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THROMBIN AFFECTS FIBRONECTIN AND PROCOLLAGEN IN THE PERICELLULAR MATRIX OF CULTURED HUMAN FIBROBLASTS

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Summary

We have studied the effects of human thrombin on the isolated pericellular matrix of cultured human lung fibroblasts. Cell-free matrices were prepared from confluent cultures of cells by extraction with sodium deoxycholate and hypotonic buffer after radiolabeling the cultures with [^{14}C]glycine. After the extraction, only a few radiolabeled polypeptides were retained on the culture dishes. These were identified as fibronectin, procollagens, and as yet unidentified polypeptides with molecular weights of 180 000, 140 000, 66 000 and 43 000. The matrices were exposed to thrombin in serum-free medium and the changes in the matrix-associated proteins were studied in autoradiograms of polyacrylamide gels. As a result of the treatment, there was massive release of both fibronectin and procollagen from the matrices into the medium. In addition, thrombin cleaved the 66 000 dalton polypeptide to a 62 000 dalton form that remained in the matrix. Collagenase treatment did not bring about the release of fibronectin or affect the 66 000 dalton protein. Some procollagen was also cleaved by thrombin; when high concentrations of thrombin were used cleavage of fibronectin took place. These effects of thrombin may operate in wounded areas in vivo.

Introduction

Thrombin is a specific serine protease which has multiple functions in blood coagulation and hemostasis (reviewed in Ref. 1). It activates plasma transglu-

Abbreviations: pro-α1(I) and pro-α2(I), procollagen type I chains.

taminase (factor XIII) and converts fibrinogen to fibrin in the final stages of blood coagulation. Fibronectin is a glycoprotein found in plasma and tissues of vertebrates. Thrombin, in mitogenic concentrations, releases fibronectin from cultured human fibroblasts and stimulates their fibronectin synthesis [2]. Fibronectin is susceptible to factor XIII-catalyzed cross-linking and it can be cross-linked to fibrin, collagen and to itself [3]. Fibronectin can bind to collagen [4] and it also has binding sites for heparin, cell surfaces (reviewed in Refs. 3 and 5) and actin [6,7].

Procollagen and fibronectin codistribute in the same fibrillar structures in the pericellular matrix of cultured cells [8]. Fibronectin in fibroblast cultures is predominantly a matrix protein [9] and the pericellular matrix can be considered in vitro connective tissue matrix. Collagen is mainly found as procollagen in cultures of human fibroblastic cells [10]. We now report on the effects of human thrombin on the isolated extracellular matrices of human fibroblasts [12] and found that it can affect certain matrix-associated proteins. Fibronectin and procollagen were released from the matrix as a result of the action of thrombin, but collagenase alone was unable to bring about the release of fibronectin. Collagen and collagen-derived peptides are chemotactic factors for human fibroblasts [11]. The ability of thrombin to release fibronectin and procollagen may be involved in wound healing in vivo.

Materials and Methods

Cell cultures, media and reagents. Diploid human embryonic lung fibroblasts (CCL-137) were obtained from ATCC, Rockville, MD. The cells were grown on plastic tissue culture dishes (Falcon Plastics, Oxnard, CA) at 37°C in Eagle's basal medium supplemented with 10% fetal calf serum, 100 I.U./ml penicillin and 50 μg/ml streptomycin. Bacterial collagenase (form III, 500 U/mg) was purchased from Advance Biofactures Corp., Lynbrook, N.Y. Highly purified human thrombin (3000 U/mg) was from Sigma Chemical Co., St. Louis, MO. Thrombin did not contain plasmin or plasminogen activators when studied in gels containing casein [28].

Fibronectin-releasing 10000 dalton polypeptides were purified from the serum-free supernatant fluids of the human fibrosarcoma cell line 8387, as described [13].

Immunological methods. Human plasma fibronectin was purified from plasma by affinity chromatography on gelatin-Sepharose according to previously published methods [4,14] and used to immunize rabbits. The antisera were adsorbed with fetal calf serum to remove the cross-reacting antibodies. Radioimmunoassays for human fibronectin were performed as described earlier [14]. Immunofluorescence for cell surface fibronectin was performed as described [14] using 3.5% paraformaldehyde for fixation. Sheep antirabbit IgG conjugated with fluorescein isothiocyanate was used (Hyland Laboratories, Costa Mesa, CA). Immunofluorescence for the different types of procollagen was performed as described in detail [8], using antibodies kindly provided by Dr. Rupert Timpl.

Isolation of extracellular matrices. Confluent 20 cm^2 cultures of CCL-137 fibroblasts were metabolically labeled with [^{14}C]glycine (113 Ci/mol, The

Radiochemical Centre, Amersham, U.K.) for 24 h with 5 mCi/l in culture media, followed by a subsequent labeling of 3 h with 5 mCi/l to label the rapidly turned-over polypeptides. The labeling was terminated by washing with phosphate-buffered saline and the cell-free matrices were then prepared by extracting the cells three times with sodium deoxycholate in hypotonic buffer (0.5% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride in 10 mM Tris-HCl-buffered saline, pH 8.0) at 0°C, as described earlier [12]. The matrices were then rapidly washed with 2 mM Tris-HCl buffer (pH 8.0) containing 1 mM phenylmethylsulfonylfluoride followed by washing with the following serum-free buffer: Dulbecco's modification of Eagle's media plus 50 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid and 0.1 mg/ml of bovine serum albumin, pH 6.8. The experiments were carried out in this buffer.

The assay for fibronectin release. Metabolically labeled isolated cell-free matrices of human lung fibroblasts were maintained in the serum-free buffer. At the onset of the assay, the medium was changed and thrombin or the 10 000 dalton fibronectin-releasing polypeptides were added into the medium of the matrices. The digestion of the matrices with 50 mg/l collagenase was performed in the serum-free buffer for 60 min prior to the assay. The treatment of the matrices was terminated by collecting the medium and washing the matrices gently with phosphate-buffered saline. The proteins in the media were precipitated with trichloroacetic acid (10% final concentration) and prepared for SDS-polyacrylamide gel electrophoresis. The matrices were dissolved in the gel sample buffer for analysis by gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gels were prepared as described by Laemmli [15]. Purified plasma fibronectin (subunit molecular weight 220 000), phopshorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000) and bromphenol blue were used as markers for molecular weight and mobility measurements.

Results

Polypeptide components of the extracellular matrices of human lung fibroblasts

Isolated cell-free matrices were studied by indirect immunofluorescence for the presence of different connective tissue components [8]. Cultures of CCL-137 cells contained fibronectin and procollagens of types I and III, but not of types II or IV (data not shown). Polypeptide analysis of the pericellular matrix showed the presence of several radiolabeled proteins (Fig. 1). The major polypeptides in the matrices were fibronectin, procollagen chains and some as yet unidentified polypeptides with molecular weights of 180 000, 140 000 and 66 000, respectively. The 180 000 protein has not been identified. The 140 000 dalton protein was evidently identical to the 140 000 dalton glycoprotein found in association with the cytoskeletons of cultured fibroblasts [16]. Frequently, a 43 000 dalton polypeptide was observed that comigrated in the gels with actin. The collagenous polypeptides were also identified by their susceptibility to bacterial collagenase (Figs. 1 and 2).

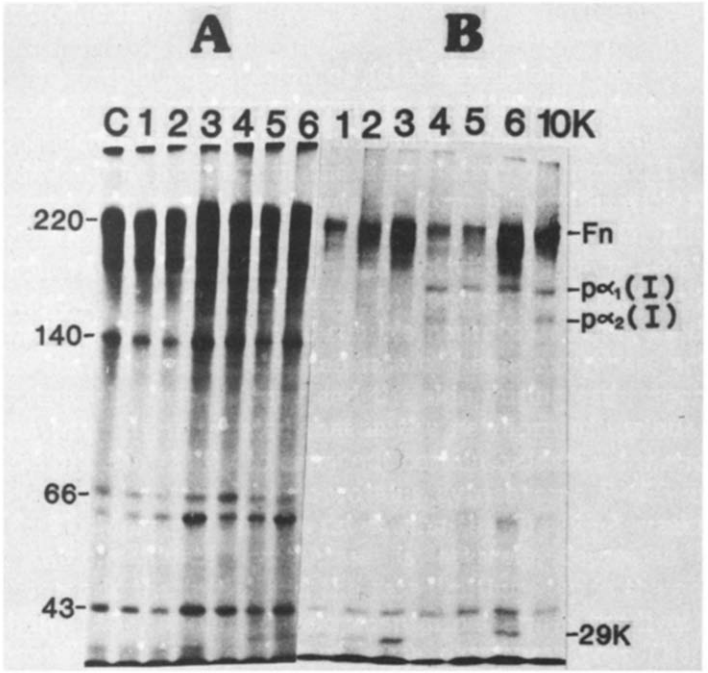

Fig. 1. Effects of thrombin and collagenase on the isolated matrices. Cultures of human lung fibroblasts were labeled metabolically and extracted to prepare the matrices. The isolated matrices were exposed to human thrombin in the serum-free buffer for 1 h. The changes in the radiolabeled proteins were observed in autoradiograms of 5% SDS-polyacrylamide gels. Panel A, matrices; Panel B, supernatant fluids. (C) Collagenase-treated control matrix; (1) collagenase treatment followed by the addition of 0.25 U/ml thrombin; (2) collagenase treatment followed by the addition of 2.5 U/ml thrombin; (3) collagenase treatment followed by the addition of 25 U/ml thrombin; (4) 0.25 U/ml thrombin only; (5) 2.5 U/ml thrombin only; (6) 25 U/ml thrombin only; (10K) polypeptides released from the untreated matrix by the fibronectin-releasing 10 000 dalton polypeptides (10 μg/ml). Fn, $p\alpha_1(I)$ and $p\alpha_2(I)$ point to the positions of fibronectin and the procollagen pro-α1(I) and pro-α2(I) chains. The position of the 29 000 dalton fragment of fibronectin is indicated (29K). Molecular weights are shown on the left. Both parts of the figure have been taken from the same gel.

Effects of thrombin on the cell-free matrices

Radiolabeled isolated matrices of cultured human fibroblasts were exposed to thrombin in serum-free media and the changes in the matrix-associated and in the released polypeptides were observed in polyacrylamide gels. Human thrombin had several effects on the matrix-associated polypeptides. Thrombin was able to cause a dose-dependent release of fibronectin from the matrices (Fig. 1) into the media. The release was massive and time-dependent but not quantitative (Fig. 2). The release could be demonstrated at 37°C and 22°C but not at 4°C (not shown). The release of fibronectin was accompanied by cleavage of the 66 000 dalton protein in the matrix (see below); the 180 000, 140 000 and 43 000 (actin) dalton proteins were not affected. Some matrix components were occasionally found in the supernatant fluids after prolonged incubation (Fig. 2, lane B2). Fibronectin molecules released from the matrices were either found as apparently intact 220 000 dalton subunits of fibronectin

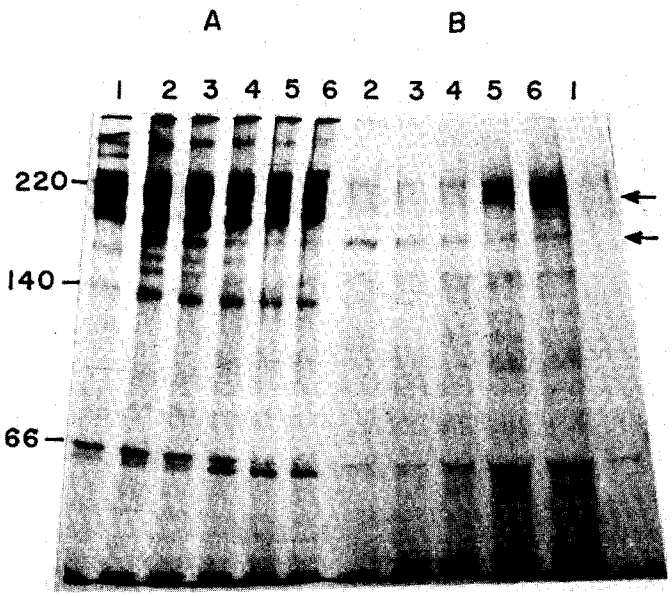

Fig. 2. Release of fibronectin from the matrix; time dependence. Radiolabeled isolated matrices were exposed to 5 U/ml thrombin for different periods of time and the released polypeptides were precipitated and analyzed in a 6% SDS-polyacrylamide gel. Autoradiogram of the gel is shown. Panel A, matrices; Panel B, released polypeptides. (1) collagenase treatment; (2) untreated matrix incubated in the buffer for 2 h; (3—6) thrombin treatments for 2 min, 10 min, 30 min and 60 min, respectively. The upper arrow points to the large fragment of fibronectin and the lower arrow to the pro-α1(I) chain. Molecular weights are indicated on the left.

(Fig. 1, lanes B1 and B4) or as approx. 200 000 dalton cleavage products (Fig. 1, lanes B3 and B6). The released fibronectin molecules were immunologically identified by their ability to compete in radioimmunoassays specific for human fibronectin, as described before [14]. Both intact and degraded forms were active. Treatment of the matrix with collagenase removed procollagen quantitatively but did not significantly affect matrix-associated fibronectin (Fig. 2, lanes A1 and B1). On the other hand, the release of fibronectin by thrombin was accompanied by the release of procollagen into the medium (Fig. 1, lanes B4—6). Using higher concentrations of thrombin we were able to demonstrate the characteristic cleavage of fibronectin into its 200 000 and 29 000 dalton fragments [17] (Fig. 1, lanes B3 and B6). This cleavage took place also in the collagenase-treated matrix, indicating that the fragment is not derived from procollagen.

Even the lower concentrations of thrombin brought about proteolysis of procollagen. The pro-α1 (I) chain was usually not affected, whereas the pro-α2(I) chain was significantly decreased in amount (Fig. 1, lanes B 4—6). No major cleavage product of collagen was detected, suggesting that there may be several sites of procollagen where thrombin acts. In contrast, procollagen polypeptides released by the fibronectin-releasing 10 000 dalton polypeptides from the matrix were unaffected and also the released fibronectin remained apparently intact (Fig. 1, lane 10K). The other matrix-associated polypeptides were not

released into the medium by thrombin, suggesting that the release and digestion of fibronectin and procollagen by thrombin involves relatively specific cleavage sites.

If the matrices were treated with trypsin, several nonspecific cleavages took place and almost all matrix-associated polypeptides were affected (Fig. 3). The 140 000 dalton protein was very resistant even to trypsin. Trypsin digested most of the 66 000 dalton protein while thrombin converted it here partially to the 62 000 dalton form. Even low concentrations of trypsin caused a cleavage in the matrix-associated fibronectin without the release (Fig. 3, lane A6). The trypsin-digested forms of fibronectin were significantly smaller than the thrombin-digested forms (Fig. 3, lanes B1 and B5). Both trypsin and thrombin could digest fibronectin in the matrix to approx. 200 000-dalton fragments that are not disulfide-linked [18]. This suggests that the region of the molecule close to the interchain disulfide bridge is not involved in the attachment of fibronectin into the matrix.

Cleavage of the 66 000 dalton protein in the matrix

A proteolytic cleavage was constantly seen in the 66 000 dalton matrix-associated protein after treatment with thrombin. This protein apparently remained in the matrix as a 62 000 dalton polypeptide after the treatment (Figs. 1 and 2). A similar cleavage was brought about by the fibronectin-releas-

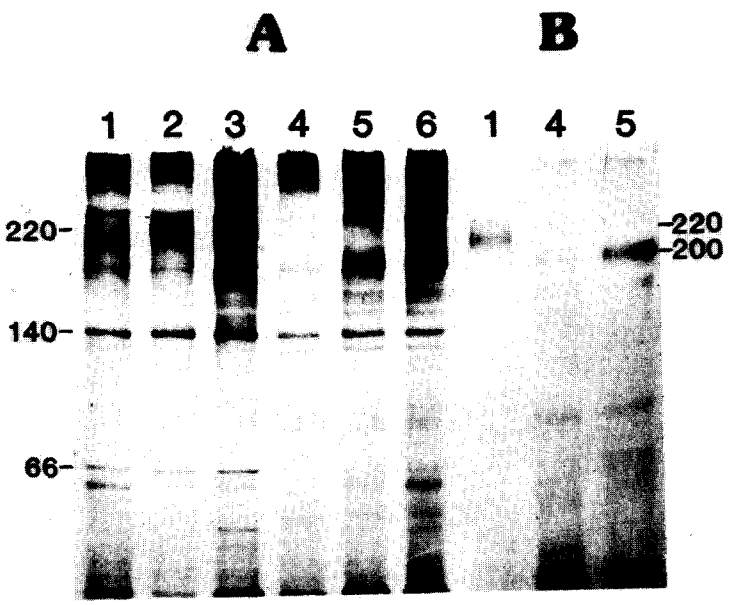

Fig. 3. Effects of trypsin and thrombin on matrix polypeptides. Radiolabeled matrices were treated with collagenase and exposed to trypsin or thrombin for 1 h. Autoradiogram of a 5% gel is shown. Panel A, matrices; Panel B, supernatant fluids. (1) 25 U/ml thrombin; (2) 2.5 U/ml thrombin; (3) 0.25 U/ml thrombin; (4) 10 μg/ml trypsin; (5) 1 μg/ml trypsin; (6) 0.1 μg/ml trypsin. Molecular weights of the protein standards ($\times 10^3$) are given on the left.

ing polypeptide in association with the release of intact fibronectin from the matrix [19]. The cleavage also appeared to occur naturally to some extent in culture conditions since some 62 000 dalton protein was frequently detected in the matrix. However, there is no direct evidence that the 62 000 dalton protein is a cleavage product of the 66 000 dalton polypeptide. Only traces of radioactivity were detected in the supernatant fluids in the 66 000 molecular weight area, indicating that the 66 000 and 62 000 dalton polypeptides are not effectively released from the matrices.

Discussion

The biological activities of thrombin include the conversion of fibrinogen to fibrin and activation of plasma protransglutaminase (factor XIII) during the final stage of blood coagulation, stimulation of cell division and enhancement of fibronectin production by cultured fibroblasts [1,2,20—22]. The effects of thrombin on cells are thought to be mediated by specific cell surface receptors that undergo a cascade of events including a proteolytic cleavage of the receptor-ligand complex and the endocytosis of the complex [23]. Using human thrombin and human fibroblasts, Mosher and Vaheri [2] were able to demonstrate an increase in the production of fibronectin in association with the decrease of cell-associated fibronectin. Although fibronectin in the extracellular matrix was greatly decreased, the loss was not considered a direct proteolytic effect. Only at high concentrations or during prolonged treatment thrombin will cleave fibronectin and yield two fragments per subunit [17] as was also observed in the present study.

Using cell-free matrices of cultured human fibroblasts [12], we studied the interactions of human thrombin and the matrix components. The composition of the extracellular matrices of cultured fibroblastic cells is partially known. The matrices contain hyaluronic acid and heparan sulfate as their major glycosaminoglycan components, and fibronectin and procollagen as major polypeptides [12,24]. Some other protein components could be identified in our gels. The 180 000 dalton protein may represent myosin and the 43 000 dalton polypeptide is apparently cellular actin that comigrates with it in electrophoresis. The protease-resistant 140 000 dalton polypeptide is evidently identical to the glycoprotein found in association with the detergent-resistant cytoskeletons of human fibroblasts [16]. The identity of the 66 000 dalton protein that has a protease-sensitive site is not known, but it may be identical to fimbrin, a microfilament-associated 68 000 dalton protein that is particularly prominent in membrane ruffles, microspikes and microvilli [25]. The fibronectin-releasing 10 000 dalton polypeptides, the action of which is inhibited by protease inhibitors [24], also cleaved the 66 000 dalton polypeptide [19]. These observations suggest that it may be one of the major targets for different proteases in cell cultures. The role of the 66 000 dalton protein as a possible linker between fibronectin and the other matrix components is unclear at present.

Thrombin released fibronectin from the matrix concomitantly with the cleavage of a 66 000 dalton protein. High concentrations of thrombin caused the cleavage of a 29 000 dalton fragment from fibronectin, both from the released and in the matrix-associated forms, but this cleavage of fibronectin was

not essential for the release. Thrombin was also able to affect procollagen molecules by releasing them into the media, evidently because of the association of procollagen molecules with the fibronectin-containing pericellular structures.

Collagenase was not effective in the release of fibronectin in human fibroblast cultures. The situation is apparently different from that found in tissues, since fibronectin could be extracted from lung alveolar basement membranes by collagenase digestion [26]. Both procollagen and fibronectin that codistribute in cultured fibroblasts are lost in transformation [8]. Although there is a high affinity between denatured collagen and fibronectin in vitro, there is no direct indication of an association between fibronectin and collagen in vivo or in cell cultures. Our approach to use fibronectin-releasing peptides [19] and thrombin has permitted sequential analysis of the organization of these matrix-associated proteins. The results presented in this study support our previous observations that collagen in the pericellular matrix of cultured human fibroblasts binds to fibronectin-containing structures [8,20].

The ability of thrombin to digest the pro-α2(I) chain of collagen may be due to denaturation of procollagen during the extraction of the cultures to prepare the matrices. Collagen, collagen-derived peptides and fibronectin are chemotactic factors for several cell types [11], and thrombin might induce directional migration of fibroblasts and other cells to sites of tissue injury or wound.

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