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Cell surface fibronectin of mouse peritoneal macrophages

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Fibronectin (FN) was detected on thioglycollate-induced mouse peritoneal macrophages by binding the ^{125}I-labeled $F(ab')_2$ fragment of rabbit anti-human plasma fibronectin. The cell surface fibronectin (sFN) was removed from the surface of the macrophage monolayer by limited trypsinization. After trypsinization, binding of ^{125}I-labeled plasma fibronectin (^{125}I-pFN) to the macrophage monolayer was increased, suggesting that the FN receptor covered with sFN was exposed by trypsinization without destroying the receptor activity. The amounts of saturation binding of ^{125}I-pFN to the macrophage monolayers before and after trypsinization were about 2.4 and 6.3 μg per 10^6 cells, respectively, indicating that the macrophage monolayer has the capacity of binding 6.3 μg FN per 10^6 cells, and the FN receptor equivalent to about 4 μg pFN per 10^6 cells is covered with sFN.

Fibronectin; Fibronectin receptor; Trypsinization; (Macrophage)

1. INTRODUCTION

Fibronectin, an adhesive glycoprotein found on the surface of many types of cells and in extracellular matrices and plasma, affects various cell behaviors such as cell adhesion, cell migration, and embryonic cell differentiation [1–3].

Plasma fibronectin is known to show biological activities on phagocytic cells of the reticuloendothelial system [4]. pFN promotes attachment of human monocytes to gelatin-coated substrate, and attachment of gelatin-coated particulate material to the cell monolayers [5]. It also promotes uptake of gelatin-coated particles by rat peritoneal macrophages [6,7]. Furthermore, substrate-bound pFN stimulates human monocytes [8] or cultured human monocytes [9,10] to promote opsonin (IgG or C3)-dependent phagocytosis of erythrocytes. Binding of the particle- or substrate-bound pFN to the FN receptor on the phagocytes has been supposed to cause these biological effects [5–10].

On the other hand, little attention has been paid to FN present on the surface of phagocytes. It was demonstrated that macrophages express FN on the cell surface [11,12], while monocytes only express slightly after in vitro long-term culture [13]. Although the cell surface FN (sFN) of macrophages appears to play a regulatory role in a macrophage function [12], no information on the quantity of sFN of macrophages is available. To investigate the role played by sFN as well as by extracellular FN in macrophage function, quantitative analysis of sFN and the capacity of the cells to bind FN is important.

In this study, we have determined the amount of sFN and the FN-binding capacity of thioglycollate-induced mouse peritoneal macrophages.

2. MATERIALS AND METHODS

2.1. *Materials*

Trypsin (bovine pancreas, 189 U/mg) and pepsin (porcine, 3600 U/mg) were purchased from Worthington Diagnostics, Freehold, New Jersey. Bovine serum albumin (BSA, crystallized and lyophilized) and soybean trypsin inhibitor (type I-S)

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Abbreviations: FN, fibronectin; sFN, cell surface fibronectin; pFN, plasma fibronectin

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were obtained from Sigma (St. Louis, MO). BSA (fraction V) was the product of Miles Laboratories (Elkhart, IN). Gelatin-Sepharose 4B was prepared by coupling gelatin (swine skin type I, Sigma) with Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) activated by BrCN [14].

2.2. *Preparation of pFN, antibody to FN and its F(ab')$_2$ fragment*

pFN was purified from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B columns according to Engvall and Ruoslahti [15] with minor modifications. Plasma was passed through a plain Sepharose 4B column before the affinity chromatography to remove any material which might adsorb to Sepharose gel. pFN bound to the gelatin-Sepharose 4B column was eluted with 4 M urea in 0.05 M Tris buffer (pH 7.5) after elution of loosely bound material with 1 M urea in the same buffer. The eluted pFN fraction was dialyzed extensively against 10 mM phosphate buffer/0.15 M NaCl, pH 7.2 (phosphate-buffered saline), containing 0.02% NaN_3, stored at 4°C, and used within 3 weeks. SDS-polyacrylamide gel electrophoresis [16] of the protein obtained indicated that virtually no proteins other than intact pFN (M_r = 220000) were present in the preparations. pFN concentration was determined spectrophotometrically using $E^{1\%}_{1cm}$ (at 280 nm) = 12.8 [7].

Anti-FN antibody was raised in a Japanese White rabbit using human pFN as an antigen. The antiserum obtained was heat-treated at 56°C for 30 min to inactivate complements, and passed through a column of Sepharose 4B coupled with dialyzed pFN-free human plasma to remove rabbit antibodies reactive with human plasma proteins other than pFN. Normal serum obtained from the preimmune rabbit was similarly processed. The resultant antiserum gave a monospecific response in the Ouchterlony gel diffusion assay against whole human plasma. The antiserum as well as normal serum was further absorbed with human erythrocytes at 4°C for 2 h. IgG was then isolated from the sera by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography. F(ab')$_2$ framents of anti-FN IgG and normal IgG (anti-FN F(ab')$_2$ and normal F(ab')$_2$, respectively) were prepared by pepsin digestion and gel filtration on a Sephadex G-200 column. Concentration of F(ab')$_2$ fragments was determined according to the method of Lowry et al. [17] using BSA as standard.

2.3. *Macrophages*

Macrophages were obtained from the peritoneal cavity of 7–10-week-old ddY male mice 4 days after an intraperitoneal injection of 2–3 ml of 3% thioglycollate medium (Difco Laboratories, Detroit, MI). Peritoneal exudate cells obtained were washed twice with Hanks' balanced salt solution by centrifugation (80 × *g*, 10 min) at 4°C. The cells were then resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 20 mM Hepes (pH 7.2), 50 U/ml penicillin and 50 μg/ml streptomycin (RPMI-Hepes medium) at 1 × 10^6 cells per ml, and 3 ml of the cell suspension was plated on a 60 mm tissue culture dish (Corning Glass Works, Corning, NY). After incubation at 37°C for 1 h, nonadherent cells were removed by washing three times with phosphate buffered saline and the macrophage monolayers were subjected to the binding assay. More than 90% of the cells were macrophages as defined by phagocytosis of latex beads (0.8 μm, Difco) and sheep erythrocytes sensitized with a subhemagglutinating dose of rabbit anti-sheep erythrocyte IgG. To determine the cell number of the macrophage monolayer, 1 ml of 2% BSA (fraction V) in RPMI-Hepes medium was added to replicate dishes, and the monolayer was scraped from the dish with a rubber policeman. The cells were dispersed by gentle pipetting, and cell number was counted under phase-contrast microscope. The cell number was usually 1.5–2 × 10^6 per dish.

Trypsinization of the macrophage monolayers was performed by the incubation with 1–100 μg/ml of trypsin in RPMI-Hepes medium (3 ml) at 4°C for 30 min. After removal of the solution, soybean trypsin inhibitor (50 μg/ml, 2 ml) in phosphate-buffered saline was added to quench the proteolysis, and the monolayers were washed twice with phosphate-buffered saline. Almost all cells remained attached to the dish under the conditions employed.

2.4. *Binding experiment*

Radiolabeling of pFN, anti-FN F(ab')$_2$, and normal F(ab')$_2$ was carried out by the chloramine-T method of Hunter [18] using Na^{125}I (375 mCi/ml, carrier-free, New England Nuclear, Boston, MA). All the glass- and plasticwares and Sephadex G-25 columns for separation of the labeled pFN from free radioactivity were coated with 2% BSA prior to use.

Intact and trypsinized macrophage monolayers were precoated with 2 ml of 2% BSA (fraction V) in RPMI-Hepes medium at 4°C for 30 min to prevent nonspecific adsorption of ^{125}I-labeled proteins. The precoated macrophage monolayers were washed once with phosphate-buffered saline, and incubated with labeled proteins (spec. act. 10000–20000 cpm/μg) in RPMI-Hepes medium containing 0.5% BSA (fraction V) at 22°C for 1 h with occasional shaking. After the incubation, the radioactive medium was removed, and the macrophage monolayers were washed 5 times with precooled phosphate-buffered saline. The washed monolayers were then solubilized in 2 ml of 4 N NaOH, and the radioactivity of the solutions was counted. Nonspecific adsorption of the radioactivity on the dish was similarly measured using the dish without macrophages, and this value was subtracted from the data as a blank. The nonspecific adsorption was usually less than 10% of the radioactivity bound to the monolayer dish. The data shown are the mean values of duplicate assays.

3. RESULTS

The presence of FN on macrophages was verified by binding the ^{125}I-labeled F(ab')$_2$ fragment of rabbit anti-pFN antibody (^{125}I-anti-FN F(ab')$_2$) to the monolayer of thioglycollate-induced mouse peritoneal macrophages. As shown in fig.1A, ^{125}I-anti-FN F(ab')$_2$ at 4 μg/ml bound to the macrophages in proportions to the number of cells, while the same concentration of the ^{125}I-labeled F(ab')$_2$ fragment of normal rabbit IgG (^{125}I-normal F(ab')$_2$) bound slightly. The binding of ^{125}I-anti-FN F(ab')$_2$ to the cells increased depending on the concentration of the antibody, and reached a plateau at 50 μg/ml antibody

(fig.1B). This specific and saturable binding of ^{125}I-anti-FN F(ab')$_2$ to the macrophages indicates that FN is present on the surface of the macrophages.

We, then, attempted to estimate the amount of sFN as a difference in the saturation levels of pFN-binding to the macrophage monolayers before and after removal of sFN from the monolayer surface. To remove sFN, the macrophage monolayer was subjected to mild trypsinization at 4°C for 30 min. The amount of sFN on the monolayer, as detected by the binding of ^{125}I-anti-FN F(ab')$_2$, was decreased on treatment with 1–100 μg/ml of trypsin (fig.2). About 85% of sFN was removed at the trypsin concentration as low as 10 μg/ml. The effect of trypsin treatment on the ability of macrophages to bind FN was then examined by the binding of ^{125}I-pFN to the trypsinized monolayer. In contrast to the binding of anti-FN-antibody, the binding of a fixed concentration of ^{125}I-pFN increased with increasing trypsin concentration (fig.2), which suggests that the FN receptor covered with sFN was exposed by the removal of sFN.

To make sure that the receptor activity is restored on removal of bound FN by trypsin, the following experiment was designed. The macrophage monolayer surface was treated with unlabeled pFN, trypsinized, and then ^{125}I-pFN binding was measured. As shown in table 1, ^{125}I-pFN (100 μg/ml) bound only slightly to the macrophage monolayer which had been treated with a large amount of unlabeled pFN (1900 μg/ml). However, when the unlabeled pFN-treated monolayer was briefly trypsinized, subsequent binding of ^{125}I-pFN was restored to 67% of the ^{125}I-pFN binding to the monolayer not pretreated with unlabeled pFN. The result in-

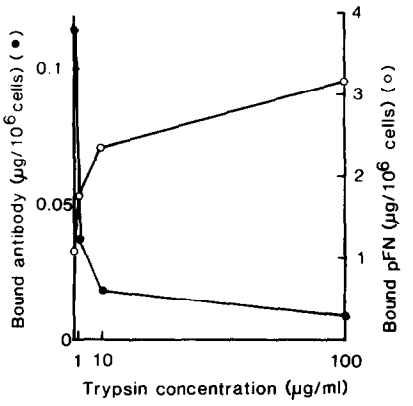

Fig.2. Binding of ^{125}I-anti-FN F(ab')$_2$ and ^{125}I-pFN to trypsinized monolayers of mouse peritoneal macrophages. Macrophage monolayers (2.0 × 10^6 cells per dish) were treated with 1, 10, and 100 μg/ml of trypsin in RPMI-Hepes medium at 4°C for 30 min. After quenching the proteolysis with trypsin inhibitor, the monolayers were incubated with ^{125}I-anti-FN F(ab')$_2$ (50 μg/ml) (•) or ^{125}I-pFN (400 μg/ml) (○) at 22°C for 1 h.

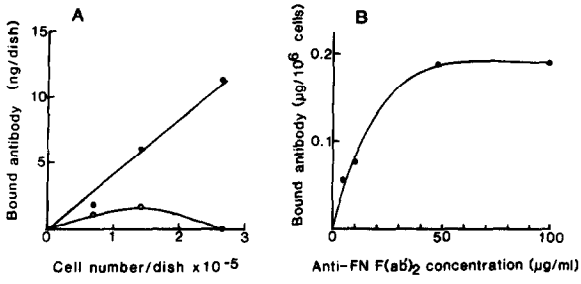

Fig.1. The presence of sFN on mouse peritoneal macrophages. (A) Binding of ^{125}I-anti-FN F(ab')$_2$ (•) or ^{125}I-normal F(ab')$_2$ (○) to macrophage monolayers of varying cell density. Macrophage monolayers were incubated with ^{125}I-anti-FN F(ab')$_2$ or ^{125}I-normal F(ab')$_2$ at 22°C for 1 h. The concentration used for ^{125}I-anti-FN F(ab')$_2$ and ^{125}I-normal F(ab')$_2$ was 4 μg/ml. (B) Concentration-dependent binding of ^{125}I-anti-FN F(ab')$_2$ to macrophage monolayers. Macrophage monolayers (2.5 × 10^5 cells per dish) were incubated with increasing concentrations of ^{125}I-anti-FN F(ab')$_2$ at 22°C for 1 h.

Table 1

Effect of trypsinization of the macrophage monolayers pretreated with unlabeled pFN on the binding of ^{125}I-pFN

Pretreatment		Bound ^{125}I-pFN (μg per 10^6 cells)
First	Second	
pFN (1900 μg/ml)	None	0.20
pFN (1900 μg/ml)	Trypsin (10 μg/ml)	0.54 (67%)[a]
None	Trypsin (10 μg/ml)	0.82 (100%)[a]

[a] Percentage of ^{125}I-pFN binding

Macrophage monolayers (1.9 × 10^6 cells per dish) were preincubated with or without unlabeled pFN (1900 μg/ml) in RPMI-Hepes medium at 22°C for 1 h. After removal of unbound pFN, the monolayers were treated with or without trypsin (10 μg/ml) as described in the legend to fig.2. The trypsinized or untreated monolayers were then incubated with ^{125}I-pFN (100 μg/ml) at 22°C for 1 h, and the binding of the radioactivity was measured

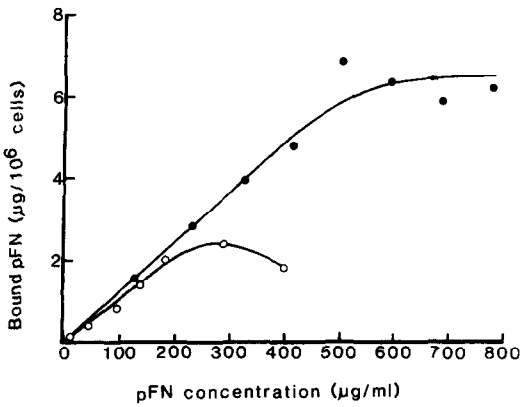

Fig.3. Binding of ^{125}I-pFN to monolayers of mouse peritoneal macrophages before and after trypsinization. Macrophage monolayers (1.5×10^6 cells per dish) treated with (•) or without (○) trypsin were incubated with increasing concentrations of ^{125}I-pFN at 22°C for 1 h. Trypsinization of macrophage monolayers was performed as described in the legend to fig.2 using 10 μg/ml of trypsin.

dicates that the FN receptor blocked with pFN can be exposed by trypsinization of the bound pFN without loss of the receptor activity.

Concentration-dependent binding of ^{125}I-pFN to the macrophage monolayers was measured before and after brief trypsinization. As shown in fig.3, the binding of ^{125}I-pFN to the macrophage monolayer before trypsinization increased with the increase in the concentration of ^{125}I-pFN up to 300 μg/ml. At 400 μg/ml, its binding rather decreased. The maximal binding of ^{125}I-pFN to the monolayer was 2.4 μg per 10^6 cells. After trypsinization of the monolayer, the binding of ^{125}I-pFN reached a plateau at the ^{125}I-pFN concentration above 500 μg/ml (fig.3). The maximal binding to the monolayer was about 6.3 μg per 10^6 cells. This value represents the total capacity of the macrophage monolayer to bind FN, and the net increase in the saturation level of the ^{125}I-pFN binding (approx. 4 μg pFN per 10^6 cells) is likely to represent the amount of sFN removed by trypsin from the monolayer surface, which approximately corresponds to the total sFN present on the monolayer surface.

4. DISCUSSION

Recent progress in the research on FN has revealed that FN interacts with cells through its cell-binding domain containing the tripeptide Arg-Gly-Asp (RGD) [19,20]. Cell surface FN receptors have also been characterized and identified as an 140 kDa glycoprotein complex using cell surface-directed monoclonal antibodies which inhibit cell adhesion to FN-coated substrata [21–25] and the synthetic peptides containing the RGD sequence [26]. Furthermore, it has been shown that many adhesive proteins contain the RGD sequence, and that a wide variety of cells including fibroblasts, myoblasts, macrophages, platelets, and leukocytes express RGD-directed receptors with considerable homology (reviewed in [27]).

In spite of the rapid progress in the field of cell adhesion, little information is available on sFN of macrophages, and we have investigated its quantity. In the present study, a key technque which enabled us to estimate the quantity of sFN on the macrophage monolayer was the limited trypsinization of the monolayer surface. It efficiently removed sFN from the cell surface, but did not appear to impair FN receptor activity. However, there are conflicting reports as to the trypsin sensitivity of FN receptors on cells. FN receptors on human monocytes [5] and rat peritoneal macrophages [7] have been reported to be trypsin sensitive, but those on fibroblastic cells have been reported to be trypsin resistant [28,29]. These differences may be due not only to the difference in the cell type but to the differences in the conditions of the enzyme treatment.

Data on the binding of ^{125}I-pFN to the trypsinized macrophages also made it possible to estimate the total capacity of the cells to bind FN. The amount of saturation binding of ^{125}I-pFN (6.3 μg per 10^6 cells) approximates to the total capacity of the cells. The number of FN receptors calculated from this value is approx. 5×10^6 per cell assuming 1 : 1 binding of pFN and FN receptor. A Scatchard plot of the binding data, however, did not give a linear line which would allow us to estimate binding constant and the receptor number. According to Hörmann and Jelinic [30], there are approx. 10^6 FN receptor sites per cell with weak affinity and approx. 10^4 sites per cell with high affinity on trypsinized guinea pig peritoneal macrophages in suspension. However, no saturation of FN binding was attained in their experiments. The number of FN receptors in fibroblastic cell suspensions was reported to be $5 \times$

10^5 per cell. The variation of cells and the difference in the conditions of the binding experiments may be reflected in these results.

It is likely that a considerably large fraction of the FN receptors on the surface of thioglycollate-induced mouse peritoneal macrophages is covered with sFN since the amount of sFN appears to be as much as 60% of the maximal binding capacity of the macrophage monolayer. sFN may be their own product since macrophages and differentiated monocytes produce FN [13,31,32] although the possibility that the sFN is derived from ascites fluid or plasma cannot be excluded.

sFN on the macrophages has been suggested to be involved in some of their functions. Remold et al. [12] reported the involvement of an FN-like component of the peritoneal macrophage surface in the response of macrophages to a migration inhibitory factor. We have previously reported that mouse peritoneal macrophages recognize oxidatively damaged erythrocytes [33], and recently found that sFN on the macrophages plays a promoting role in the recognition of the oxidatively damaged erythrocytes (manuscript in preparation). These observations indicate that effects of sFN on macrophages have to be taken into consideration in studying the effects of extracellular FN on macrophage functions.

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