Involvement of calcium signaling in the fibronectin-stimulated macrophage recognition of oxidatively damaged erythrocytes

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Abstract

Macrophages recognize oxidatively damaged autologous erythrocytes, and cell surface fibronectin of macrophages enhances the recognition (Beppu et al., FEBS Lett. 295 (1991) 135–140). In the present study, mechanisms of enhanced macrophage recognition of oxidatively damaged erythrocytes by fibronectin were investigated. Monolayers of thioglycollate-induced mouse peritoneal macrophages with cell surface fibronectin recognized autologous erythrocytes oxidized with an iron catalyst ADP/Fe³⁺. The macrophage recognition of the oxidized erythrocytes was inhibited partially by pretreatment of the macrophage monolayers with a Ca²⁺ channel blocker (diltiazem), calmodulin inhibitors (W-7, trifluoperazine, chlorpromazine and dibucaine), an inhibitor of myosin light chain kinase (ML-9), a microfilament formation inhibitor (cytochalasin B), phospholipase A₂ inhibitors (4-bromophenacyl bromide, mepacrine) and cyclooxygenase inhibitors (indomethacin and aspirin). Monolayers of macrophages depleted of fibronectin by trypsinization lost the ability of recognizing oxidized erythrocytes, but acquired the ability when stimulated with a fibronectin-coated coverslip. The recognition of fibronectin-stimulated trypsinized macrophages was also inhibited by the above inhibitors. On treatment with Ca ionophore A23187, trypsinized macrophages acquired the ability to recognize oxidized erythrocytes. The recognition of Ca ionophore-stimulated trypsinized macrophages was inhibited by the above inhibitors except the Ca²⁺ channel blocker. These results indicate that the Ca²⁺ signaling including Ca²⁺ influx, calmodulin activation and myosin light chain phosphorylation are involved in the fibronectin stimulation of the recognition of macrophages for oxidized erythrocytes. Involvement of microfilament formation and arachidonate cascade in the fibronectin stimulation was also suggested. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Oxidative damage of cells involved in lipid and protein oxidation is one of the possible means of cellular damages that can take place in vivo [1–3]. Binding of autologous anti-band 3 IgG to the cell surface sugar chains of band 3 protein aggregates generated during aging or oxidative damage of human erythrocytes has been suggested to be involved in the removal of the cells from the circulation [4]. In addition, recognition of oxidatively damaged erythrocytes in the circulation by macrophages is an important event for phagocytic removal of effete or
senescent erythrocytes from the circulation [5,6]. We have found that mouse macrophages selectively recognize oxidatively damaged erythrocytes in the absence of serum [7–9].

Macrophages have the receptors, members of the integrin family [10–12], for fibronectin, an adhesive glycoprotein on the surface of many types of cells and in extracellular matrices and plasma. Fibronectin is detected on thioglycollate-induced mouse peritoneal macrophages [13], and it has been shown that cell surface fibronectin plays an important role in the opsonin-independent macrophage recognition of oxidatively damaged erythrocytes [14]. Macrophages plated on a coverslip precoated with fibronectin are found to be more active in recognizing the oxidized erythrocytes, and those depleted of cell surface fibronectin by trypsinization lose their recognizing ability. The effect of fibronectin is shown to be due to specific binding of Arg-Gly-Asp (RGD)-containing tripeptide sequence of fibronectin to the fibronectin receptors on the macrophage surface.

In the present study, in order to elucidate the role of fibronectin in the thioglycollate-induced macrophage recognition of oxidatively damaged erythrocytes, we investigated the effect of Ca$^{2+}$ signaling on the fibronectin-stimulated recognition activity of the mouse macrophages for the oxidized mouse erythrocytes. We will report here that fibronectin-stimulated Ca$^{2+}$ signaling was involved in the fibronectin-stimulated macrophage recognition of the oxidatively damaged erythrocytes.

2. Materials and methods

2.1. Materials

ADP monopotassium salt was purchased from Oriental Yeast (Tokyo). Trypsin (bovine pancreas, 223 units/mg) was obtained from Worthington Biochemical (Freehold, NJ). Hanks’ balanced salt solution (HBSS) and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) were obtained from Nissui Pharmaceutical (Tokyo) and Dojindo (Kumamoto), respectively. RPMI 1640 medium and penicillin-streptomycin solution were obtained from Gibco (Grand Island, NY). Soybean trypsin inhibitor (type I-S), calmodulin inhibitor trifluoperazine dihydrochloride and Ca ionophore A23187 were purchased from Sigma (St. Louis, MO). Ca$^{2+}$ channel blocker diltiazem hydrochloride, calmodulin inhibitor chlorpromazine hydrochloride, cyclooxygenase inhibitors indomethacin and aspirin, phospholipase A$_2$ inhibitors 4-bromophenacyl bromide (BPP) and quinacrine dihydrochloride (mepacrine), and calmodulin inhibitor dibucaine hydrochloride were obtained from Wako Pure Chemical Industries (Osaka). Calmodulin inhibitor W-7, protein kinase C inhibitor H-7, myosin light chain kinase inhibitor ML-9 and microfilament formation inhibitor cytochalasin B were obtained from Seikagaku (Tokyo).

Plasma fibronectin was purified from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B columns according to the method of Engvall and Ruoslahti [15] with minor modifications as described [10]. Purified plasma fibronectin was stored in 10 mM phosphate buffer/0.15 M sodium chloride (pH 7.2) containing 0.02% sodium azide, at 4°C, and used within a month. Plasma fibronectin concentration was determined spectrophotometrically using $E (1\%, 1\ cm) = 12.8$ [16].

2.2. Coating coverslip with fibronectin

A round glass coverslip (18 mm diameter) that had been treated with 5% nitric acid at 100°C for 45 min was loaded with 0.2 ml of a solution of fibronectin (67 µg/ml) in 10 mM phosphate buffer (pH 7.2) containing 0.02% sodium azide, at 4°C, and used within a month. Plasma fibronectin concentration was determined spectrophotometrically using $E (1\%, 1\ cm) = 12.8$ [16].

2.3. Macrophages

Macrophages were obtained from the peritoneal cavity of 7–10-week-old male ddY mice 4 days after an intraperitoneal injection of 2–3 ml of 3% thioglycollate medium (Difco, Detroit, MI). The peritoneal exudate cells obtained were washed twice with HBSS by centrifugation (80 × g, 10 min) at 4°C. The cells were resuspended at appropriate concentrations in RPMI 1640 medium supplemented with 20 mM HEPES (pH 7.2), 50 units/ml penicillin and 50 µg/ml streptomycin (RPMI-HEPES medium) at 1–1.5 × 10$^6$ cells/ml.
2.4. Trypsinization of macrophages

A macrophage suspension in RPMI-HEPES medium (1–1.5×10^6 cells/ml) was incubated with trypsin (100 µg/ml) at 25°C for 30 min, centrifuged (80×g, 10 min) at 4°C, and the supernatant was removed. A solution of soybean trypsin inhibitor (100 µg/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 (DPBS(−)), and resuspended in RPMI-HEPES medium (2×10^6 cells/ml).

2.5. Macrophage monolayer

The cell suspension (0.2 ml) of macrophages or trypsinized macrophages was loaded onto a round glass coverslip (18 mm diameter) plain or one that had been coated by fibronectin. After incubation at 37°C for 1 h, non-adherent cells were removed by washing three times with DPBS(−), and adherent monolayers were subjected to the assay for erythrocyte recognition. 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 [14]. The cell density of the monolayers was not significantly different from that of intact macrophages.

2.6. Erythrocytes

Erythrocytes were obtained from the same mouse individuals from which macrophages were obtained. Mouse blood was collected by cardiac puncture using an acid-citrate-dextrose preservative solution, and erythrocytes were isolated. Oxidized erythrocytes were obtained according to the method previously described [14]. An erythrocyte suspension (20% hematocrit) in DPBS was mixed with an equal volume of a solution of 3.4 mM ADP/0.2 mM FeCl_3 in 0.15 M sodium chloride, 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.

2.7. Assay for the macrophage recognition

For the investigation of the effect of the inhibitors on the recognition of the fibronectin-stimulated macrophages, the macrophage monolayer on a plain coverslip or the trypsinized macrophage monolayer on a fibronectin-precoated coverslip was incubated with a 200 µl solution of each of the inhibitors in RPMI-HEPES medium at the indicated concentration at 37°C for 1 h. The monolayer was washed three times with DPBS(−). For the investigation of the effect of the inhibitors on the recognition of the Ca ionophore-stimulated macrophages, the trypsinized macrophage monolayer on a plain coverslip was incubated with a 200 µl solution of 0.1 µM Ca ionophore A23187 in RPMI-HEPES medium at 37°C for 2 h. The monolayer was washed three times with DPBS(−). The monolayer was immediately treated with a 200 µl solution of each of the inhibitors at 37°C for 1 h followed by washing with DPBS(−).

A 2% cell suspension of erythrocytes (200 µl) in RPMI-HEPES medium was added to the macrophage monolayer. After incubation at 37°C for 1 h, non-adhering erythrocytes were removed by gentle washing with DPBS(−). The monolayer was fixed with 1.25% glutaraldehyde. The number of the macrophages binding one or more erythrocytes on their surface was scored for the random fields of the coverslip under a phase contrast microscope. At least 200 macrophages were examined for binding of erythrocytes, and the percentage of macrophages that bound one or more erythrocytes (% adhesion) was determined. The results were expressed as the mean of the triplicate experiments.

3. Results

Thioglycollate-induced mouse peritoneal macrophage monolayer recognized autologous mouse erythrocytes oxidized with an iron catalyst ADP/Fe^{3+} at 1.7 mM/0.1 mM and 37°C for 1 h more effectively than unoxidized erythrocytes. The percentages of the number of macrophages that recognized oxidized

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Fig. 1. Inhibition of adhesion of oxidized erythrocytes to a monolayer of macrophages by Ca$^{2+}$ signal inhibitors, Ca$^{2+}$ channel blocker (A), calmodulin inhibitor (B) and myosin light chain kinase inhibitor (C). A thioglycollate-induced mouse peritoneal macrophage suspension was plated on a coverslip at 37°C for 1 h, and the resultant macrophage monolayer was treated with 200 μl of the solution of each of the inhibitors at 37°C for 1 h followed by the assay for adhesion of oxidized mouse erythrocytes at 37°C for 1 h. The percentages of the number of macrophages that adhered to oxidized erythrocytes were 30-40%, and those that adhered to unoxidized erythrocytes were 10-20%. The macrophage adhesion ratio of oxidized erythrocytes in the absence of the inhibitors was made 100. The level of macrophage adhesion for unoxidized erythrocytes was not affected in the presence of the inhibitors, and the dotted line indicates the level of macrophage adhesion of unoxidized erythrocytes.

Fig. 2. Inhibition of adhesion of oxidized erythrocytes to a monolayer of macrophages by arachidonate cascade inhibitors, phospholipase A$_2$ inhibitors (A) and cyclooxygenase inhibitors (B). Details are the same as in the legend of Fig. 1.
erythrocytes were 30–40%, whereas those recognized unoxidized erythrocytes were 10–20%. The macrophage recognition was lowered by depletion of the cell surface fibronectin by trypsinization, and the ability of recognition was recovered by pretreatment of the trypsinized macrophage monolayer with fibronectin precoated on the coverslip. The percentages of the number of fibronectin-stimulated trypsinized macrophages that recognized oxidized erythrocytes were 30–40%, whereas those of the unstimulated trypsinized macrophages that recognized oxidized erythrocytes were 10–20%. These observations were consistent with those in the previous studies [14].

Effect of pretreatment of the intact macrophage monolayers with the inhibitors of Ca$^{2+}$ signaling on the recognition of oxidized erythrocytes was examined (Fig. 1). The macrophage recognition of oxidized erythrocytes was decreased by pretreatment of the macrophage monolayers with a Ca$^{2+}$ channel blocker, diltiazem, in a dose-dependent manner (Fig. 1A). By contrast, the recognition of unoxidized erythrocytes was not affected in the presence of the inhibitors, and the dotted line indicates the level of fibronectin-unstimulated trypsinized macrophages.

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**Fig. 3.** Inhibition of adhesion of oxidized erythrocytes to a monolayer of macrophages by protein kinase C inhibitor (A) and microfilament formation inhibitor (B). Details are the same as in the legend of Fig. 1.

**Fig. 4.** Inhibition of adhesion of oxidized erythrocytes to a monolayer of fibronectin-stimulated trypsinized macrophages by Ca$^{2+}$ signal inhibitors, Ca$^{2+}$ channel blocker (A), calmodulin inhibitor (B) and myosin light chain kinase inhibitor (C). A trypsinized thioglycollate-induced mouse peritoneal macrophage suspension was plated on a fibronectin coated-coverslip at 37°C for 1 h, and the resultant macrophage monolayer was treated with 200 μl of the solution of each of the inhibitors at 37°C for 1 h followed by the assay for adhesion of oxidized mouse erythrocytes at 37°C for 1 h. The percentages of the number of fibronectin-stimulated trypsinized macrophages that adhered to oxidized erythrocytes were 30–40%, and those of fibronectin-unstimulated trypsinized macrophages were 10–20%. The macrophage adhesion ratio of oxidized erythrocytes in the absence of the inhibitors was made 100. The level of fibronectin-unstimulated trypsinized macrophage adhesion for unoxidized erythrocytes was not affected in the presence of the inhibitors, and the dotted line indicates the level of fibronectin-unstimulated trypsinized macrophages.
erythrocytes in the lower level was unchanged by the diltiazem pretreatment. The macrophage recognition of oxidized erythrocytes was decreased by the pretreatment with 100 μM diltiazem to about 50% of the recognition obtained without the pretreatment. The depressed level of the macrophage recognition of oxidized erythrocytes with 100 μM diltiazem was similar to the level of recognition of unoxidized erythrocytes (Fig. 1A, dotted line). The macrophage recognition of oxidized erythrocytes was decreased by pretreatment with calmodulin inhibitors, W-7, trifluoperazine, chlorpromazine and dibucaine, in dose-dependent manners, whereas that of unoxidized erythrocytes was not affected by the pretreatment (Fig. 1B). The recognition of oxidized erythrocytes was decreased by pretreatment with a myosin light chain kinase inhibitor, ML-9 (Fig. 1C), in a dose-dependent manner, whereas that of unoxidized erythrocytes was not affected by the pretreatment.

Effect of pretreatment of the intact macrophage monolayers with the inhibitors of arachidonate cascade on the recognition of oxidized erythrocytes was examined (Fig. 2). The macrophage recognition of oxidized erythrocytes was decreased by pretreatment of the macrophage monolayers with phospholipase

Fig. 5. Inhibition of adhesion of oxidized erythrocytes to a monolayer of fibronectin-stimulated trypsinized macrophages by arachidonate cascade inhibitors, phospholipase A2 inhibitors (A) and cyclooxygenase inhibitors (B). Details are the same as in the legend of Fig. 4.

Fig. 6. Inhibition of adhesion of oxidized erythrocytes to a monolayer of fibronectin-stimulated trypsinized macrophages by protein kinase C inhibitor (A) and microfilament formation inhibitor (B). Details are the same as in the legend of Fig. 4.
A2 inhibitors, BPB and mepacrine (Fig. 2A), and with cyclooxygenase inhibitors, indomethacin and aspirin (Fig. 2B), in dose-dependent manners, whereas that of unoxidized erythrocytes was not affected by the pretreatments.

The recognition of oxidized erythrocytes was not decreased by a protein kinase C inhibitor, H-7, even at the concentration at 100 µM (Fig. 3A), and the recognition was decreased by pretreatment with a microfilament formation inhibitor, cytochalasin B, in a dose-dependent manner (Fig. 3B).

Fig. 7. Enhancement of adhesion of oxidized erythrocytes to a monolayer of trypsinized macrophages by Ca ionophore A23187. An intact (○) or a trypsinized (●) thioglycollate-induced mouse peritoneal macrophage suspension was plated on a coverslip at 37°C for 1 h, and the resultant macrophage monolayer was treated with 200 µl of 0.1 µM Ca ionophore A23187 solution at 37°C for the indicated period followed by the assay for adhesion of oxidized mouse erythrocytes at 37°C for 1 h.

A2 inhibitors, BPB and mepacrine (Fig. 2A), and with cyclooxygenase inhibitors, indomethacin and aspirin (Fig. 2B), in dose-dependent manners, whereas that of unoxidized erythrocytes was not affected by the pretreatments.

The recognition of oxidized erythrocytes was not decreased by a protein kinase C inhibitor, H-7, even at the concentration at 100 µM (Fig. 3A), and the recognition was decreased by pretreatment with a microfilament formation inhibitor, cytochalasin B, in a dose-dependent manner (Fig. 3B).

Fig. 8. Inhibition of adhesion of oxidized erythrocytes to a monolayer of Ca ionophore A23187-stimulated trypsinized macrophages by Ca2+ signal inhibitors, Ca2+ channel blocker (A), calmodulin inhibitor (B) and myosin light chain kinase inhibitor (C). A trypsinized thioglycollate-induced mouse peritoneal macrophage suspension was plated on a coverslip at 37°C for 1 h, and the resultant macrophage monolayer was treated with 200 µl of 0.1 µM Ca ionophore A23187 solution at 37°C for 2 h followed by treatment with the solution of each of the inhibitors at 37°C for 1 h. The assay for adhesion of oxidized mouse erythrocytes was conducted at 37°C for 1 h. The percentages of the number of A23187-stimulated trypsinized macrophages that adhered to oxidized erythrocytes were 30-40%, and those of unstimulated trypsinized macrophages were 10-20%. The macrophage adhesion ratio of oxidized erythrocytes in the absence of the inhibitors was made 100. The level of Ca ionophore-unstimulated trypsinized macrophage adhesion of unoxidized erythrocytes was not affected in the presence of the inhibitors, and the dotted line indicates the level of Ca ionophore-unstimulated trypsinized macrophages.
The recognition of oxidized erythrocytes by fibronectin-stimulated trypsinized macrophages was also inhibited by the Ca\(^{2+}\) channel blocker (Fig. 4A), the calmodulin inhibitors (Fig. 4B) and the myosin light chain kinase inhibitor (Fig. 4C). The levels of the recognition reached those of the recognition by fibronectin-unstimulated trypsinized macrophages. The recognition by fibronectin-stimulated trypsinized macrophages was slightly inhibited by the phospholipase A\(_2\) inhibitors (Fig. 5A) and also inhibited by the cyclooxygenase inhibitors (Fig. 5B). The recognition was not inhibited by the protein kinase C inhibitor (Fig. 6A), and the recognition was decreased by pretreatment with the microfilament formation inhibitor in a dose-dependent manner (Fig. 6B). The results were similar to those obtained from intact macrophages (Figs. 1–3).

It was found that macrophages depleted of fibronectin by trypsinization acquired the ability of recognition of oxidized erythrocytes on treatment with Ca ionophore A23187. Fig. 7 shows that incubation of the monolayer of trypsinized macrophages with 0.1 μM Ca ionophore A23187 at 37°C for more than 1 h increased the recognition by 2-fold, whereas the incubation of the monolayer of intact macrophages with Ca ionophore did not increase the recognition. Increased Ca ionophore-stimulated recognition was lost 5 h after washing out the ionophore, indicating that the augmentation was reversible (data not shown). Hence, Ca\(^{2+}\) influx into the macrophage cells was important for the recognition of fibronectin-stimulated macrophages for oxidized erythrocytes.

Inhibitory effects of several inhibitors on the Ca ionophore-stimulated trypsinized macrophage recognition of oxidized erythrocytes were examined. The recognition was not inhibited by the Ca\(^{2+}\) channel blocker (Fig. 8A), but the recognition was inhibited by each of the calmodulin inhibitors (Fig. 8B) and the myosin light chain kinase inhibitor (Fig. 8C). The level of recognition reached to the level of the Ca ionophore-unstimulated trypsinized macrophages. While this recognition was not inhibited by the phospholipase A\(_2\) inhibitors (Fig. 9A), it was inhibited by the cyclooxygenase inhibitors (Fig. 9B).

4. Discussion

It has been shown that thioglycollate-induced mouse peritoneal macrophages plated on a coverslip precoated with fibronectin are more active in binding
oxidized erythrocytes [14]. This effect of the fibronectin-precoated coverslip is found to be due to specific binding of a RGD-containing sequence of fibronectin to the fibronectin receptors on the macrophages. Removal of fibronectin originally present on the macrophage surface by trypsinization, prior to attachment to the unprecoated coverslip, results in diminution of their ability of recognition of oxidized erythrocytes, but the diminished ability is restored when trypsinized macrophages are plated on a fibronectin-precoated coverslip, indicating that the cell surface fibronectin is effective for the macrophage recognition.

In the present study, we found that Ca$^{2+}$ signaling was involved in the enhancement of the fibronectin-stimulated recognition for oxidized erythrocytes. This was clearly demonstrated by the following lines of evidence. The recognition of oxidized erythrocytes by the monolayers of intact macrophages attached to the plain coverslip or trypsinized macrophages attached to the fibronectin-precoated coverslip was inhibited by the pretreatment of the monolayers with Ca$^{2+}$ signal inhibitors, the Ca$^{2+}$ channel blocker, calmodulin inhibitors and the myosin light chain kinase inhibitor (Figs. 1 and 4). The recognition of oxidized erythrocytes by the monolayers of trypsinized macrophages attached to the plain coverslip was enhanced by Ca ionophore A23187 (Fig. 7), and the Ca ionophore-stimulated recognition was inhibited by calmodulin inhibitors and the myosin light chain kinase inhibitor (Fig. 8). The Ca$^{2+}$ channel, calmodulin and myosin light chain kinase inhibitors appeared to inhibit the effect of fibronectin selectively because these inhibitors did not inhibit the recognition of the ability of the fibronectin-unstimulated or Ca ionophore-unstimulated trypsinized macrophages (Figs. 4 and 8).

There are several lines of evidence showing that calcium ions participate in the signal transduction into the cells through the receptors on the cell surface. Integrins including fibronectin receptors have been shown to transmit biochemical signals into the cells upon binding at the extracellular sites with the adhesion molecules [17,18]. Elevation of intracellular free Ca$^{2+}$ upon binding with integrin ligands including fibronectin has been reported for endothelial cells [19,20], epithelial cells [21] and platelets [22]. Inhibition by the Ca$^{2+}$ channel blocker of the fibronectin-stimulated macrophage recognition of oxidized erythrocytes observed here (Figs. 1 and 3) indicates that the binding of fibronectin to the fibronectin receptors on the surface of the macrophages elevated the Ca$^{2+}$ influx. This was consistent with the observation that Ca$^{2+}$ influx elevated by Ca ionophore activated the recognition of fibronectin-unstimulated trypsinized macrophages (Fig. 7). Intracellular free Ca$^{2+}$ may be elevated in the fibronectin-stimulated macrophages because the calmodulin inhibitors inhibit the recognition of oxidized erythrocytes.

It is likely that arachidonate cascade, phospholipase A$_2$ and cyclooxygenase, was also involved in the fibronectin-stimulated recognition because their inhibitors prevented the fibronectin-stimulated recognition (Figs. 2 and 4). While the Ca ionophore-stimulated recognition was not inhibited by the phospholipase A$_2$ inhibitors, it was inhibited by the cyclooxygenase inhibitors (Fig. 9). This result indicates that activation of arachidonate cascade by fibronectin was not always through Ca$^{2+}$ signaling. Activation of the arachidonate cascade by fibronectin may be an independent pathway from Ca$^{2+}$ signaling. Formation of microfilament from actin may also be involved in the fibronectin-stimulated recognition of oxidized erythrocytes (Figs. 3 and 6).

In the recognition of mouse thioglycollate-induced macrophages for mouse erythrocytes oxidized with an iron catalyst, aggregated poly-$N$-acetyllactosaminyl saccharide chains of band 3 protein are suggested to be involved [9]. The receptor on the macrophage surface for oxidized erythrocytes may be the lactoferrin receptor, which has been found on the surface of human macrophages [23–25]. Fibronectin- or Ca ionophore-unstimulated trypsinized macrophage recognition of oxidized erythrocytes observed here (Figs. 4, 5, 8 and 9) may be due to the lactoferrin receptor on the macrophage surface. However, Ca$^{2+}$ signaling stimulated by fibronectin or Ca ionophore, observed here, may not be due to the activation of the above lactoferrin receptor, because the inhibitors did not inhibit the recognition ability of the fibronectin- or Ca ionophore-unstimulated trypsinized macrophages. It is likely that in the fibronectin- and the Ca ionophore-stimulated macrophages, one or more other specific receptors for oxidized erythrocytes are created.
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