Role for Moesin in Lipopolysaccharide-Stimulated Signal Transduction

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Moesin is a 78-kDa protein with diverse functions in linking the cytoskeleton to the membrane while controlling cell shape, adhesion, locomotion, and signaling. The aim of this study was to characterize the expression and localization of moesin in mononuclear phagocytes by using confocal microscopy, flow cytometry, immunoprecipitation, and Western blotting and to analyze the function of moesin as a lipopolysaccharide receptor, utilizing an antisense oligonucleotide approach to knock down the moesin gene. Results revealed that moesin is expressed on the surface of monocytes/macrophages and surface expression is increased after lipopolysaccharide stimulation. The total protein mass of moesin is increased in monocytes after lipopolysaccharide stimulation. Immunoprecipitation showed that moesin coprecipitates with TLR4, a well-known lipopolysaccharide receptor, suggesting an early role of moesin in the formation of the initiation complex for lipopolysaccharide signaling. Two antisense and two control sense oligonucleotides were synthesized and introduced every 4 h for 48 h in adherent macrophage-like cells. Cells were then stimulated with lipopolysaccharide for 4 h, and the supernatants were assayed for tumor necrosis factor alpha (TNF-α) production. Cell lysates were assayed for moesin expression by Western blotting immediately after the 48-h treatment period and also after 116 h of recovery to assess the return of moesin expression and function. Moesin gene expression was completely suppressed after 48 h of incubation with antisense oligonucleotides. The antisense elimination of moesin gene expression led to a significant reduction of lipopolysaccharide-induced TNF-α secretion. Restoration of moesin gene expression led to restoration of TNF-α production. These data suggest an important role for moesin in lipopolysaccharide-induced TNF-α production, highlighting its importance in lipopolysaccharide-mediated signal transduction.

The pathology associated with gram-negative bacterial infections is tightly linked to the direct and indirect biological impact of bacterial endotoxin (lipopolysaccharide [LPS]). Once released into the host circulation, LPS has been shown to cause a wide variety of pathophysiological events, including cell injury, septic shock, and death (27). Microbial endotoxin is a potent inducer of cytokine synthesis, and although proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-12 are in general part of the innate immune response to infection, they can play crucial roles in the adverse development of systemic toxicity and septic shock (36). Peripheral mononuclear phagocytes are the host cells mainly responsible for binding and eliminating bacterial toxins. Activation with LPS leads to a significant increase in TNF-α protein synthesis in mononuclear phagocytes and macrophages (8). In addition, purified TNF-α has been shown to induce many of the deleterious effects of LPS in vivo (45). Despite intense research and numerous advances, the outcome of gram-negative sepsis has remained relatively unchanged, with a high mortality rate (28). Gaining complete understanding of the nature of LPS signaling in host cells will provide further insight into the design of lifesaving anti-inflammatory therapies.

There is a growing consensus that LPS stimulates cells through binding of specific membrane receptors, thereby initiating a cascade of signal transduction events (for a recent review, see reference 11). The molecules involved in LPS-mediated cell signaling include CD14, a 55-kDa glycosylphosphatidylinositol-linked membrane protein, and LPS binding protein (LBP) (50). Both are considered central to cellular activation by LPS and comprise the CD14-dependent pathway (4, 13, 35). Recently, several other molecules have been suggested to participate in transduction of LPS signals, including β2 integrins (CD11b/CD18) and TLR4 and TLR2, members of the Toll-like receptor (TLR) family (for reviews, see references 15 and 42, respectively). It was also shown that all three receptors (CD14, CD11b/CD18, and TLR4) act in concert to elicit the LPS response in murine macrophages (34). TLR-induced signal transduction includes the important transcription factors NF-κB, p38/MAPK, and Jun N-terminal kinase. These signals give rise to increased expression of proinflammatory proteins, including MyD88, IRAKs (IRAK-4, IRAK, and IRAK-2), Traf-6, and TAK-1 (31). Activation of NF-κB is required for cytokine release in response to LPS, and MyD88, IRAK, IRAK-2, and TRAF6 were shown to inhibit both IL-1- and LPS-induced NF-κB-luciferase activity, suggesting that a signal transduction molecule in the LPS receptor complex may belong to the IL-1 receptor/TLR superfamily, and the LPS signaling cascade is similar to IL-1 signaling in mononuclear phagocytes (52). However, the exact mechanism of action, sequence of events in formation of ligand-receptor complexes, and downstream propagation of the signal are still not completely understood.

Studies in our laboratory of LPS binding and induction of a biologic response by LPS have revealed the presence of a new putative LPS receptor identified as moesin (membrane-or-
organizing extension spike protein) (44). In these experiments, it was demonstrated that antibody to moesin completely eliminated the mononuclear phagocyte response to LPS without affecting the cell response to other stimulatory ligands. In additional experiments, moesin homozygote knockout mice were found to exhibit a threefold reduction in LPS-induced inflammation after subcutaneous LPS injection, suggesting a role for moesin in the LPS-induced inflammatory response (1). The ERM family of proteins comprises three structurally related proteins, ezrin, radixin, and moesin (23, 38). These proteins exhibit ~85% amino-terminal identity with talin and merlin, and all belong to the band 4.1 superfamily (22). ERM proteins are often coexpressed in various tissues and cells where they are localized in filopodia, microvilli, microspikes, retraction fibers, membrane ruffles, and adhesion contacts. Such microextensions and membrane formations are considered important for cell-cell and cell-substrate recognition, for signal transduction, and for motility (2). Moesin binds F-actin and functions as a membrane-cytoskeleton linking protein together with radixin and ezrin (17, 48). Moesin is widely expressed, especially in macrophages, lymphocytes, fibroblasts, epithelial cells, neuronal cells, and certain types of tumor cells. In areas of inflammation, Masumoto et al. (26) has shown moesin to be expressed in both the center and periphery of neutrophils. Moesin has been reported to have diverse biological functional activities, ranging from a structural protein to a receptor (5, 16, 37, 40, 47, 51).

In this study, we investigated the cell surface expression of moesin by immunofluorescence staining and flow cytometry, the role of moesin in LPS receptor signaling by immunoprecipitation, and the role of moesin in LPS signal transduction by an antisense oligonucleotide approach. We report that moesin is expressed on the cell surface of differentiated THP-1 cells and expression increased after LPS stimulation, that moesin immunoprecipitates with TLR4, and that THP-1 cells specifically lacking moesin are incapable of responding to LPS. The inability to respond to LPS was restored after the decay of the antisense oligonucleotide.

**MATERIALS AND METHODS**

**Materials.** Vitacell RPMI 1640 cell culture medium was obtained from the American Type Culture Collection (Manassas, Va.). Opti-MEM I reduced serum medium, fetal bovine serum (FBS), macrophage serum-free medium (M-SFM), and LipofectAMINE were obtained from Invitrogen Life Technologies (Carlsbad, Calif.). Phorbol 12-myristate 13-aceta (PMA), phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, aprotinin, benzamidine, and all belong to the band 4.1 superfamily (22). ERM proteins, ezrin, radixin, and moesin (23, 38). These proteins exhibit ~85% amino-terminal identity with talin and merlin, and all belong to the band 4.1 superfamily (22). ERM proteins are often coexpressed in various tissues and cells where they are localized in filopodia, microvilli, microspikes, retraction fibers, membrane ruffles, and adhesion contacts. Such microextensions and membrane formations are considered important for cell-cell and cell-substrate recognition, for signal transduction, and for motility (2). Moesin binds F-actin and functions as a membrane-cytoskeleton linking protein together with radixin and ezrin (17, 48). Moesin is widely expressed, especially in macrophages, lymphocytes, fibroblasts, epithelial cells, neuronal cells, and certain types of tumor cells. In areas of inflammation, Masumoto et al. (26) has shown moesin to be expressed in both the center and periphery of neutrophils. Moesin has been reported to have diverse biological functional activities, ranging from a structural protein to a receptor (5, 16, 37, 40, 47, 51).

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**Cell culture, differentiation, and stimulation.** The human promonocytic cell line THP-1 was obtained from the American Type Culture Collection. Cells were cultured in Vitacell RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine adjusted to contain 1.5 g of sodium bicarbonate/liter, 4.5 g of glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.2 mM antiprotease/ethanol. Cells were then plated in 24-well tissue culture plates at a density of 5 × 10^5 and stimulated to differentiate and adhere with 20 ng of PMA/ml for 24 h (46). For all procedures, cells were cultured at 37°C in a 5% CO2 atmosphere. For Western immunoblotting assays, cells were cultured with 0.1 μg of LPS/ml. For immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis, differentiated THP-1 cells were stimulated with 10 ng of LPS/ml. The peak response from LPS stimulation was predetermined based on pilot dose-response experiments. Cells were incubated for different times.

**Determination of expression of moesin on THP-1 cell surfaces by confocal immunofluorescence microscopy.** A moesin monoclonal antibody (IgG1) was labeled using an Alexa Fluor 488 protein labeling kit (final concentration, 2.5 μg/ml) according to the manufacturer's instructions. As a negative control, an isotype-matched antibody was used. THP-1 cells were seeded in a chamber slide system and treated with 20 ng of PMA/ml for 24 h. After differentiation, the nonadherent cells were washed with M-SFM and the adherent cells were stimulated with LPS in M-SFM. Cells were labeled with the moesin antibody labeled with Alexa Fluor 488. Brieﬂy, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. For membrane permeabilization, cells were incubated with 0.1% Triton X-100 in PBS for 5 min at room temperature after fixation. The cells were then incubated with 10% normal goat serum in PBS for 30 min followed by incubation with either moesin antibody or an isotype-matched control antibody in PBS with 10% normal goat serum for 60 min at room temperature protected from light. Cell samples were then washed twice with PBS and fixed again in 4% parafomaldehyde solution in PBS for 15 min at room temperature. The ProLong antifade mounting kit was used, and the cells were examined using a laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany).

**FACS analyses.** Differentiated THP-1 cells were stimulated in culture with several concentrations of LPS (10, 100, and 1000 ng/ml) at different time points (resting, 1, 5, 10, 30, and 60 min, and 2, 6, 24, and 48 h). All staining and washing procedures were performed at 4°C with staining buffer (2% FBS in PBS). Cells were incubated with moesin monoclonal antibody (10 μg/ml) for 30 min. After being washed, cells were further incubated with FITC-conjugated secondary antibody (40 μg/ml; Santa Cruz Biotech) for 30 min. For control experiments, cells were incubated either with an isotype-matched control primary antibody or with only FITC-conjugated secondary antibody. Data were acquired and analyzed by an FACSscan instrument using CellQuest software (BD Bioscience).

**Immunoprecipitation.** THP-1 cells were grown and differentiated in six-well plates with 20 ng of PMA/ml for 24 h. Some of the cells were additionally primed with LPS for 17 h. Primed and resting cells (2 × 10^6 each) were lysed in modified radioimmunoprecipitation buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg of leupeptin/ml, 5 μg of aprotinin/ml, 5 μg of leupeptin/ml, 5 μg of aprotinin/ml, 5 μg of dithiothreitol/ml, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) with protease inhibitor cocktail and incubated on ice for 30 min. Cells were then mechanically disrupted by homogenization, and lysates were preclarified by a 30-min centrifugation at 5,000 × g at 4°C. Supernatants were preabsorbed with goat serum and protein A-agarose beads for 1 h followed by overnight immunoprecipitation with goat antimoesin polyclonal antibody according to the manufacturer's instructions. Beads were washed three times with radioimmunoprecipitation buffer, resuspended in PBS, and boiled in Laemmle sample buffer for 5 min, followed by Western blot analyses. Western blots were developed with a biotinylated mouse anti-TLR4 antibody or goat antimoesin antibody. Either streptavidin-HRP conjugate or donkey anti-goat–HRP conjugate was used as the developing agent. Proteins were visualized on X-ray film by using the Pierce chemiluminescence system (Pierce Biotechnology, Rockford, Ill.), Results were confirmed in three independent experiments.

**Sense and antisense phosphorothioate-modified oligonucleotides.** Sense and antisense phosphorothioate-modified oligonucleotides used in this study were based on two different coding regions from the mouse moesin gene and were identical to those previously used by Takeuchi et al. (41) and Paglini et al. (32). The first antisense sequence used was a 24-mer sequence corresponding to positions 173 to 194 in the initiation site of the mouse moesin gene: 5′ ACCGA CACGATGCGTCTCGCAGCT 3′ (41). The second antisense sequence was a 15-mer sequence, corresponding to positions 1463 to 1477 of the mouse moesin gene: 5′ TTGAGCATATTCTTCCAAA 3′ (32). Sense sequences from both gene location were also synthesized and used as controls. The oligonucleotides were synthesized, phosphorothioate modified to prevent intra-

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cellular degradation, and purified by reverse-phase high-performance liquid chromatography (Keystone Laboratories, Camarillo, Calif.). The antisense experiments were performed in 24-well plates in duplicate or triplicate, depending on the availability of cells. THP-1 cells (5 × 10⁴) were plated and stimulated with PMA for 30 h. The cells were washed two times with serum-free RPMI 1640 Vitacell medium, and the same medium (200 μl) was added to each well. Separately, 50 μl of Opti-MEM medium containing antisense oligonucleotides, 2 μM each, and 2% LipofectAMINE was added according to the manufacturer’s protocol, and cells were incubated at 37°C in a 5% CO₂ atmosphere. Fifty microliters of oligonucleotide-LipofectAMINE mixture was added every 4 h for 48 h in serum-free RPMI 1640 medium. The cells were assayed at different time points. Before each transfection, the old medium was replaced with fresh medium. The cells treated with antisense oligonucleotides were labeled antisense cells (THP-1AS). Sense controls contained sense oligonucleotides at the same concentration under the same conditions and were labeled THP-1S. Negative controls contained no oligonucleotides and were labeled wild type (THP-1W). After the final transfection, cells were washed with Vitacell medium containing 30% FBS and incubated in the same medium for 3 h. The medium was changed with RPMI 1640 Vitacell medium containing 10% FBS, and cells were incubated for an additional 24 h at 37°C in a 5% CO₂ atmosphere. Cells were then stimulated with LPS at 100 ng/ml in medium containing 5% human serum for 4 h, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting.

Restoration of moesin gene expression and LPS-induced signaling. The antisense experiment was performed as described above in triplicate. After 48 h, cells were washed with serum-free medium and THP-1AS cells were incubated with 250 μg of serum-free medium containing sense oligonucleotides and LipofectAMINE for 4 h at the same concentration and conditions as those described above. Cells were then washed with Vitacell medium containing 30% FBS and incubated in the same medium for 3 h. The medium was changed with RPMI 1640 Vitacell medium containing 10% FBS, and the cells were incubated for another 99 h (a total of 106 h) at 37°C in a 5% CO₂ atmosphere. This medium was replaced with 250 μl of RPMI 1640 medium containing 5% human serum and LPS at a concentration of 100 ng/ml, and the cells were incubated for 4 h at 37°C in an atmosphere of 5% CO₂. After incubation, the medium was aspirated and saved at −20°C for further analysis of TNF-α expression. Quantification and viability of cells were assessed either by trypan blue exclusion or a light microscope or by DNA concentration measurement, where cell number and viability were determined by direct quantification of DNA using a DNA/azol reagent (Invitrogen) and comparison to a standard curve. THP-1 cells treated with antisense oligonucleotides and rescued were labeled THP-1ASR.

ELISA for TNF-α. The release of TNF-α from cells was assessed by a commercially available enzyme-linked immunosorbent assay (ELISA) performed according to the manufacturer’s instructions (Duoset ELISA Development System; R&D Systems, Minneapolis, Minn.). Briefly, 96-well microplates were coated with mouse anti-human TNF-α capture antibody and incubated overnight at room temperature. The plates were washed with PBS containing 0.05% Tween 20 and then blocked by PBS with 1% bovine serum albumin and 5% sucrose. After the addition of diluted samples and standard TNF-α dilutions, plates were incubated for 2 h at room temperature. Biotinylated goat anti-human TNF-α was used as the detection antibody, and streptavidin-horseradish peroxidase was added as the conjugate to each well. Equal proportions of hydrogen peroxide and tetramethylbenzidine were used as the substrate solution, and the reaction was stopped by adding 2 N sulfuric acid. All samples and standards were run in duplicate, and optical density was determined with a V-max microplate reader (Molecular Devices, Sunnyvale, Calif.) at a wavelength of 450 nm. Samples above the standard determination range for optical density readings were assessed again and read at an appropriate dilution.

SDS-PAGE and Western immunoblotting. The protein contents of cell lysates were determined by the Bradford assay (9). To detect moesin or ezrin, 100 μg of protein from each sample was used. Each cell lysate sample was placed in Laemmli sample buffer (21) and loaded on SDS-PAGE gels (4 to 20% gradient). After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, Mass.) in a Tris-glycine–20% methanol buffer for 1.5 h at 4°C and 50 mA. Membranes were blocked overnight in Tris-buffered saline (TBS) containing 5% dry milk, incubated for 1 h at room temperature with a 1:500 dilution of goat polyclonal antisera to moesin and a 1:250 dilution of mouse antiezrin antibody, washed, and incubated for 1 h with a 1:2,500 dilution of peroxidase-conjugated bovine anti goat-serum for moesin or anti-mouse antibody for ezrin. Proteins were visualized using an ECL system (Pierce Biotechnology).

To investigate moesin up-regulation, after stimulation cells were lysed with cold lysis buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1% [vol/vol] protease inhibitor cocktail solution [Sigma]). Supernatant was collected by centrifugation at 16,000 × g for 15 min at 4°C. Samples were mixed in Laemmli sample buffer and then boiled for 3 to 5 min. Five micrograms of protein per lane was separated by SDS–10% PAGE. Proteins were transferred onto a PVDF membrane and blocked in 5% skim milk in TBS. The blocked membrane was incubated with moesin monoclonal antibody (1:1,000 dilution) in 2.5% skim milk in TBS. After being washed with TBS-Tween, the membrane was subsequently incubated with the appropriate HRP-conjugated secondary antibody (1:10,000 dilution) in TBS-Tween. Immunodetection was accomplished as described above. Semiquantitative analysis of the autoradiograms was assessed by the Bio-Rad imaging system, using their software (Bio-Rad, Hercules, Calif.).

RESULTS

Extracellular expression and subcellular distribution of moesin in differentiated THP-1 cells. We examined the expression of moesin on differentiated THP-1 cells before and after LPS stimulation. Expression of moesin was observed with both permeabilized and nonpermeabilized cells (Fig. 1). Upon LPS stimulation (10 ng/ml), fluorescence intensity increased for both the permeabilized and the nonpermeabilized conditions (Fig. 1). The cell staining pattern for permeabilized cells was uniform, whereas it was peripheral in the nonpermeabilized cells, suggesting staining only on the cell surface. Fluorescence intensity began to decay after 2 h under both conditions. Control cells incubated with isotype-matched antibody showed weak or no fluorescence (data not shown). These findings, for the first time, suggest that moesin is expressed on the surface of differentiated macrophage-like THP-1 cells. Expression increased upon LPS stimulation, suggesting either a conformational change in the moesin molecule or recruitment of moesin to the cell surface or both.

FACS analysis. Flow cytometry was used to quantify moesin cell-surface expression before and after LPS stimulation of differentiated THP-1 cells. Results shown in Fig. 2 confirm that moesin is expressed on the surface of differentiated THP-1 cells. When cells were stimulated with LPS (10 ng/ml), there was a significant increase in the expression of moesin on the cell surface. This expression increased after 5 min of stimulation and reached a peak level after 1 h.

Western immunoblotting analyses of moesin up-regulation. To investigate whether increased expression of moesin on the cell surface included an increase in total protein, Western blotting was performed. Analyses showed that LPS stimulation (100 ng of LPS/ml for 1 h) resulted in a significant increase in the moesin protein level (Fig. 2, inset).

Immunoprecipitation. To evaluate the participation of moesin in propagating the LPS signal, immunoprecipitation with antimoesin antibody was performed. TLR4 was demonstrated to coprecipitate with moesin when both antibodies were used (Fig. 3). Immunoprecipitation in the absence of specific antibody failed to reveal any specific bands except IgG bands (negative control).

Selective suppression of moesin gene expression in THP-1 cells. Cross-linking studies have shown previously that moesin binds to LPS and possibly functions as an LPS receptor or cofactor (1, 44). To confirm a biological role for moesin in LPS signaling, we performed functional studies utilizing synthetic oligonucleotides to selectively suppress the moesin gene in the promonocytic cell line THP-1. Due to the difficulties in suppressing the moesin gene that were previously reported (41),
we used two different antisense sequences at concentrations and durations that have been reported to be sufficient (32). Figure 4 illustrates the region of the moesin gene used for synthesizing antisense and sense oligonucleotides. To confirm the suppression of moesin gene expression and monitor the production of moesin protein in the cells, Western blots were developed with antimoesin antibody. Cell lysates from cells treated with antisense or sense oligonucleotides and controls with no oligonucleotides added, taken at different time points after transfection, were analyzed (Fig. 5). Western blotting of cells treated with antisense oligonucleotide showed that moesin protein expression diminished at 36 h and was virtually undetectable at 48 h. No change in the density of moesin bands for 16 and 24 h compared to the sense oligonucleotide treatment and controls was observed.

In a separate experiment, THP-1AS cells were allowed to recover for an additional 106 h after cessation of antisense oligonucleotide additions to evaluate the return of moesin expression and function. After 106 h, THP-1AS cells partially regained gene function and moesin message was partially expressed (Fig. 6). To show that moesin antisense oligonucleotides suppressed the expression of the moesin gene selectively, and not other members of the ERM family of proteins, Western blotting with antiezrin antibody was performed. The results revealed that at 48 h there was no suppression of ezrin gene expression. Similar results were obtained when the Western blot was developed with antiradixin antibody (data not shown). These data demonstrate that the antisense sequences used in these studies selectively knock down moesin gene expression.

Moesin antisense oligonucleotides eliminate TNF-α cytokine expression upon LPS stimulation in THP-1 cells. ELISA was used to assess TNF-α production by THP-1AS, THP-1S, and THP-1W cells at 4, 24, and 48 h after transfection (Fig. 7A). Results revealed an 82% reduction in TNF-α secretion at 48 h in the THP-1AS cells (analysis of variance with Bonferroni correction for multiple comparisons, P < 0.05), whereas
are results from three separate experiments. Densitometric analysis of the bands showed a twofold increase in the also showed an increase in moesin after 1 h of LPS stimulation (inset). sin unstimulated, 158; moesin stimulated, 260; isotype control unstimu-

lation was observed after 1 h (mean for LPS-stimulated cells (1 h) with moesin antibody. A signi
cant increase in moesin expression on the cell surface after LPS stimulation was observed after 1 h (mean fluorescence intensity: moe-

sin unstimulated, 158; moesin stimulated, 260; isotype control unstimu-

lated, 75; and isotype control stimulated, 78). Western blot analysis also showed an increase in moesin after 1 h of LPS stimulation (inset). Densitometric analysis of the bands showed a twofold increase in the intensity of the band. The histograms, immunoblot, and densitometry are results from three separate experiments.

the controls (THP-1S and THP-1W) showed no change. When THP-1AS cells were allowed to recover and reexpress moesin, the TNF-α response returned to normal levels (Fig. 7B).

DISCUSSION

In this study, we used confocal immunofluorescence microscopy, immunoprecipitation, and an antisense oligonucleotide approach to study moesin expression and the role of moesin in the transduction of LPS receptor signals. Data from confocal immunofluorescence staining and FACS analyses suggest for the first time that moesin is expressed on the surface of differentiated macrophage-like THP-1 cells. Exposure of THP-1 cells to LPS resulted in a significant increase of moesin protein level as measured by Western blotting, implying possible up-regulation of moesin mRNA as well. Furthermore, increased expression suggests either a conformational change in the moesin molecule or recruitment of moesin to the cell surface or both. Finally, moesin was shown to be associated with TLR4 in immunoprecipitation experiments. Antisense moesin oligonucleotides were able to eliminate moesin gene expression for given time periods, which rendered THP-1 cells unresponsive to LPS stimulation as measured by TNF-α secretion. We were also able to show that upon reversing the effect of the antisense oligonucleotides, LPS signaling and TNF-α production in THP-1 cells were partially restored, implicating moesin in LPS signal transduction in THP-1 monocytes.

Previous studies in our laboratory used a cross-linking strategy to investigate LPS-membrane interactions. These studies revealed that LPS binds to two proteins, the well-established receptor for LPS, CD14, and a novel LPS binding molecule identified as moesin (44). Antibody to moesin was found to eliminate LPS-induced responses at all LPS concentrations. In addition, the inhibition was specific for the LPS-moesin interaction, since events mediated by unrelated receptors (N-formyl-methionyl-leucyl-phenylalanine and IL-1) were not inhibited by antimoesin IgG. The response to the gram-positive bacterium Staphylococcus aureus was also not abolished by moesin antibody, confirming that the effects were unique to LPS-elicited responses. In addition, moesin antibody inhibition experiments revealed a dramatic reduction of TNF-α production at all LPS concentrations tested. Conversely, CD14 antibodies were able to completely eliminate the TNF response only at lower concentrations of LPS. The observation that at lower concentrations, CD14 antibody partially reduced binding of LPS to moesin suggests that these two receptors may physically interact for optimal binding of LPS and transduction of the signal.

The human gene encoding moesin was cloned (22), and its sequence was shown to contain neither a signal peptide nor a transmembrane domain, suggesting that the protein is not integrally incorporated into the plasma membrane, thus making it available for free movement upon activation and phosphorylation. However, moesin is a very dynamic molecule with the apparent capability of quick activation and relocation upon cellular stimulation. Several studies have proposed a role for moesin as a receptor since it is expressed on the cell surface. Keresztes et al. (19) suggested that upon adhesion of porcine neutrophils to a plastic surface, moesin is translocated to the plasma membrane and the extracellular surface. Our laboratory proposed that moesin is a receptor for LPS based on direct cross-linking and functional studies (44). Ariel et al. (5) implied that moesin can be expressed on the cell surface not only by stimulation with phytohemagglutinin and phorbolmyristate acetate but also by stimulation with calyculin A, a specific inhibitor of phosphatases 1 and 2A, capable of inducing moe-
sin phosphorylation (29).

We successfully used an antisense oligonucleotide approach to specifically abolish moesin gene expression and observed changes in the biological response of THP-1 cells upon stimulation with E. coli LPS. Previous observations (32, 41) revealed that inhibition of moesin gene expression was difficult, which led us to use two different antisense oligonucleotides (32). In fact, our data demonstrate that it takes at least 48 h of antisense oligonucleotide cell treatment (fresh oligonucleotide added every 4 h) in order to accomplish complete inhibition of the moesin gene. Our Western blot data show that moesin was completely suppressed in THP-1 cells for 48 h. When such cells were stimulated with LPS, an 82% decrease of TNF-α secretion was observed compared to controls.

At present, the most extensively studied model of LPS sig-

naling is CD14/LBP, or the so-called CD14-dependent pathway. CD14, being a membrane-anchored protein, cannot transduce the LPS signal, and another molecule(s) is needed as a coreceptor, linker, or transducer. Recent studies of the human
counterpart of Drosophila Toll receptors, using mostly genetic approaches, revealed that TLR4 is the main Toll-like molecule involved in LPS signaling (3, 18, 24, 43). It has also been shown that the MD-2 adapter molecule can link LPS and TLR4 and thus amplify the response (7, 39). LPS can bind directly to MD-2 without LBP participation, and it is physically associated with TLR4 and MD-2 in the presence of CD14 (10).

As a structural protein, moesin plays a role as a cellular stabilizer together with ezrin and radixin, linking the cellular actin cytoskeleton to the membrane and thus participating in numerous events connected with changes in cell shape, from establishing cell contacts and adhesion to signal transduction (for a recent review, see reference 14). Moesin has been characterized as an essential protein for the formation of focal adhesions in response to active Rho in permeabilized fibroblasts (25). There is increasing evidence for involvement of moesin as a key molecule in adhesion events during the inflammation process in different types of cells. Recently it has been shown that moesin interacts with vascular adhesion molecule 1 during leukocyte adhesion and transendothelial migration (6). The role of moesin in inflammation has been confirmed in studies showing binding of N-terminal moesin in PMA-stimulated lymphocytes to the cytoplasmic tail of L selectin and this binding to be regulated by protein kinase C. In addition, moesin has been implicated in the regulation of T-cell adhesion and has been shown to be present as a receptor

FIG. 3. Western blot of THP-1 cell lysate immunoprecipitated with antimoesin antibody. (A) Western blot of THP-1 cell lysate immunoprecipitated with antimoesin antibody and developed with goat antimoesin antibody. THP-1 cells (2 x 10^7) were differentiated with PMA (20 ng/ml) and stimulated with 100 ng of E. coli LPS/ml for 17 h. Lysed cells were pretreated with goat serum and protein A/G-agarose beads for 1 h at 4°C, used subsequently as a negative control, and immunoprecipitated further with goat antimoesin antibody for 24 h. Thirty microliters of each sample was loaded on 4 to 20% gradient gels and subjected to SDS-PAGE. A Jurkat cell lysate was used as the positive control. The Western blot was developed with antimoesin antibody as the primary antibody and HRP-conjugated streptavidin.

FIG. 4. Map of the moesin DNA sequence used for the design of antisense and sense oligonucleotides.

FIG. 5. Time-dependent inhibition of moesin expression in LPS-stimulated THP-1 cells treated with moesin antisense oligonucleotides. Differentiated THP-1 cells were cultured in 24-well plates in triplicate in the absence of oligonucleotides (THP-IW) or in presence of two different types of sense (THP-1S) and antisense (THP-1AS) moesin oligonucleotides. Oligonucleotides were added every 4 h for a total of 48 h at a final concentration of 2 μM each. At each time point, cells were stimulated with E. coli LPS (100 ng/ml) for 4 h after changing the medium containing 5% human serum. Equal amounts of protein from cell lysates obtained at each time point were loaded on SDS-polyacrylamide gels and transferred to PVDF membranes, and moesin expression was analyzed by Western blotting with antimoesin antibody.
expressed on the surface of T cells (5). It was shown in the same study that moesin is involved in binding IL-2 peptides and subsequent adhesion of activated T cells to the extracellular matrix. Finally, moesin homozygote knockout mice were found to exhibit a threefold reduction in LPS-induced inflammation after subcutaneous LPS injection, suggesting a role for moesin in the LPS-induced inflammatory response (1). Current experiments and the antisense approach not only show the expression of moesin on the cell surface but also suggest involvement of moesin protein in receptor/signal transduction events following exposure of human monocytic cells to LPS. These data, extending our previous observations made with knockout mice, represent a more convenient human model, in which an antisense in vitro approach could be reproducibly used to determine mechanisms underlying moesin up-regulation, and strongly support a role of moesin in LPS signaling.

This is the first study to show association of moesin with TLR4 protein. Immunoprecipitation of THP-1 cell lysates with moesin antibody revealed the presence of TLR4, a widely accepted LPS receptor molecule. Earlier, our lab demonstrated binding of moesin to LPS and CD14 (44). The current work demonstrates that moesin binds to TLR4, implicating a role for moesin in the formation of the protein-receptor complex. Moesin may be solely involved in the formation of the receptor complex, or it may have a function as an independent receptor, since it has been shown to bind directly to LPS. The temporal sequence of complex formation is still unknown, and further studies are under way to investigate the initiation of the LPS signal transduction cascade. Since TLR4 is tightly connected to innate immunity and moesin is responsible for cytoskeleton changes, the association of moesin with TLR4 reveals a new mechanism and illustrates additional diversity in the biological activity of these two proteins. Moesin is most probably involved in early membrane events involved in the propagation of the LPS signal together with CD14 and TLR4/MD2 molecules. In addition, immunoprecipitation studies demonstrated association of moesin with TLR4 and confirmed participation of moesin in LPS signaling in THP-1 cells. Further studies will reveal the sequence of initiation and propagation of the LPS signal.

The diversity of moesin as a biological molecule is readily visualized by its rich structural domain and capability for numerous different interactions with other proteins. At its N terminus, moesin and other proteins from this family have a so-called FERM domain (band 4.1, ezrin, radixin, moesin homology domain), containing sequences of three subdomains, F1 to F3 (33). F1 has similarity to ubiquitin (49), F2 has similarity to acyl coenzyme A binding protein (20), and F3 has similarity to PTB (phosphotyrosine-binding module), PH (plekstrin homology domain), and VH1 (enabled/VASP homology 1) (12). The cluster of acidic amino acids, especially in the PH homology domain, has been shown to be important in interactions of ERM proteins with lipid layers of the membrane (30). Although moesin lacks a membrane-spanning domain, crystallographic studies recently showed that these proteins are able to interact and link with various lipid fractions in the membrane.
On the basis of these observations, moesin may be viewed not only as a structural protein but also as a receptor. Several research groups, including our laboratory, have suggested the expression of moesin on the cell surfaces of different cell types, e.g. macrophages, lymphocytes, fibroblasts, and endothelial and epithelial cells. It would appear from these studies that moesin could play an important role in signal transduction. Moreover, we report in this study direct visualization of moesin on the cell surface by fluorescent antibody techniques and that the expression of moesin is up-regulated by exposure of cells to LPS. Based upon our understanding of the folding of the FERM domain of moesin, it would appear that moesin is folded upon itself in its inactive form. One interpretation of our data suggests that moesin unfolds upon cell activation, leading to increased expression of an extracellular domain. In summary, the data presented here provide further evidence for the role of moesin as an LPS receptor. Interference with moesin gene expression in human THP-1 monocytes with antisense oligonucleotides led to elimination of the LPS responses in antisense oligonucleotide-treated cells. When gene function was restored, cytokine production was also restored.

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REFERENCES


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