Volume 295, number 1,2,3, 135–140 FEBS 10528 December 1991

Augmentation of macrophage recognition of oxidatively damaged erythrocytes by substratum-bound fibronectin and macrophage surface fibronectin

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Received 21 October 1991

Thioglycollate-induced mouse peritoneal macrophages plated on a coverglass bind oxidized mouse erythrocytes in the absence of serum. Macrophages plated on a coverglass pre-coated with fibronectin (FN) were more active in binding of the oxidized erythrocytes. This effect of FN-coated coverglass was due to specific binding of an RGD-containing sequence of FN to FN-receptors on the macrophage, since GRGDSP hexapeptide in solution inhibited this effect, and GRGDSP-coated coverglass exhibited the same effect as FN-coated coverglass. Removal of FN originally present on the macrophage surface by trypsinization, prior to attachment to the coverglass, resulted in diminution of their ability of recognition of the oxidized erythrocytes, but the diminished ability was restored when the trypsinized macrophages were plated on a FN-coated coverglass, indicating that the cell surface FN is required for the macrophage recognition. Attachment to the coverglass was necessary for the cell surface FN to be effective. These results suggest that solid-phase FN, produced either by deposition of soluble FN to substratum or attachment of macrophage surface FN to substratum, activates the macrophages and augments their ability to recognize the oxidized erythrocytes.

Macrophage recognition; Oxidized erythrocyte; Fibronectin; Cell surface fibronectin; Macrophage activation

1. INTRODUCTION

Recognition and phagocytic removal of effete or senescent erythrocytes from the circulation is an important function of macrophages to maintain homeostasis [1,2]. It is believed that macrophages also have an ability to recognize damaged cells [1]. Oxidative damage of cells involved in lipid and protein oxidation is one of the possible means of cellular damage that can take place in vivo [3–5], and we have previously demonstrated that mouse macrophages recognize the oxidatively damaged mouse erythrocytes in vitro [6]. The macrophages recognized the oxidized erythrocytes in the absence of serum opsonins, and thus, they directly recognized the membrane changes of the erythrocytes caused by oxidation [6].

Considering the in vivo situation, the ability of macrophages to recognize these cells could be affected by various surrounding factors. Tissue macrophages interact with connective tissue or other cells on which various adhesive proteins exist [1]. Fibronectin (FN) is the most abundant adhesive glycoprotein in tissue [7–9], and solid-phase FN, like the substratum-bound form of FN, has been shown to promote the complement receptor-mediated phagocytosis of erythrocytes coated with C3b or C3bi, the cleavage products of the third components of complement (C3), by cultured human monocytes [10,11]. In addition, macrophages themselves express FN on cell surfaces [12–14], and this cell surface FN may affect the activity of macrophages to recognize the oxidized erythrocytes.

We have investigated the effect of FN on the opsonin-independent macrophage recognition of oxidized erythrocytes, and show here that the recognition is augmented by substratum-bound FN, and that cell surface FN also plays an augmenting role in the recognition when macrophages are attached to substratum.

Abbreviations: FN, fibronectin; DPBS, Dulbecco's phosphate-buffered saline.

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2. MATERIALS AND METHODS

2.1. *Materials*

ADP monopotassium salt was purchased from Oriental Yeast Co., Tokyo. Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) peptides were the products of Iwaki Glassware Co., Tokyo. Trypsin (bovine pancreas, 189 U/mg) was obtained from Worthington Diagnostics, Freehold, NJ. Arg-Gly-Asp (RGD) peptide, bovine serum albumin (BSA, γ-globulin free) and soybean trypsin inhibitor (type I-S) were from Sigma Chemical Co., St. Louis, MO. Hanks' balanced salt solution and HEPES were from Nissui Pharmaceutical Co., Tokyo, and Dojindo Laboratories, Kumamoto, respectively. RPMI 1640 medium and fetal bovine serum were from Gibco Laboratories, Grand Island, NY. Plasma FN was purified from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B columns according to Engvall and Ruoslahti [15] with minor modifications as described previously [14]. Purified plasma FN was stored in 10 mM phosphate buffer/0.15 M NaCl (pH 7.2) containing 0.02% NaN_3, at 4°C, and used within a month. Plasma FN con-

centration was determined spectrophotometrically using $E^{1\%}_{1cm}$ (at 280 nm) = 12.8 [16].

2.2. *Coating of coverglass with FN and synthetic peptides*

Round coverglass (18-mm diameter) was loaded with a solution (0.2 ml) of FN (1 mg/ml), or synthetic peptides GRGDSP or GRGESP (50 μg/ml) in 10 mM phosphate buffer/0.15 M NaCl (pH 7.2). After 2–15 h, the coverglass was rinsed several times with Dulbecco's phosphate-buffered saline (DPBS) and used immediately.

2.3. *Macrophages*

Macrophages were obtained from the peritoneal cavity of 7–12-week-old ddY male mice 4 days after intraperitoneal injection of 2–3 ml of 3% thioglycollate medium (Difco Laboratories, Detroit, MI). The 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 supplemented with 20 mM HEPES (pH 7.2), 50 U/ml penicillin and 50 μg/ml streptomycin (RPMI–HEPES medium) at 2 × 10^6 cells/ml, and 0.2 ml of the cell suspension was loaded onto a round coverglass (18-mm diameter). After incubation at 37°C for 1 h, nonadherent cells were removed by washing three times with Ca^{2+}- and Mg^{2+}-free DPBS, and adherent cell monolayers were subjected to the assay for erythrocyte recognition, or further incubated at 37°C for 20 h in RPMI–HEPES medium supplemented with 5% heat-inactivated fetal bovine serum. The macrophage monolayer was rinsed with DPBS before use. More than 80% of the cells were macrophages as defined by phagocytosis of latex particles (0.8 μm, Difco) and sheep erythrocytes sensitized with a subhemagglutinating dose of rabbit anti-sheep erythrocyte IgG.

Trypsinization of the macrophage monolayers was performed by incubation of the cell monolayers with 100 μg/ml of trypsin in RPMI–HEPES medium (0.2 ml for each coverglass) at 4°C for 30 min. After removal of the solution, soybean trypsin inhibitor (0.5 mg/ml, 0.2 ml for each coverglass) in RPMI–HEPES medium was added to quench the proteolysis, and the monolayers were washed twice with Ca^{2+}-, Mg^{2+}-free DPBS. Under the conditions employed, there were no loss of attached cells and no morphological alterations of the cells, and it was confirmed that most of the FN was removed from the cell surface [14].

Trypsinization of macrophages in suspension was carried out similarly. A suspension of thioglycollate-induced mouse peritoneal exudate cells in RPMI–HEPES medium (2 × 10^6 cells/ml) was incubated with trypsin (100 μg/ml) at 4°C for 30 min, centrifuged (80 × *g*, 10 min) at 4°C, and the supernatant was removed. A solution of soybean trypsin inhibitor (0.5 mg/ml) in RPMI–HEPES medium was added to the cell pellet, and the cell suspension was allowed to stand for 10 min at room temperature to ensure the quenching of the proteolysis. The cells were washed twice with Ca^{2+}-, Mg^{2+}-free DPBS, resuspended in PPMI–HEPES medium (2 × 10^6 cells/ml), and loaded onto a coverglass to prepare macrophage monolayers as described above. The cell density of the monolayers was not significantly different from that of intact macrophages.

2.4. *Target erythrocytes*

Erythrocytes were obtained from the mouse individuals from which macrophages were obtained. Prior to harvesting the peritoneal exudate cells, mouse blood was collected by cardiac puncture using acid-citrate-dextrose preservative solution, and erythrocytes were isolated and subjected to oxidation as described below. For the assay using 20-h-cultured macrophages, erythrocytes were freshly prepared from other mouse individuals on the day of their use.

Oxidation of erythrocytes was carried out using an iron catalyst, ADP/Fe^{3+} chelates (ADP/Fe^{3+}) as described [6]. An erythrocyte suspension (20% hematocrit) in DPBS was mixed with an equal volume of a solution of 3.4/0.2 mM ADP/Fe^{3+} (a mixture of 3.4 mM ADP and 0.2 mM $FeCl_3$ in 0.15 M NaCl), and incubated at 37°C for 1 h with gentle shaking. The cells were collected by centrifugation (650 × *g*, 5 min), washed three times with DPBS, and resuspended in RPMI–HEPES medium at a concentration of 2%. Control cells were prepared in a similar manner and incubated at 37°C for 1 h in the same buffer.

2.5. *Assay for macrophage adhesion and phagocytosis*

A 2% cell suspension of erythrocytes (200 μl) in RPMI–HEPES medium was added to the macrophage monolayer. After incubation at 37°C for 2 h, nonadhering erythrocytes were removed by gentle washing with DPBS, and the number of the macrophages binding one or more erythrocytes on their surface was scored for the random fields of the coverglass under phase contrast microscopy. The surface-bound erythrocytes were then lysed by treatment with 0.83% NH_4Cl/17 mM Tris-HCl (pH 7.6) for 5 min. The cells on the coverglass were fixed with methanol, dried and stained with 3% Giemsa's solution (E. Merck, Darmstadt) for 20 min. The number of the macrophages ingesting one or more erythrocytes was scored microscopically as described below. At least 300 macrophages were examined for binding and ingestion of erythrocytes, and the percentage of macrophages that bound one or more erythrocytes (% adhesion) and the percentage of macrophages that ingested one or more erythrocytes (% phagocytosis) were determined. Results were expressed as the mean of the triplicate experiments.

3. RESULTS

Mouse erythrocytes were oxidized with an iron catalyst ADP/Fe^{3+} (1.7/0.1 mM) [6], and the oxidized cell suspension was loaded onto the macrophage monolayers prepared by plating the thioglycollate-induced mouse peritoneal exudate cell suspensions at 37°C for 1 h onto a coverglass precoated or not with FN. After incubation at 37°C for 2 h, the proportions of the macrophages binding and ingesting the erythrocytes (% adhesion and % phagocytosis, respectively) were determined.

Only a small fraction of macrophages plated onto uncoated coverglass bound untreated erythrocytes (intact erythrocytes) and erythrocytes incubated in DPBS at 37°C for 1 h (incubated erythrocytes) that were prepared as a control for the oxidized erythrocytes, but a considerable number of the macrophages bound the oxidized erythrocytes (Table I, 35.6% of the total macrophages.) This demonstrates that macrophages recognize the oxidized erythrocytes. The macrophages plated onto FN-coated coverglass bound the oxidized erythrocytes better (52%) than those plated onto uncoated ones, while only a slight increase in binding was observed for intact erythrocytes. This indicates that the coverglass-bound FN augmented the ability of macrophages to recognize the oxidized erythrocytes. Moreover, the number of the macrophages that bound the incubated erythrocytes significantly increased (41.2%). It is likely that coverglass-bound FN enabled the macrophages to recognize the erythrocytes which may have undergone minute damage during the 1 h incubation at 37°C in the buffer.

The recognition-promoting effect of the coverglass-bound FN was reflected not only by the increase in the proportion of the macrophages binding the erythrocytes but also by the increase in the number of erythrocytes attached to each macrophage. While 3–5 oxidized ery-

Table I

Adhesion and phagocytosis of oxidized erythrocytes by macrophages plated on FN-coated coverglass for 1 h

Macrophage		% Adhesion			% Phagocytosis		
Pretreatment in suspension	Attached coverglass	Intact erythrocyte[a]	Incubated erythrocyte[b]	Oxidized erythrocyte[c]	Intact erythrocyte[a]	Incubated erythrocyte[b]	Oxidized erythrocyte[c]
None	Plain	8.4 ± 1.3	14.0 ± 3.3	35.6 ± 3.5	3.5 ± 0.7	5.0 ± 2.9	6.5 ± 1.9
None	FN-coated	13.7 ± 1.2	41.2 ± 2.3	52.0 ± 2.9	6.9 ± 2.5	5.8 ± 1.5	5.9 ± 1.9
Trypsinized	Plain	10.0 ± 2.1	16.7 ± 2.4	14.7 ± 2.9	6.2 ± 1.9	6.2 ± 1.2	5.7 ± 2.5
Trypsinized	FN-coated	12.1 ± 1.2	48.6 ± 8.2	47.6 ± 4.1	6.0 ± 0.5	4.6 ± 1.9	6.4 ± 1.9

Thioglycollate-induced mouse peritoneal cell suspensions, with or without brief trypsinization, were plated on coverglass or FN-coated coverglass at 37°C for 1 h, and the resultant macrophage monolayers were subjected to assay for adhesion and phagocytosis of oxidized mouse erythrocytes as described in Materials and Methods.

[a] Untreated mouse erythrocytes.
[b] Mouse erythrocytes incubated at 37°C for 1 h.
[c] Mouse erythrocytes treated with 1.7/0.1 mM ADP/Fe^{3+} at 37°C for 1 h.

throcytes were usually observed on the macrophages plated on plain coverglass, many erythrocyte rosettes were observed around the macrophages plated on FN-coated coverglass (data not shown).

Macrophages express FN on their surface [12–14], and considerable amounts of FN were detected on thioglycollate-induced mouse peritoneal macrophages [14]. We then investigated whether or not the macrophage FN plays a role in the recognition of the oxidized erythrocytes. Since FN of the macrophages is removable by limited trypsinization of the cells [14], effect of FN removal from the macrophage surface on the recognition of the oxidized erythrocytes was examined. A suspension of briefly trypsinized peritoneal exudate cells were plated onto coverglass and assayed for recognition of the oxidized erythrocytes. As shown in Table I, the ability of the macrophages to recognize the oxidized erythrocytes were remarkably diminished on trypsinization (14.7%). However, their ability was restored when the trypsinized macrophages were plated onto FN-coated coverglass (47.6%). This strongly suggests that FN on macrophages is required for the recognition of the oxidized erythrocytes, and that even though FN is removed by trypsinization, the substrate-bound FN can compensate the loss. The trypsinized macrophages plated onto FN-coated coverglass were also capable of recognizing the incubated erythrocytes (48.6%) as effectively as untrypsinized macrophages (41.2%).

Phagocytosis of intact, incubated, oxidized erythrocytes was scarcely observed for the macrophages plated on either types of coverglass (Table I).

To test the effect of long-term contact of macrophages with FN, macrophage monolayers were cultured for an additional 20 h following the 1 h-plating, and subjected to the assay for the recognition and phagocytosis of the oxidized erythrocytes. Results similar to those in Table I were obtained for the macrophage recognition (Table II). In addition, a small increase in phagocytosis of the oxidized erythrocytes was observed for the macrophages plated onto FN-coated coverglass, suggesting that the prolonged macrophage culture on FN-coated coverglass potentiated the avidity of macrophages to ingest the bound erythrocytes.

The effect of FN of the macrophage surface on the recognition of the oxidized erythrocytes shown above may be due to FN molecules exposed to the fluid phase at the upper side of the macrophage monolayer, or to

Table II

Adhesion and phagocytosis of oxidized erythrocytes by macrophages plated on FN-coated coverglass for 1 h followed by culture for 20 h

Macrophage		% Adhesion			% Phagocytosis
Pretreatment in suspension	Attached coverglass	Intact erythrocyte[a]	Incubated erythrocyte[b]	Oxidized erythrocyte[c]	Oxidized erythrocyte[a]
None	Plain	11.4 ± 1.3	8.9 ± 0.4	18.8 ± 3.2	6.1 ± 0.5
None	FN-coated	14.5 ± 1.3	19.8 ± 4.8	48.5 ± 8.2	11.3 ± 3.4
Trypsinized	Plain	10.2 ± 1.7	8.7 ± 4.2	10.0 ± 1.4	6.3 ± 0.9
Trypsinized	FN-coated	12.9 ± 2.2	22.3 ± 5.6	47.2 ± 2.5	9.4 ± 2.3

Experiments were performed similarly to those in Table I except that the macrophage monolayers were cultured for an additional 20 h before use.

[a] [c] See the footnotes in Table I.

Table III

Effect of trypsinization of macrophages before and after attachment to substratum on their ability to recognize oxidized erythrocytes

Experiment	Treatment of macrophage			% Adhesion	
	Pretreatment in suspension	Attached to coverglass	Treatment of monolayer	Intact erythrocyte[d]	Oxidized erythrocyte[e]
A	None	Plain	None[a]	7.6 ± 2.2	19.8 ± 0.4
B	None	Plain	Trypsinized[b]	9.3 ± 3.4	21.2 ± 2.4
C	Trypsinized	Plain	None[a]	7.6 ± 1.0	9.5 ± 2.2
D	Trypsinized	Plain	Addition of FN[c]	10.3 ± 2.6	9.7 ± 1.4
E	Trypsinized	FN-coated	None[a]	9.8 ± 1.5	32.1 ± 1.9

Macrophage monolayers prepared as described in the legend to Table I were further treated with trypsin (Expt. B) or FN (Expt. D) or medium alone (Expts. A, C, E), and subjected to assay for adhesion of oxidized erythrocytes.

[a] Incubated in RPMI-HEPES medium at 20°C for 1 h.
[b] Incubated with trypsin (100 μg/ml) at 4°C for 30 min followed by the addition of soybean trypsin inhibitor (500 μg/ml) as described in Materials and Methods.
[c] Incubated with FN in RPMI-HEPES medium (600 μg/ml, 0.2 ml for each coverglass) at 20°C for 1 h, followed by rinsing with DPBS.
[d] Untreated mouse erythrocytes.
[e] Mouse erythrocytes treated with 1.7/0.1 mM of ADP/Fe^{3+} at 37°C for 1 h.

FN molecules attached to the substratum at the lower side of the monolayer. To determine which was correct, the effect of removal of FN from the monolayer surface was examined (Table III).

The macrophage monolayer attached to uncoated coverglass was briefly trypsinized (Expt. B) under the conditions that caused removal of most of the FN from the upper surface of the monolayer [14]. The trypsinized monolayer macrophages were as active in recognizing the oxidized erythrocytes (21.2%) as before trypsinization (Expt. A, 19.8%), while the macrophages trypsinized in suspension before attachment were much less active (Expt. C, 9.5%). The results exclude the former possibility. Consistent with this result, addition of soluble FN, at a concentration that would saturate the FN-receptors on the macrophage upper surface (600 μg/ml) [14], to the trypsinized macrophages spread on uncoated coverglass (Expt. D) was unable to augment the macrophage recognition (9.7%), indicating that ligation of the FN-receptors exposed to the fluid phase was not effective. Thus, ligation of the FN receptors from the solid-phase (Expt. E) was necessary for the macrophage activation. It is likely that the cell surface FN on the side of macrophage attachment to the substratum is 'fixed' on the substratum, and exhibits the activity as 'solid-phase FN'.

It is known that interaction of FN with cell surface FN-receptors is mediated by specific binding of Arg-Gly-Asp (RGD)-containing sequences in the cell-binding domain of FN molecule to FN-receptor [17,18]. To confirm involvement of RGD-containing sequences in the macrophage-activating effect of solid-phase FN, the effect of a synthetic hexapeptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), a sequence contained in the RGD region of FN molecule [17], on macrophage activation was tested.

During attachment of macrophages to FN-coated coverglass, GRGDSP or an inactive control hexapeptide GRGESP, in which Glu is substituted for Asp, was added to prevent interaction of the solid-phase FN with the cell surface FN-receptors. After washing the monolayer, the ability of the macrophages to recognize the oxidized erythrocytes was examined. GRGDSP inhibited the augmentation of the macrophage recognition of the oxidized erythrocytes by coverglass-bound FN, whereas GRGESP did not (Table IV). Similar results were obtained in the activation of trypsinized macrophages by the solid-phase FN (Table IV). These data demonstrate that the GRGDSP sequence of the solid-

Table IV

GRGDSP hexapeptide inhibits augmentation of macrophage recognition of oxidized erythrocytes by coverglass-bound FN

Treatment of macrophage			% Adhesion
Pretreatment in suspension	Attached to coverglass	Inhibitor	
None	Plain	None	40.2 ± 3.3[a]
None	FN-coated	None	73.7 ± 7.9
None	FN-coated	GRGESP	72.6 ± 0.6
None	FN-coated	GRGDSP	46.7 ± 10.7
Trypsinized	Plain	None	23.9 ± 1.2
Trypsinized	FN-coated	None	61.5 ± 5.3
Trypsinized	FN-coated	GRGESP	56.4 ± 10.1
Trypsinized	FN-coated	GRGDSP	29.5 ± 3.3

Thioglycollate-induced mouse peritoneal exudate cells, or those treated with trypsin, were loaded on FN-coated coverglass in the absence or presence of GRGDSP (1 mM) or GRGESP (1 mM) hexapeptides, and incubated at 37°C for 1 h. The resultant macrophage monolayers were subjected to assay for adhesion of oxidized erythrocytes.

[a] Adhesion to intact erythrocytes was 2.9 ± 0.8%.

Table V

Coverglass-bound GRGDSP hexapeptide augments macrophage recognition of oxidized erythrocytes

Attached to coverglass	% Adhesion
Plain	17.6 ± 2.6[a]
GRGESP-coated	16.2 ± 1.7
GRGDSP-coated	26.5 ± 7.3
FN-coated	28.6 ± 5.1

Thioglycollate-induced mouse peritoneal exudate cells were loaded onto coverglass pre-coated for 15 h with or without GRGDSP (50 μg/ml), GRGESP (50 μg/ml) or FN (1 mg/ml). After incubation at 37°C for 1 h, the resultant macrophage monolayers were subjected to assay for adhesion of oxidized erythrocytes.

[a] Adhesion to intact erythrocytes was 3.9 ± 3.3%.

phase FN is involved in the augmentation of the macrophage activity. Consistently, the GRGDSP-coated coverglass potentiated the macrophage recognition of the oxidized erythrocytes, while the GRGESP-coated coverglass did not (Table V), indicating that specific binding with the GRGDSP sequence was sufficient for the macrophages to be activated.

4. DISCUSSION

The present results indicated that solid-phase FN augments the ability of macrophages to recognize and ingest oxidized erythrocytes. It is conceivable that solid-phase FN in vitro, such as FN in tissue or in extracellular matrix, functions similarly to remove oxidized cells.

It is known that FN is present on the surface of macrophages [12–14], but little is known about its effect on cell function. Interestingly, cell surface FN of the macrophages also exhibited an augmenting effect on macrophage recognition of the oxidized erythrocytes. Cell surface FN attached to substratum appears to be involved in the activation of macrophages for the following reasons: (i) removal of FN from the entire surface of the macrophages by trypsin before attachment to substratum abrogated the ability of the macrophages to recognize the oxidized erythrocytes, (ii) ligation of the FN-receptors on the monolayer of the trypsinized macrophages from fluid phase had no effect, and (iii) removal of FN by trypsinization only from the surface of the macrophage monolayer did not reduce the macrophage activity of oxidized erythrocyte recognition (see Table III). This suggests that attachment of macrophages onto substratum is necessary for the cell surface FN to be effective in the activation of the macrophages. It is likely that, at the side of macrophage attachment to substratum, FN is fixed on the substratum surface in a manner similar to how it coated the coverglass, and exhibits the activity as solid-phase FN.

The macrophage-activating effect of cell surface FN appeared to be lower than that of the substratum-bound FN since attachment of macrophages having cell surface FN to FN-coated coverglass resulted in further augmentation of the macrophage recognition of the oxidized erythrocytes. The density of cell surface FN attached to substratum may be lower than that of FN coated on it, or there may be some qualitative difference between cell surface FN and plasma FN coated on substratum.

The specific interaction of FN and FN-receptor of macrophages is obviously involved in the macrophage activation by solid-phase FN since synthetic GRGDSP peptide inhibited the activation. Activation of macrophages by coverglass-bound GRGDSP indicates that this specific interaction is sufficient to cause the macrophage activation. This result is consistent with the previous report of Wright et al. [19] for the activation of C3-dependent activation of cultured human monocytes. The inhibitory effect of free GRGDSP indicates that the soluble form of this peptide competes with solid-phase FN for FN-receptor, but is ineffective in the macrophage activation. Consistently, FN was effective only in substratum-bound form since ligation of the FN-receptors by the addition of soluble FN to the trypsinized macrophages did not restore the macrophage activity that had been lost by trypsinization, while attachment of trypsinized macrophages to the FN-coated coverglass did.

Why is solid-phase FN effective, and the soluble form is not? The most probable explanation may be that FN molecules fixed on solid surfaces can cross-link the FN receptors extensively as a result of multipoint attachment to the cell surface FN-receptors. It is known that multipoint attachment or cross-linkage formation of functional cell-surface receptors is effective in triggering the signal for the cell activation by ligands in various cell systems including lymphocyte activation [20–22], mast cell activation [23,24], and fat cell stimulation [25,26].

The biochemical mechanism involved in the activation of macrophages by FN has not been elucidated, and further study is required to answer this question.

Acknowledgements: This work was supported in part by The Mochida Memorial Foundation For Medical and Pharmaceutical Research.

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