FIBRONECTIN RECEPTOR ON CULTURED HUMAN SKIN FIBROBLASTS OF NORMAL SUBJECTS AND PATIENTS WITH HEREDITARY CONNECTIVE TISSUE DISEASES

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Abstract

We investigated the binding of ¹²⁵I-labeled fibronectin to cultured human skin fibroblasts from normal subjects and patients with hereditary connective tissue diseases. Scatchard analysis of data from binding experiments showed that the fibronectin receptor (FNR) was a single class receptor of the Arg-Gly-Asp receptor family, its binding to fibronectin being inhibited by Arg-Gly-Asp-Ser. The maximum number of binding sites (Bmax) and dissociation constant (Kd) of FNR of fibroblasts from 3 normal subjects were 1.82 ± 0.11 × 10⁴ sites/cell and 17.5 ± 0.8 × 10⁻⁹ mol/L, respectively. The Bmax values were markedly decreased in patients with hereditary connective tissue diseases (0.79–1.25 × 10⁴ sites/cell in 5 with Ehlers-Danlos syndromes of various types, 1.00 × 10⁵ sites/cell in one with osteogenesis imperfecta and 1.17 × 10⁴ sites/cell in one with Marfan syndrome). However, the Kd values of the FNR of these patients were within the normal range. These results suggest a pathophysiological role of the FNR in hereditary connective tissue diseases.

Key words: fibronectin; fibronectin receptor; hereditary connective tissue diseases

I. Introduction

The extracellular matrix (ECM) contains cell-adhesion molecules (CAMs) regulating cell-cell adhesion and substrate-adhesion molecules (SAMs) regulating the adhesion of cells to their substrate. These molecules maintain the structure of the tissue. Fibronectin, one of the SAMs, has various physiological functions, such as in transfer, differentiation and growth of the cells, and acts through the fibronectin receptor (FNR). FNR is a member of a receptor family generally called integrins that recognizes the Arg-Gly-Asp (RGD) sequence. It is a heterodimer consisting of two subunits, α and β, with a molecular weight of about 140 kd. FNR has been demonstrated on the surface of various cells, such as cultured human skin fibroblasts, rat hepatocytes, baby hamster kidney cells, and blood cells. FNR frequently increases in tissues in which morphogenetic proliferation and differentiation are active, and transforming growth factor-β accelerates its appearance and promotes accumulation and synthesis of the ECM. In contrast, the level of FNR is reduced in mature cells and in cells transformed by tumor viruses.
Hereditary connective tissue diseases (ECM-related diseases), such as Ehlers-Danlos syndrome, osteogenesis imperfecta and Marfan syndrome are thought to be disorders of collagen fibers and have been studied with respect to abnormalities of collagen molecules, but the pathogenesis of these diseases is still uncertain. In this study, we examined the FNR on cultured human skin fibroblasts of patients with hereditary connective tissue diseases and found a decrease in the number of binding sites of the FNR on fibroblasts from these patients.

II. Materials and Methods

Human skin fibroblasts. The human skin fibroblast cell lines CRL 1180, CRL 1215, CRL 1176, CRL 1409, CRL 1195 (Ehlers-Danlos syndrome), CRL 1248 (osteogenesis imperfecta), CRL 1271 (Marfan syndrome) and CRL 7288 (normal) were obtained from the American Type Culture Collection (Rockville, MD). Fibroblasts (N-1, N-2) were also obtained from skin biopsy specimens from normal volunteers.

Cell culture. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Laboratories). For use in binding assays, the cultured cells were suspended by treatment with 0.25% trypsin, washed 3 times with 0.01 mol/L phosphate-buffered saline solution, pH 7.0, containing 1% bovine serum albumin and 0.05% sodium azide (incubation buffer) and adjusted to 5–6 × 10⁵ cells/ml in the same buffer. All experiments were performed between passages 5 and 21 and 1 to 3 days after the cells reached confluence.

The viability of human skin fibroblasts in suspension was about 99%, as determined by the trypan blue dye exclusion test.

Purification and radioiodination of fibronectin. Fibronectin was purified and radioiodinated as reported previously. Briefly, human plasma fibronectin was extracted from fresh-frozen plasma and purified by the method of Engvall and Ruoslahti. The fibronectin was labeled with iodine -125 by the chloramine-T method. The specific activity of the ¹²⁵I-labeled fibronectin was about 2.0–2.5 μCi/μg (74.0–92.5 KBq/μg).

Binding assay. 200 μl samples of fibroblast suspension were incubated with 100 μl of incubation buffer or unlabeled fibronectin and 100 μl of ¹²⁵I-labeled fibronectin at the optimum temperature and for the optimum period, as described later. Then each incubation mixture was transferred to Eppendorf tubes (Brinkmann Instruments Inc., Westbury, N.Y.) containing 400 μl incubation buffer and 400 μl di-n-butyl phthalate, and centrifuged at 12,000 × g for 1 minute at room temperature. The radioactivity of the precipitate was measured in a gamma counter (model ARC 300; Aloka, Tokyo). All binding assays were carried out in duplicate. Specific binding was calculated by subtracting nonspecific binding from total binding, and the data were analyzed by Scatchard analysis.

III. Results

Time course of binding. As shown in Fig. 1, the binding of ¹²⁵I-labeled fibronectin to fibroblasts in suspension increased time-dependently at 4, 22 and 37°C. The specific binding reached a plateau after 30 minutes at 4°C and 22°C, and after 15 minutes at 37°C, and decreased after 60 minutes at all three temperatures. There was no significant difference between the maximum specific bindings or the subsequent slopes of decrease in specific binding with time at 22°C and 37°C. Nonspecific
Fig. 1. Time course of specific binding of $^{125}$I-labeled fibronectin to fibroblasts. Fibroblast-bound radioactivity in counts per minute is plotted as a function of incubation time in minutes. Fibroblasts, 3 μg/ml $^{125}$I-labeled fibronectin, and incubation buffer in the presence (nonspecific binding) or absence (total binding) of 1.5mg/ml unlabeled plasma fibronectin in a total volume of 400 μl were mixed in microcentrifuge tubes at 4°C (panel A), 22°C (panel B), and 37°C (panel C). After incubation for the times indicated, $^{125}$I-labeled fibronectin-bound fibroblasts were washed and their radioactivity was counted as described in the Methods.

binding was highest at 4°C. On the basis of these findings, all subsequent binding assays were carried out for 30 minutes at 37°C.

Binding of $^{125}$I-labeled fibronectin to cultured fibroblasts of normal subjects. To examine the saturation of $^{125}$I-labeled fibronectin binding, the fibroblasts were incubated at 37°C for 30 minutes with increasing amounts of $^{125}$I-labeled fibronectin ranging from 1.2 to 9.3 μg/ml, in the presence or absence of excess unlabeled fibronectin (100 times the amount of $^{125}$I-labeled fibronectin). The curves for specific binding of $^{125}$I-labeled fibronectin to fibroblasts of a control subject are shown in Fig. 2. Scatchard analysis of the data gave a straight line with a correlation coefficient (r) of −0.982, indicating the presence of a single class of binding sites. The mean value for the maximum number of binding sites ($B_{max}$) and the dissociation constant ($K_d$) for fibroblasts of 3 normal subjects were 1.82 ± 0.11 × 10⁵ sites/cell and 17.5 ± 0.8 × 10⁻⁹ mol/L, respectively, indicating that the FNR on the fibroblasts has high binding affinity (Table I).

Effect of Arg-Gly-Asp-Ser (RGDS) on binding of $^{125}$I-labeled fibronectin to fibroblasts. The fibroblasts were incubated with various concentrations of $^{125}$I-labeled fibronectin at 37°C for 30 minutes in the presence or absence of RGDS. The specific binding of $^{125}$I-labeled fibronectin to fibroblasts from a normal subject and the Scatchard analyses of the data are shown in 1a and 1b of Fig. 3, respectively. RGDS inhibited the specific binding of $^{125}$I-labeled fibronectin to fibroblasts and lowered the binding affinity, but did not affect the number of binding sites. Similar results were obtained with fibroblasts from a patient with Ehlers-Danlos syndrome Type I (IIa and IIb).
Fig. 2. Binding of $^{125}$I-labeled fibronectin to fibroblasts in suspension and Scatchard analysis of data on specific binding in a normal subject. $^{125}$I-labeled fibronectin bound to fibroblasts (A) is plotted as a function of the concentration of $^{125}$I-labeled fibronectin added (specific activity 2.0 μCi/μg). Fibroblasts were incubated at 37°C for 30 minutes with increasing concentrations of $^{125}$I-labeled fibronectin. Specific binding (●) was calculated by subtracting nonspecific binding (△) from total binding (●). The Scatchard plot (B) gives a straight line with a correlation coefficient (r) of −0.982. The slope and intercept indicate a $K_d$ value of 18.3 × 10⁻⁹mol/L and a $B_{max}$ value of 1.70 × 10⁶sites/cell.

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Fig. 3. Effect of RGDS on bindings of 125I-labeled fibronectin to fibroblasts in a normal subject (I) and a patient with Ehlers-Danlos syndrome type I (II). Fibroblasts (5 x 10^5 cells/ml) were incubated at 37°C for 30 minutes in the absence (●) or presence of various concentrations of RGDS (○, 300 μmol/L; △, 500 μmol/L). Specific binding of 125I-labeled fibronectin (specific activity 2.0 μCi/μg) to fibroblasts is plotted as a function of concentrations of 125I-labeled fibronectin (I a, II a). Scatchard analyses of these data are shown in panels I b and II b. In panel I b, the slope for A (●, r = -0.988) indicates a K_d value of 18.1 x 10^-9 mol/L and a B_max value of 1.76 x 10^5 sites/cell. The slope for B (○, r = -0.968) indicates a K_d value of 34.9 x 10^-9 mol/L and a B_max value of 1.76 x 10^5 sites/cell. The slope for C (△, r = -0.892) indicates a K_d value of 54.6 x 10^-9 mol/L and a B_max value of 1.76 x 10^5 sites/cell. In panel II b, the slope for A (●, r = -0.971) indicates a K_d value of 17.4 x 10^-9 mol/L and a B_max value of 0.99 x 10^5 sites/cell. The slope for B (○, r = -0.976) indicates a K_d value of 32.3 x 10^-9 mol/L and a B_max value of 0.99 x 10^5 sites/cell. The slope for C (△, r = -0.922) indicates a K_d value of 51.3 x 10^-9 mol/L and a B_max value of 0.99 x 10^5 sites/cell.

in Fig. 3). Thus the binding of 125I-labeled fibronectin to fibroblasts of the normal subject and the patient was inhibited competitively by RGDS.

**B_max and K_d values for FNR on fibroblasts of normal subjects and patients with hereditary connective tissue diseases.** Table I shows the B_max and K_d values of the FNR on fibroblasts from normal subjects and patients with hereditary connective tissue diseases. The B_max values in 5 patients with Ehlers-Danlos syndrome, one patient with osteogenesis imperfecta and one patient with Marfan syndrome were 0.79-1.25 x 10^6 sites/cell, 1.00 x 10^6 sites/cell and 1.17 x 10^6 sites/cell, respectively. These values were all lower than those of control subjects, but indicated no significant differences for different diseases or different types of Ehlers-Danlos syndrome. The K_d values for the FNR
on fibroblasts from all the patients were within the normal range.

IV. Discussion

We have been studying the pathophysiology of hereditary connective tissue diseases from the viewpoint of adhesion proteins and their receptors, and previously reported decreased $B_{\text{max}}$ values for the FNR on polymorphonuclear leukocytes of patients and some healthy members of families of patients with diseases including Ehlers-Danlos syndrome, osteogenesis imperfecta and Marfan syndrome. In this study, the $B_{\text{max}}$ and $K_d$ values for the FNR on normal fibroblasts were $1.82 \pm 0.11 \times 10^5$ sites/cell and $17.5 \pm 0.8 \times 10^{-9}$ mol/L, respectively. These data were comparable to those in the studies of McKeown-Longo and Mosher. We observed decreased $B_{\text{max}}$ values for the FNR on fibroblasts of patients with Ehlers-Danlos syndrome, osteogenesis imperfecta and Marfan syndrome compared with those of normal subjects, but without any change in $K_d$ values. The results of this study with cultured skin fibroblasts support our previous results with polymorphonuclear cells. Furthermore, we found that RGDS, as a partial competitive inhibitor, decreased the binding of $^{125}$I-labeled fibronectin to the fibroblasts of a normal subject and a patient with Ehlers-Danlos syndrome without any change in the number of binding sites. This finding implies that there is no difference between normal subjects and patients in the binding characteristic of FNR on the fibroblasts. The reason for the decrease in the number of FNR on the fibroblasts of patients with hereditary connective tissue diseases is unknown, but seems likely to be due to reduced FNR gene expression judging from our previous results for FNR on polymorphonuclear cells in members of families of patients with these diseases. Incidentally, the FNR on human fibroblasts is classified as an $\alpha_s \beta_1$-integrin. Our data on the inhibition by RGDS of $^{125}$I-labeled fibronectin binding to FNR suggested that these receptors may be related to $\alpha_s \beta_1$-integrin. However, further examination by flow cytometry or Western blot analysis is required to define the $\alpha_s$ and $\beta_1$ molecules on cells.

The relation between the decrease in the number of FNR in patients with hereditary connective tissue diseases and the pathogenesis of these diseases is of great interest. Hereditary connective tissue diseases have been studied mainly with respect to abnormalities of collagen, a protein specific to connective tissue, but recently studies on adhesion molecules in the connective tissue have also been reported. According to Edelman’s morphoregulator hypothesis, cells are controlled mechanochemically by adhesion molecules including CAMs and SAMs during the differentiation of embryonic tissues into mature tissues. CAMs and SAMs affect the expression of tissue-specific proteins and are also important in morphogenesis. On the basis of this hypothesis, disorders of differentiation of embryonic tissues into mature tissues may be caused not only by defects in tissue-specific proteins but also by functional defects in CAMs, SAMs and their receptors. Gillery et al. showed that the binding of fibroblasts to collagen fibers through fibronectin is necessary for the contraction process of the collagen fibers during morphogenesis of collagen lattices. Ruoslahti and Adams and Watt proposed that components of the ECM, such as fibronectin and collagen, play roles in transmitting information through integrin family receptors by means of their cell adhesion and consequently have effects on gene expression inducing functional or morphological changes in the cells and tissues. Werb et al. observed that the aggregation of FNR with an increase in its density is necessary for fibronectin to transmit information to the cells. These studies suggest that a decrease in the number of FNR on cells and consequent aberrant signal transmission would result in functional or morphological disorders of the cells and tissues.
To understand the pathogenesis of hereditary connective tissue diseases, further studies are required on abnormalities not only of collagen molecules, but also of the ECM including fibronectin and FN.

References