Functional and possible physical association of scavenger receptor with cytoplasmic tyrosine kinase Lyn in monocytic THP-1-derived macrophages

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Abstract Acetyl LDL (modified low-density lipoprotein), which is thought to be taken up through scavenger receptor A (SR-A), rapidly induced the appearance of phosphotyrosine proteins in monocytic THP-1-derived macrophages in vitro. The two alternative forms of Lyn (p^{53} and p^{56}) were found to be tyrosine-phosphorylated within 30 s after the stimulation with acetyl LDL. The catalytic activity of Lyn measured by an in vitro kinase assay had also increased in acetyl LDL-stimulated THP-1-derived macrophages. Furthermore, Lyn could be co-immunoprecipitated with SR-A from the cell lysate. These observations suggest a functional and possible physical association of SR-A with Lyn in THP-1-derived macrophages, and also imply a possible involvement of Lyn in SR-A signal transduction.

Key words: THP-1-derived macrophage; Lyn; Scavenger receptor; Acetyl low-density lipoprotein

1. Introduction

The scavenger receptor (SR-A) is mainly expressed on macrophages and is thought to be the molecule which binds and uptakes modified LDL (low-density lipoprotein) through the endocytotic process which results in foam cell formation [1]. This interaction between SR-A and modified LDL also induces the production of some cytokines such as TNF [2] and interleukin-1 (IL-1) [3]. Furthermore, it has been reported that modified LDL induces the cell growth of murine peritoneal exudate macrophages [4] and exhibits a mitogenic activity on human monocyte-derived macrophages [5]. Recently, it was demonstrated that the binding of lipopolysaccharide (LPS) to SR-A on macrophages induces the release of various cytokines and nitric oxide (NO), which suggests that SR-A might play an important role in host defense mechanism of macrophages [6,7]. While the expression of SR-A in other cell types is relatively limited, we previously reported that SR-A is expressed on renal cell carcinoma (RCC) cells and the acetyl LDL stimulation promotes lipid accumulation in RCC [8]. Furthermore, we demonstrated that acetyl LDL exhibits a growth-stimulating effect on RCC and promotes the production of IL-6, which functions as an in vitro autocrine growth factor in RCC [9]. These observations indicate that the result of the binding of modified LDL to SR-A is not only the deposition of cholesteryl ester through the endocytotic process, but also the transmission of signals which result in the production of some cytokines or growth-promotion. However, it is still not clear how the signals being sent through SR-A are intracellulary transmitted and induce a variety of biological responses. For this reason, we investigated in the present study the protein tyrosine phosphorylation induced by acetyl LDL engagement on SR-A. We were able to demonstrate that a cytoplasmic tyrosine kinase Lyn is rapidly activated after acetyl LDL stimulation of PMA-treated THP-1 cells (THP-1-derived macrophages). Furthermore, Lyn was found to be co-immunoprecipitated with SR-A. These observations demonstrate a functional and possible physical association between SR-A and Lyn.

2. Materials and methods

2.1. Cells

THP-1 cells (Japanese Cancer Research Resources Bank) were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) (Flow Laboratories Inc., USA). For PMA priming, THP-1 cells were treated with 0.2 μM phorbol 12-myristate-13-acetate (PMA) (Sigma) for 24 h.

2.2. Antibodies

Anti-SR-A antibody: The polyclonal rabbit anti-human SR-A antibody was generated by immunization with a synthetic peptide (MEQWDHFHNQQEDTDSC) carrying the sequence of the N terminal residues (amino acids 1–17) [10] conjugated with keyhole limpet hemocyanin (KLH). Prior to use, the antibody was purified from the serum by protein-A Sepharose chromatography [11]. Mouse anti-Lyn monoclonal antibody [12] was provided by Tadashi Yamamoto (Institute of Medical Science, University of Tokyo, Japan). Mouse anti-phosphotyrosine antibody PY20 was obtained from ICN Immunobiologicals.

2.3. Immunoblotting

PMA-treated THP-1 cells were lysed with a lysis buffer (1% Triton-X100, 150 mM NaCl, 1 mM EGTA, 0.2 mM orthovanadate, 10 mM Hepes, pH 7.4, 1 mM methyl sulfonyl fluoride) for 30 min on ice. Postnuclear supernatants were collected after centrifugation at $15000 \times g$ for 30 min at 4°C. The supernatant containing 50 μg of cellular proteins was mixed with the same volume of ×2 sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% Bromophenol Blue) and slightly (boiled for 30 s in the presence of 2-mercaptoethanol [2-ME]) or normally reduced (boiled for 3 min in

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the presence of 2-ME), separated by SDS-PAGE by means of a 10% gel and electrotransferred onto Nitroplus 2000 (Micron Separations, Inc.). The membranes were blocked with TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 10% skim milk for 30 min at room temperature (RT). Primary antibody (1 μg/ml in TBS–milk) incubation was performed for 1 h at RT. The secondary antibody incubation used alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG (1:1000 dilution in TBS–milk) for 2 h at RT. The membranes were washed with TBS and then incubated for 15 min at RT with a buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.4. Immunoprecipitation

PMA-treated THP-1 cells (1×10^7) were lysed with 1 ml of the cold lysis buffer. The crude lysate was centrifuged at $15000\times g$ and the supernatant was incubated with 1 μg of the anti SR-A or anti-Lyn antibody for 1 h on ice, followed by conjugation with protein A sepharose beads for 1 h at 4°C. The beads were washed 4 times with the lysis buffer.

2.5. In vitro kinase assay

To evaluate the autophosphorylation activity of Lyn, Lyn was immunoprecipitated from PMA-treated THP-1 cells with anti-Lyn antibody and subjected to an in vitro kinase assay [13]. PMA-treated THP-1 cells (1×10^7) were lysed with 1 ml of the lysis buffer for 5 min on ice, and centrifuged at $18000\times g$ for 10 min. The supernatant was incubated with 1 μg of anti-Lyn antibody for 1 h on ice, followed by conjugation with protein A sepharose beads for 1 h at 4°C. The beads were washed 4 times as follows: twice with 1 ml of the cell lysis buffer, once with 1 ml of the kinase buffer (20 mM PIPES, pH 7.0), and once with 1 ml of the reaction buffer (kinase buffer containing 10 mM $MnCl_2$+10 mM $MgCl_2$). They were finally suspended in 40 μl of the reaction buffer. The in vitro kinase assay was carried out by incubating the beads with 2 μl (10 μCi) of [^{32}P-γ]ATP (Amersham) for 2 min at 30°C. After the reaction, the beads were washed 3 times with 1 ml of the washing buffer (1% Triton-X100, 0.5% deoxycholate, 0.1% SDS, 0.1% NaCl, 10 mM phosphate buffer, pH 7.5, 5 mM EDTA) and mixed with 40 μl of the loading buffer (2% SDS, 0.1 M Tris-HCl, pH 6.8, 20% glycerol, 5% 2-ME, 0.1% Bromophenol Blue), denatured for 5 min at 97°C, and placed on ice. The samples were loaded onto a 10% SDS–polyacrylamide gel and electrophoresed, then dried and autoradiographed.

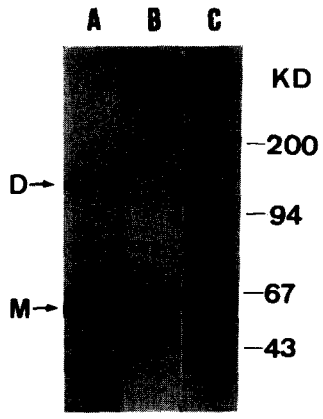

Fig. 1. Immunoblotting analysis of scavenger receptor proteins expressed in PMA-treated THP-1 cells with anti-SR-A antibody. Cellular proteins were extracted as described in Section 2 and the expressions of SR-A were evaluated by immunoblotting with the anti-SR-A antibody. Lane A, PMA-treated THP-1 cells, slightly reduced specimen; lane (B), PMA-treated THP-1 cells, normally reduced specimen; lane (C), untreated THP-1 cells, normally reduced specimen. The indicated bands represent monomeric (M) and dimeric (D) forms of SR-A. The positions of standard protein markers (arrows) are indicated in kilodaltons (kDa).

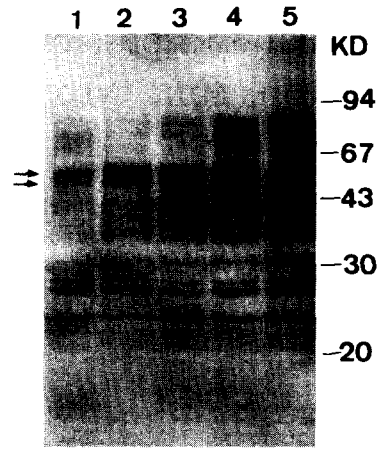

Fig. 2. Rapid induction of tyrosine phosphorylation by addition of acetyl LDL. PMA-treated THP-1 cells (1×10^7/ml) were incubated in FCS-free RPMI-1640 medium with acetyl-LDL (100 μg/ml) for the times indicated below. Then the cells were lysed and phosphotyrosine containing proteins were evaluated by immunoblotting with anti-phosphotyrosine antibody. Incubation time: lane 1, untreated control; lane 2, 30 s; lane 3, 1 min; lane 4, 3 min; lane 5, 5 min.

3. Results and discussion

Detection of SR-A on THP-1-derived macrophages by anti-SR-A antibody. It has been demonstrated that type 1 and type 2 SR-A proteins can be coexpressed on monocyte-derived macrophages with a higher expression of type 1 than of type 2 SR-A isoform [14]. To evaluate the level of SR-A isoform expression on the THP-1-derived macrophages used in this study, the cell lysate was immunoblotted with the anti SR-A antibody as described in Section 2. As shown in Fig. 1, the anti SR-A antibody could detect both the dimer and monomer of type 1 SR-A isoform when slightly reduced (lane A) and only the monomer when normally reduced (lane B) in the cell lysate of PMA-treated THP-1 cells, while no corresponding protein was detected in the cell lysate of untreated THP-1 cells, which normally do not express SR-A [15] (lane C). Pre-immune serum could not detect these proteins (data not shown).

3.1. Protein tyrosine phosphorylation in THP-1-derived macrophages following acetyl LDL engagement

To evaluate the role of protein tyrosine phosphorylation in the SR-A signal transduction, THP-1-derived macrophages were stimulated with acetyl LDL for various durations, after which the cell lysates were subjected to anti-phosphotyrosine (APT) immunoblotting (Fig. 2). Acetyl LDL engagement was found to rapidly induce the tyrosine phosphorylation of a variety of cellular proteins, with that of the approximately 53 and 56 kDa proteins predominantly observed within 30 s after the stimulation and retaining intense APT reactivity for at least 5 min.

3.2. Lyn is tyrosine-phosphorylated and activated in response to acetyl LDL stimulation

Because our preliminary observation showed that Lyn is strongly expressed in THP-1-derived macrophages (Fig. 4, lane 1) and the predominant tyrosine-phosphorylated proteins (with approximate sizes of 53 and 56 kDa) following acetyl LDL stimulation had molecular sizes similar to those of the

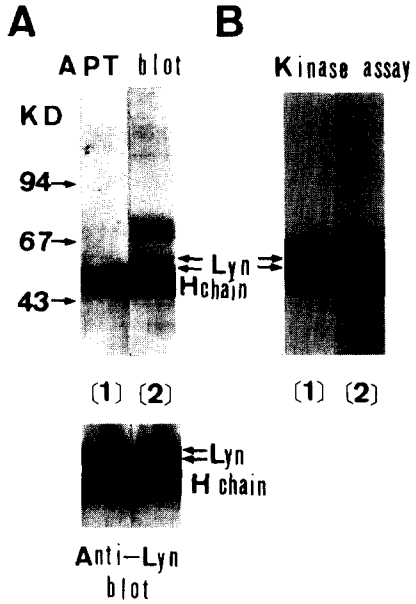

Fig. 3. Tyrosine phosphorylation and activation of Lyn by addition of acetyl-LDL. PMA-treated THP-1 cells (1×10^7/ml) were incubated in FCS-free RPMI-1640 medium in the absence (lane 1) or presence of acetyl-LDL (100 μg/ml) for 1 min (lane 2) and lysed with lysis buffer. The lysates were subjected to immunoprecipitation with the anti-Lyn antibody which was covalently coupled to protein A-Sepharose. The immunoprecipitates were washed and then examined by anti-phosphotyrosine immunoblotting (A, upper panels). The immunoprecipitates were washed and then examined by anti-Lyn immunoblotting (A, lower panels). B: Kinase activity of Lyn examined by in vitro kinase assay before (lane 1) and after (lane 2) 1 min of stimulation. The positions of p^{56Lyn} and p^{53Lyn} are indicated by arrows. IgG heavy chain was detected in (A) by immunoblotting originated from the antibody used for the immunoprecipitation.

two alternative forms of Lyn (Fig. 2), we next set out to determine whether Lyn is indeed tyrosine-phosphorylated in response to acetyl LDL stimulation. The cell lysates of THP-1-derived macrophages before acetyl LDL stimulation and after 1 min of stimulation were immunoprecipitated with the anti-Lyn antibody and subjected to APT immunoblotting. As shown in Fig. 3A, both forms of Lyn proved to be tyrosine-phosphorylated after 1 min of acetyl LDL stimulation compared with the base-line level of phosphorylation observed before the stimulation. It is generally known that the activation of a cytoplasmic tyrosine kinase can be evaluated by both its tyrosine phosphorylation and the increase in its catalytic activity measured by an in vitro kinase assay [16]. Indeed, it has been reported that the increased catalytic activity of Lyn is accompanied by its tyrosine phosphorylation (probably of the autophosphorylation site) upon B cell receptor cross-linking on B cells [12,17], or high-affinity Fcε receptor (FcεRI) cross-linking on mast cells [18,19]. Because a rapid tyrosine phosphorylation of Lyn in THP-1-derived macrophages was observed in response to acetyl LDL stimulation, we next evaluated the autokinase activity of Lyn before and after stimulation by using an in vitro kinase assay. The cell lysates of THP-1-derived macrophages obtained before and after 1 min of acetyl LDL stimulation were immunoprecipitated with anti-Lyn antibody followed by in vitro kinase assay performed as described in Section 2 (Fig. 3B). The autokinase activity of Lyn from the stimulated cells was found to have become approximately 4 times higher than that from the unstimulated cells. These observations indicate that Lyn in THP-1-derived macrophages is functionally responsive to acetyl LDL stimulation.

3.3. Co-immunoprecipitation of Lyn with SR-A

It has been reported that Lyn is involved in the cytoplasmic signal transductions of B cell receptor [12,17], high-affinity Fcε receptor [18,19], granulocyte colony-stimulating factor (G-CSF) receptor [20] and IL-2 receptor [21]. These associations between Lyn and receptors are not only functional, but physical associations have also been observed. Though the precise molecular mechanism of the activation of cytoplasmic tyrosine kinases by ligand–receptor interactions is not known, it has been speculated that the conformational change or dimerization of receptors induces the autophosphorylation or transphosphorylaton of the accompanying tyrosine kinase, resulting in the increase of its catalytic activity [16]. To evaluate the possible physical association of Lyn and SR-A, unstimulated or acetyl LDL-stimulated THP-1-derived macrophages were lysed, immunoprecipitated with the anti-SR-A antibody, and immunoblotted with the anti-Lyn antibody. As shown in Fig. 4, both forms of Lyn (p^{53}, p^{56}) were found to be co-immunoprecipitated with SR-A in both of the lysates from unstimulated (lane 3) and acetyl LDL-stimulated THP-1-derived macrophages (lane 4), while no Lyn protein was detected in the immunoprecipitate with pre-immune serum (lane 2). This observation suggests that Lyn is physically associated with SR-A. Because any common motifs seen in signal transducing molecules have not been identified in the cytoplasmic domain of SR-A, it is at present not clear how Lyn can functionally and physically interact with SR-A. It is conceivable that this interaction may be indirect and that a still unknown third molecule may mediate the interaction between Lyn and SR-A.

It was demonstrated that the in vitro growth of murine peritoneal exudate macrophages was induced by acetyl-LDL or oxidized LDL [4]. Moreover, the experiments using human monocyte-derived macrophages showed the followings, i.e., key role of lysophosphatidylcholine (lysoPC) in the growth of macrophages by oxidized LDL and the fact that the en-

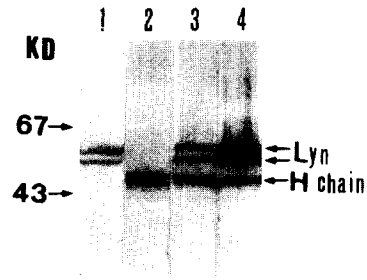

Fig. 4. Co-immunoprecipitation of p^{56Lyn} and p^{53Lyn} with scavenger receptor. PMA-treated THP-1 cells (1×10^7) were incubated in FCS-free RPMI-1640 medium in the absence (lane 3) or presence (lane 4) of acetyl LDL (100 μg/ml) for 1 min and lysed with 0.2% Triton-X100 lysis buffer. Samples of the cleared lysates were immunoprecipitated with the anti-SR-A antibody (lanes 3, 4) or non-immune rabbit IgG as a negative control (lane 2), separated by SDS/PAGE, blotted with anti-Lyn antibody. As a positive control, whole-cell lysate of PMA-treated THP-I cells was examined by anti-Lyn immunoblotting (lane 1). The positions of p^{56Lyn} and p^{53Lyn} are indicated by arrows. IgG heavy chain was detected in lanes 2–4 by immunoblotting originated from the antibody used for the immunoprecipitation.

docytic uptake of lysoPC through the scavenger receptor is crucial for the oxidized LDL-induced growth of macrophages [5]. These results demonstrated that the signals through scavenger receptors play an important role in the in vitro growth of macrophages stimulated with modified LDL. Other observations [2,3,6,7], as described in Section 1, have also suggested that the binding of modified LDL or other ligands to SR-A generates the transmission of some intracellular signals, which result in growth promotion, cytokine production, and host defense mechanisms of macrophages, in which Lyn is expressed. Furthermore, Lyn has been demonstrated to be essential for intracellular signal transductions through several cell-surface receptors [12,17–21]. Our present study has demonstrated both functional and physical associations of SR-A with Lyn in THP-1-derived macrophages. This finding suggests a possible involvement of Lyn in the intracellular signal transduction through scavenger receptors. Further studies, including experiments using macrophages in Lyn-knockout mice, may clarify the exact role of Lyn in this receptor system.

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