factor XIII was 0.1 unit/ml. The reaction mixtures were incubated at 37°C for 6 h. The formed gel was rinsed twice with Hanks' balanced salt solution (HBSS) before addition of the cells.

In some experiments, fibrinogen dissolved in NaCl/Tris-HCl (2 mg/ml) was placed in a culture dish. It was incubated at 22°C for 20 min, washed twice with HBSS, and used as a fibrinogen-treated substratum.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis

Prior to electrophoresis, samples were reduced for 10 min at 100°C in 4 M urea, 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol as described by Mosher (13). Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate was performed using the method of Laemmli (7). The gel was stained with Coomassie brilliant blue and destained by diffusion (18).

Cells

L cells were grown in stationary culture in Eagle's minimal essential medium (Eagle's MEM) supplemented with 10% fetal bovine serum (FBS) and 60 μg of kanamycin per ml.

The Kinetics of Cell Attachment

L cells were dispersed from the monolayer with 0.25% trypsin (Flow Laboratories Co.) and 0.02% ethylenediaminetetraacetate (EDTA) in phosphate-buffered saline (PBS). The detached cells were harvested by centrifugation, washed once with HBSS containing bovine serum albumin (BSA) at 10 mg/ml, and resuspended in Eagle's MEM. L cells (5×10^5 cells) in 1.5 ml

of Eagle's MEM were placed in fibrinogen- or fibrin-treated culture dishes. The dishes were incubated at 37°C in a 5% CO_2 atmosphere. The number of adherent cells was determined at various times by counting the number of non-adherent cells recovered in the medium and subtracting this number from the number of cells initially placed in the dish (16).

Cell Growth in Serum-Free Medium

For growth assay, L cells (4×10^4 cells) suspended in Eagle's MEM supplemented with 1% BSA were placed in culture dishes that had been treated with either fibrinogen or fibrin, and incubated at 37°C in a 5% CO_2 atmosphere. At the end of the incubations the medium was decanted and the cells were released with 0.25% trypsin and 0.02% EDTA in PBS. The number of released cells was counted in a hemocytometer.

RESULTS

Cross-Linking of Fibrin by Activated Factor XIII

The fibrinogen molecule is composed of three pairs of disulfide-linked nonidentical polypeptide chains ($\alpha_2\beta_2\gamma_2$) (11, 12). Thrombin catalyzes the conversion of fibrinogen into fibrin, but cross-linking is not introduced in this polymerizing reaction. On the other hand, activated Factor XIII catalyzes the formation of intermolecular ϵ -(γ -glutamyl)lysine cross-linking between side chains, converting noncovalently linked fibrin into a covalent linked form (9, 10, 15).

Fig. 1 shows the SDS-polyacrylamide gel electrophoretic patterns of reduced purified bovine fibrinogen, non-cross-linked fibrin and cross-linked fibrin. In the present experiment, cross-linking formation by activated Factor XIII was confirmed.

Time Course of Fibroblast Attachment

L cells attached more rapidly to fibrin-treated substrata than to fibrinogen-treated substrata (Fig. 2). Furthermore, clear differences were observed in the rate of cell attachment between non-cross-linked fibrin and cross-linked fibrin used as substrata. The results indicate that not only the formation of fibrin matrix but also the introduction of cross-linking by activated Factor XIII affect fibroblast attachment.

Fig. 1 Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of fibrinogen, non-cross-linked fibrin, and cross-linked fibrin. Samples were reduced for 10 min at 100°C in 4 M urea, 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol prior to electrophoresis, and 7.5 μ g was subjected to electrophoresis on 7.5% gel.

Fig. 2 Time course of L cell attachment to the substrata treated with fibrinogen (Δ), non-cross-linked fibrin ($\circ$), and cross-linked fibrin ($\bullet$)

Morphology of Attached Cells

Fig. 3 shows the morphology of attached cells on the substrata treated with fibrinogen, non-cross-linked fibrin, or cross-linked fibrin. On

fibrinogen-treated, or non-cross-linked fibrin-treated substrata, only partial or no cell spreading was observed. In contrast, when cells were seeded on cross-linked fibrin-treated substrata, they spread rapidly. Within 30 min, all cells spread fully. It is therefore likely that the introduction of cross-linking by activated Factor XIII markedly affects both the rate of cell attachment and morphology.

Relationship between Conversion of Fibrinogen into Fibrin and Fibroblast Growth

Cell growth on the substrata treated with fibrinogen, non-cross-linked fibrin, or cross-linked fibrin were compared. As shown in Fig. 4, the growth rate of L cells depends on the provided substrata. L cells failed to proliferate on culture dishes in the BSA-supplemented, serum-free medium.

On the other hand, different growth response of L cells to the conversion of fibrinogen into fibrin was observed. In the experiment presented in Fig. 4, L cells grew best on the substrata treated with cross-linked fibrin and became confluent (Fig. 5).

DISCUSSION

The present study demonstrates that the conversion of fibrinogen into fibrin directly affects not

fibrinogen

non-cross-linked fibrin

cross-linked fibrin

Fig. 3 L cell spreading on the substrata treated with fibrinogen, non-cross-linked fibrin, and cross-linked fibrin. Cells were incubated at 37°C for 30 min on the substrata treated with fibrinogen, non-cross-linked fibrin, or cross-linked fibrin. Then they were photographed with an Olympus IMT inverted microscope and an Olympus PM-10 AD 35 mm camera system. $\times 100$

only the rate of fibroblast attachment and fibroblast morphology but also growth on the substrata, and that these effects are due to the introduction of cross-linking between fibrin fibers by activated Factor XIII.

Fibrinogen molecules are converted to fibrin by thrombin, followed by the introduction of intermolecular γ -glutamyl- ϵ -lysine bonds by activated Factor XIII. The formation of fibrin by thrombin little affected cell attachment (Fig. 2) or morphology (Fig. 3). In contrast, cells rapidly attached and subsequently spread on the substrata treated with cross-linked fibrin. Furthermore, introduction of cross-linking by activated Factor XIII stimulated cell proliferation (Fig. 4).

Several investigators reported that thrombin and activated Factor XIII directly affect the growth of some types of cells (2, 6). But neither enzyme had much effect on the attachment, morphology, or growth of L cells in the concen-

Fig. 4 L cell growth on the substrata treated with fibrinogen, non-cross-linked fibrin, and cross-linked fibrin. L cells (4×10^4 cells) suspended in Eagle's MEM supplemented with 1% BSA were placed in culture dishes that had been treated with fibrinogen (Δ), non-cross-linked fibrin (○), or cross-linked fibrin (●), or were untreated (▽). All were incubated at 37°C in a 5% CO₂ atmosphere.

fibrinogen

non-cross-linked fibrin

cross-linked fibrin

Fig. 5 Morphological appearance of L cells maintained on the substrata treated with fibrinogen, non-cross-linked fibrin, or cross-linked fibrin. Cultures were placed and maintained as described in Fig. 4. Pictures were taken at 115 h. $\times 100$

trations used in this study (data not shown). Thus the possibility can be ruled out that thrombin or activated Factor XIII directly stimulate the cells. We conclude that the introduction of cross-linking between fibrin fibers directly promotes the cellular responses. Our results suggest that the formation of fibrin stabilized by cross-linking plays a significant role not only in stoppage of blood flow but also in subsequent migration and proliferation of fibroblasts.

Cross-linked fibrin is mechanically stronger than non-cross-linked fibrin and is also more resistant to enzymatic dissolution. Doolittle (3, 4) suggested that no morphological difference is found between the two in electron microscopy, and that cross-linking does not seem to introduce gross structural rearrangement.

Detailed interaction mechanism between the introduction of cross-linking and alteration of cellular behavior remains unknown. Further studies are presently being carried out to clarify not only how cross-linked fibrin interacts with the cell surface and cytoskeleton to affect their attachment, morphology, and proliferation, but also what difference in biological roles exists between the formation of γ -dimer and α -polymer subunits of cross-linked fibrin.

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