

Augmentation of Lipopolysaccharide (lps) Receptor Expression by Interferon γ Correlates with Susceptibility to lps-Induced Apoptosis in Human Monoblastic Cell Line u937

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Abstract

Apoptosis, accompanied with cytoplasmic shrinkage, nuclear condensation and caspase activation induced by LPS, is thought to proceed via activation of LPS receptor, which is comprised of the toll-like receptor (TLR) 4 and MD-2. We report that LPS induced apoptosis in the human monoblastic cell line U937 that was pretreated with interferon γ (U937IFN), but not in cells that were untreated or treated with retinoic acid (U937RA). To reveal the reason why interferon LipoPolySaccharide (LPS); Apoptosis; LPS receptor; Differentiation; Monocyte LipoPolySaccharide (LPS); Apoptosis; LPS receptor; Differentiation; Monocyte γ (IFN) confers apoptotic susceptibility to U937 cells, we focused on the LPS receptor. IFN stimulated the expression of cell surface TLR4 and the transcription level of MD-2 mRNA. By transfecting a designed antisense oligonucleotide (ASO) into U937IFN cells before exposing to LPS, the expression level of TLR4 was lowered to levels comparable to those in U937 cells; ASO transfected-cells showed no cleavage of poly (ADP-ribose) polymerase (PARP) and exhibited no morphological changes. Addition of an anti-TLR4 monoclonal antibody to the cultures of U937IFN cells along with LPS resulted in reduction of the Ac-DEVD-MCA hydrolysis and inhibition of the PARP cleavage. Neither a loss of function form of FADD, an adaptor molecule for death receptor signaling, nor a neutralization antibody against TNF- α or Fas prevented LPS-triggered apoptosis, indicating that the death ligand and death receptor were not activated. These results suggest that IFN induces expression of the LPS receptor, resulting in increased susceptibility to LPS-induced apoptosis.

Keywords: LipoPolySaccharide (LPS); Apoptosis; LPS receptor; Differentiation; Monocyte

Introduction

Cells acquire various functions and change their activities in response to various extracellular agents that induce or suppress cellular proteins. Some of these agents also trigger increased or decreased expression level of apoptotic molecules, resulting in changes in apoptotic susceptibility. Kikuchi, et al. previously reported that human monoblastic cell line U937 differentiates into CD11b-positive monocyte/macrophage-like cells upon treatment with interferon γ (IFN) or all-trans retinoic acid (RA), and that its susceptibility to Fas- and tumor necrosis factor (TNF)-mediated apoptosis changes; IFN-treated cells become highly susceptible, whereas RA-treated cells become Fas-resistant [1]. Other researcher has reported that RA decreases apoptotic susceptibility to Fas because this reagent reduces expression of Fas on the U937 cell surface [2]. On the other hand, IFN results in accumulation of Fas on the cell surface, and consequently the large number of Fas molecules contributes to increased apoptotic sensitivity [3,4]. Caspase-8, which is activated at the cytoplasmic domain of Fas, is also induced by IFN and promotes a state of susceptibility to apoptosis [5].

IFN-pretreated U937 cells (U937IFN) infected *in vitro* with *Shigella flexneri* undergo nuclear fragmentation and digestion of poly (ADP-ribose) polymerase (PARP), typical apoptotic properties [6]. Recently, it was found that dead bacterial bodies or avirulent *Shigella* strains also induce apoptosis in U937IFN cells [7]. Undifferentiated U937 cells or RA-pretreated cells (U937RA) did not show this virulence-independent apoptosis. It is conceivable that U937IFN cells acquired apoptotic sensitivity to infectious stimuli and those apoptosis-inducing elements in bacteria are independent of virulence proteins, cell invasion and intracellular propagation. Quite recently, we reported that lipopolysaccharide (LPS), one of bacterial cell surface components, induces apoptosis to U937IFN cells (Kuwabara and Imajoh-Ohmi, submitted). The LPS receptor consists of TLR4 and MD-2, and induces apoptosis in mammalian cells. However, the relationship between the LPS receptor and IFN-dependent susceptibility to LPS-induced apoptosis is still unknown.

In this study, we investigated whether IFN enhances the amount of LPS receptor on the cell surface and whether the sensitivity of LPS-induced apoptosis depends on the expression level of the LPS receptor. Expression levels of TLR4 and MD-2 were compared between U937, U937IFN and U937RA cells. Neutralization by an anti-TLR4 antibody, and knock down of expression by an antisense oligonucleotide (ASO), were examined. We also tested whether LPS-induced apoptosis activates the death receptor and its ligand. U937IFN cells, but not U937 cells or U937RA cells, exhibited LPS-mediated apoptosis. The amount of TLR4 on the cell surface and the mRNA level of MD-2 were increased by IFN treatment. In the presence of the anti-TLR4 antibody, LPS failed to activate caspase. When TLR4 on the surface of U937IFN cells was decreased by ASO, LPS-induced apoptosis was prevented. LPS did not affect the death receptor and ligand. Our results suggest that an apoptotic susceptibility shift accompanying cell differentiation closely relates to the expression level of the LPS receptor.

Materials and Methods

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), LPS (*Shigella flexneri* serotype 1A), RA, and Rhodamine123 were obtained from Sigma Aldrich Japan (Tokyo, Japan). Recombinant human interferon- γ (IFN) was kindly provided by Shionogi Pharmaceuticals (Osaka, Japan). An anti-TLR4 monoclonal antibody (MoAb), an anti-FADD MoAb and two anti-Fas MoAbs (CH-11 for the cytotoxic assay and ZB4 for the neutralization assay) were purchased from MBL (Nagoya, Japan). The anti-human TNF- α antibody was purchased from R & D systems (Minneapolis, MN, USA). Carbobenzoxy-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) and acetyl-Asp-Glu-Val-Asp-(4-Methyl-Coumaryl-7-Amide) (Ac-DEVD-MCA) were obtained from Bachem (Torrance, CA, USA) and the Peptide Institute (Osaka, Japan), respectively.

Cell Culture and Conditions for Differentiation

Human monoblastic U937 cells (provided by the Japanese Cancer Research Resources Bank) were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, (100 units/ml) penicillin G and 200 μ g/ml streptomycin at 37°C under a humidified atmosphere of 5% (v/v) CO₂. For U937 differentiation, cells (less than 2 x 10⁵ cells/ml) were treated with IFN (100 units/ml) or RA (1 μ M) for two days. These treated cells were denoted as U937IFN and U937RA, respectively.

Immunoblotting Analysis

Whole cells (1 x 10⁶ per sample) were washed with ice-cold PBS and treated with 10% (w/v) TCA. After centrifugation, the precipitates were sonicated in 100 μ l whole cells (1 x 10⁶ per sample) were washed with ice-cold PBS and treated with 10% (w/v) TCA. After centrifugation, the precipitates were sonicated in 100 μ l of SDS-sample buffer and heat denatured. Samples (corresponding to 1 x 10⁵ cells) were subjected to SDS-PAGE and transferred onto a PVDF membrane. The membrane was soaked in Tris-buffered saline (TBS) containing 20 mg/ml BSA for 1h at room temperature and incubated with antibodies overnight at 4°C. After removing excess antibodies, the membrane was incubated with an anti-rabbit antibody conjugated to alkaline phosphatase (Promega, Madison, WI, USA). Antibodies were visualized by the enzymatic reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT). The specificities of the PARP antibody has been described previously [8]. Samples (corresponding to 1 x 10⁵ cells) were subjected to SDS-PAGE and transferred onto a PVDF membrane.

The membrane was soaked in Tris-buffered saline (TBS) containing 20 mg/ml BSA for 1h at room temperature and incubated with antibodies overnight at 4°C. After removing excess antibodies, the membrane was incubated with an anti-rabbit antibody conjugated to alkaline phosphatase (Promega, Madison, WI, USA). Antibodies were visualized by the enzymatic reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT). The specificities of the PARP antibody has been described previously [8].

Caspase Assay

After exposure to LPS or an anti-Fas antibody, cells were washed once with ice-cold PBS and then disrupted by sonication in 10mM Tris-HCl (pH 8.0) 1mM EDTA (TE buffer). Cell lysates were prepared by centrifugation and incubated in assay buffer [20mM Tris-HCl (pH 7.5), 0.5mM EDTA, 5mM 2-mercaptoethanol] at 37°C. After 15 min, 10 μ M Ac-DEVD-MCA was added and incubation was continued for an additional 15 min. Caspase activity was measured by the generation of 7-amino-4-methylcoumarin (AMC) (Excitation: 365nm and Emission: 460nm). One unit was defined as the amount of enzyme capable of generating one nmol of AMC in 1h.

Viability Assay

Following sensitization with LPS (100 ng/ml), cells were incubated at 37°C in a CO₂ incubator. Viable cell numbers were estimated by an assay using MTT [9,10]. Briefly, cells (10⁵ cells/100 μ l) were incubated with medium containing 0.5mg/ml MTT for 4h. The formazan product was solubilized with isopropanol containing 0.04N HCl, and absorbance at 590 nm was measured for each well. Viability was expressed as (OD₅₉₀ of sensitized cells / OD₅₉₀ of control cells) x 100.

Analysis of Cell Surface Molecules by Flow Cytometry

Cells (5 x 10⁵) were washed with ice-cold PBS, resuspended in 100 μ l of PBS containing 3% (w/v) human γ -globulin (Jackson ImmunoResearch Laboratories, Inc.), and incubated for 10 min on ice. Cells were probed with saturating amounts of an anti-TLR4 MoAb or with isotype-matched nonbinding control MoAbs for 120 min at 4°C. After several washes with PBS, the MoAb bound to the cell surface was labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Igs (TAGO, Inc.) for 60 min at 4°C. Analyses were then performed on a FACScan flow cytometer (Becton Dickinson, Mountainview, CA, USA). The results were calculated with CELLQuest software (Becton Dickinson).

RT-PCR Analysis

Total RNA was isolated from U937 cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For the RT reaction, 1 μ g of total RNA was reverse transcribed into cDNAs by using oligo(dT) [11] primers in a 25- μ l reaction. MD-2 and β -actin cDNA were amplified from the cDNA mixture by PCR using the following primer sets: MD-2 sense 5'-atgtaccattctgtttttcc-3', antisense 5'-ctaattgaattaggtgg-3'; β -actin sense 5'-gaggagcaccctgtgctgta-3', antisense 5'-ctagaagcattgtgctggacgatggagggccc-3'. To quantify the level of mRNA expression, RT-PCR products separated on a 1% agarose gel were analyzed on an FLA-3000 (Fujifirm, Tokyo, Japan). ImageGauge software (Fujifirm) was used to compare the density of the products. Results are expressed as relative changes in RNA amounts that have been normalized to the amount of β -actin, which acted as an internal standard.

Antisense Oligonucleotide Experiment

The antisense and control oligonucleotides directed to knock down TLR4 expression were designed and manufactured by Biognosik, Germany. In experiments aimed at establishing the time course of oligonucleotide uptake by U937 cells, a fluorescein-labeled phosphorothioate oligonucleotide was added to the culture and incubated for various time durations ranging from 0–48h. The oligonucleotides (TLR4 antisense: 5'- cgaggcagacatcat -3', control: 5'- gtcctatagaacg -3') were found to be effective, and thus were delivered to cells by Oligofectamine (Invitrogen, Carlsbad, CA, USA) and Opti-Mem (Invitrogen) according to the manufacturer's instructions. After transfection with the oligonucleotides, the cells were incubated with IFN at 37°C for 48h. The transfections had no detectable effect on cell differentiation.

Preparation of Recombinant Protein and Protein Transfection

The dominant negative form of FADD, a 1-79 amino acid residue deletion mutant, was amplified by RT-PCR using the forward primer: ccgctcgaggagcactcgaggcggg and the reverse primer: cgcgatccttatcgctcgctgctctccttcttccataggacgcttcggaggtagatgc. The 5'-terminal end of the reverse primer corresponded to the protein transduction motif TAT. The PCR fragment was cloned into an expression vector pIVEX2.4b. The TAT-containing recombinant protein was expressed

using the Rapid Translation System (Roche diagnostics, Tokyo, Japan), according to the manufacturer's instructions. Subsequently, recombinant protein was applied to a column of Ni-NTA agarose (Qiagen, Tokyo, Japan) and polymyxin B agarose (Sigma). After cells were kept in RPMI1640 with 2% serum for 1h, TAT-recombinant protein was added to cells to a final concentration of 0 to 3 μ M, and the cells were incubated for 1h. Subsequently, apoptosis was induced by a cytotoxic anti-Fas antibody (50ng/ml) or by LPS (100ng/ml).

Statistical Analysis

Student's *t* test was used to analyze significant differences. *P* values < 0.05 were regarded as significant.

Results

U937 cells undergo changes in susceptibility to LPS-induced apoptosis upon differentiation. We examined whether U937 cells become susceptible to apoptosis in response to IFN treatment. Cell viability was assessed by an MTT assay. U937IFN cells treated with LPS showed a time-dependent loss of cell viability (Figure 1). The percentage of viable U937IFN cells was 65% after 24h of LPS stimulation, and this was similar in cells treated with an anti-Fas antibody (55% in 24h; Figure 1). However, the viability of U937 and U937RA cells did not decrease, even after cells were exposed to LPS.

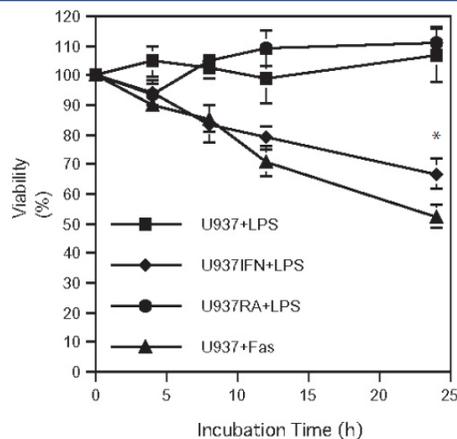


Figure 1. Sensitization of U937IFN cells with LPS reduces cell viability in a time-dependent manner. U937 cells were incubated with LPS (100ng/ml) or anti-Fas antibody CH-11 (50ng/ml) for the indicated time durations. Metabolic activity was measured using the MTT assay. The percentage of cytotoxicity was calculated as described in the Materials & Methods. Vertical bars represent averages of triplicate measurements \pm standard deviation (SD). *Values (diamond) significantly different from U937 cells (square). ($P < 0.001$).

Since the MTT assay cannot distinguish between different types of cell death, apoptosis or non-apoptotic cell death, the morphological features of the LPS-mediated cell death were examined. U937 cells were exposed to LPS for 9h and stained with hematoxylin. Whereas U937 and U937RA cells showed no apparent changes, the U937IFN cells exhibited cytoplasmic shrinkage and nuclear condensation (Figure 2A). These morphological features were similar to those seen in cells treated with the anti-Fas antibody (Figure 2A)

The activity of caspase in LPS-exposed cells was also examined. To detect caspase-3/-7 activity, we assessed apoptotic cleavage of the 116 kDa poly(ADP-ribose)polymerase (PARP), one of substrates of the executioner

caspase, into an 85kDa fragment by immunoblotting analysis. Similar to the effect of treating with the anti-Fas antibody, LPS induced proteolysis of PARP in U937IFN cells (Figure 2B). In contrast to U937IFN cells, LPS failed to induce cleavage of PARP in U937 or U937RA cells (Figure 2B). The caspase activity in the cytoplasmic fraction of U937IFN cells was also assessed by using the synthetic peptide substrate Ac-DEVD-MCA. Z-VAD-fmk-sensitive caspase activity in U937IFN cells increased threefold at 6h and by a factor of eight at 9h (Figure 2C). These results suggest that U937 cells have altered susceptibility to apoptosis caused by LPS, depending on their differentiation state.

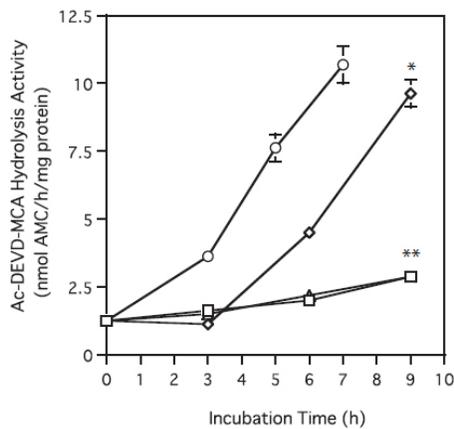
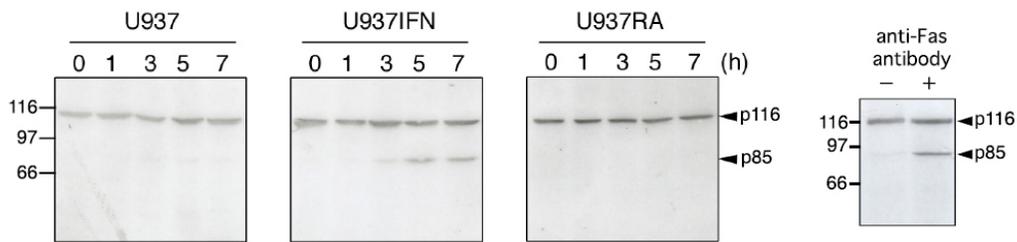
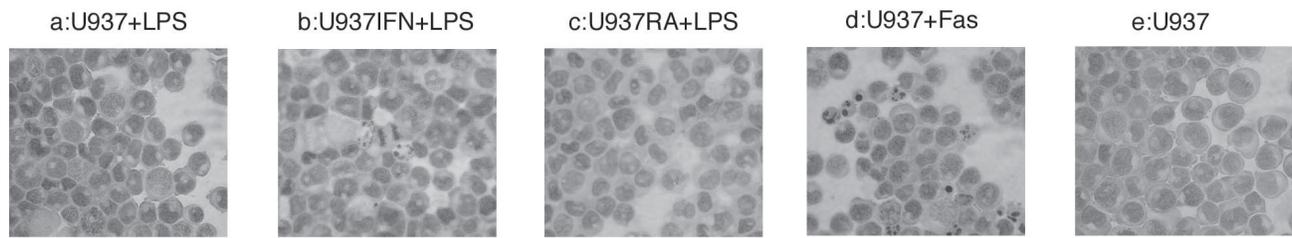


Figure 2. Apoptotic changes in U937IFN cells exposed to LPS. U937 cells were exposed to LPS (100ng/ml) or to the anti-Fas antibody CH-11 (50ng/ml). (A) Light microscopic analysis of U937 cells. LPS- or CH-11-treated cells were mounted on a slide glass by cytocentrifugation and stained with Carrazi's Hematoxylin (Muto Pure Chemicals, Tokyo) as described previously (28). a:U937 cells + LPS, b:U937IFN cells + LPS, c:U937RA cells + LPS, d:U937 cells + cytotoxic anti-Fas antibody, e:U937 cells. Arrowheads indicate the apoptotic cells. (B) Cleavage of the native p116 PARP protein into p85 was analyzed by immunoblotting with anti-PARP antibody. U937, U937IFN and U937RA cells were cultured with LPS (100ng/ml) for the indicated times, and with cytotoxic anti-Fas antibody (50ng/ml) for 5h, and samples were prepared for SDS-PAGE. The molecular masses (in KDa) of standard marker proteins are indicated. (C) Caspase activity in the cytoplasmic fraction prepared from LPS-treated cells was determined by using Ac-DEVD-MCA as a caspase-3/-7 substrate. The AMC released due to caspase activity was measured fluorometrically. One unit was defined as the amount of enzyme capable of generating 1 nmol of AMC in 1 h. The symbols indicate medium alone (squares), LPS (diamonds), anti-Fas antibody (circles), and LPS plus 50 μ M of z-VAD-fmk (triangles). *Values (diamond) significantly different from control condition (square). ($P < 0.001$). **Values (triangle) significantly different from U937IFN cells (diamond). ($P < 0.001$).

IFN enhances expression of the LPS receptor. U937 cells differentiate into CD11b positive cells after pretreatment with IFN or RA. However, the expression level of this marker protein is different in response to IFN or RA treatment [8]. It appears that the two reagents lead U937 cells to different states. It is conceivable that the degree of susceptibility to apoptosis is defined by some distinct features of these cells. We decided to investigate alterations of LPS receptor expression among three differentiation states of U937 cells. Using an anti-TLR4 antibody and flow cytometric analysis, the amount of cell surface TLR4 was assessed. As shown in (Figure 3A), exposure of U937 cells to IFN increased TLR4 expression on the cell

surface. This indicated enhanced expression of TLR4 at the surface of U937IFN cells (average relative mean fluorescence intensity (MFI), 97), with a 4.5-fold increase in MFI compared with U937 cells (average relative MFI, 21.5). Since TLR4 forms a complex with MD-2, then is translocated to the cell surface where it recognizes LPS, we analyzed the expression level of MD-2. Because we did not have methods to quantify the MD-2 protein, the MD-2 mRNA level was compared by reverse transcription-polymerase chain reaction (RT-PCR). U937IFN cell mRNA expression was double that of the other cells (Figure 3B). These results suggest that susceptibility to apoptosis is closely related to the expression level of the LPS receptor.

Fig. 3A

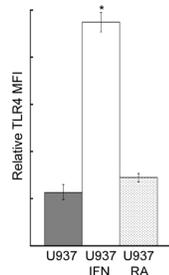
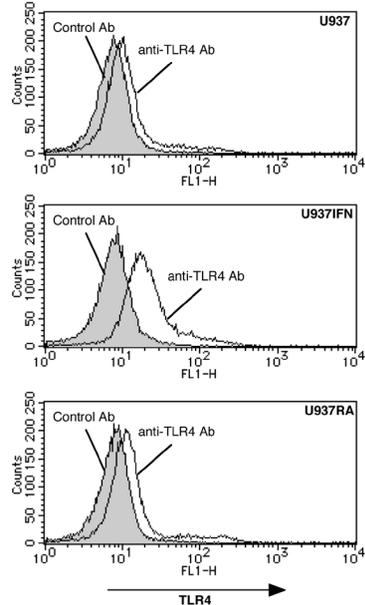


Fig. 3B

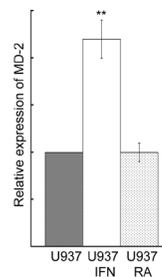
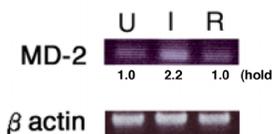


Figure 3. IFN stimulates TLR4 expression by U937 cells. U937 cells were induced to differentiate by incubation with IFN or RA for 48h. (A) Greater TLR4 levels were found on the surface of U937IFN cells. Untreated U937 cells and the differentiated U937 cells were probed with an anti-TLR4 MoAb or a control mouse antibody. The antibodies were followed by incubation with a FITC-conjugated anti-mouse IgG and then analyzed by FACScan. Relative MFI value of TLR4 in relationship to those obtained from the U937 cells. *Values significantly different from U937 cells. ($P < 0.001$). (B) Enhancement of MD-2 transcription by IFN treatment. MD-2 transcription levels were analyzed by PCR following reverse transcription of total RNA from untreated U937, U937IFN and U937RA cells. Amplified β -actin mRNA was used as an internal loading control. Graph depicts densitometry analysis of three independent experiments. **Values significantly different from U937 cells. ($P < 0.05$)

LPS receptor triggers apoptosis in the U937IFN cells. Since U937IFN cells showed a large amount of the LPS receptor, we tried to transfect the gene of TLR4 in order to express this receptor in undifferentiated U937 cells. However, transfectants were not established. Therefore, U937IFN cells were transfected with an antisense oligonucleotide (ASO) to reduce the TLR4 level. As shown in (Figure 4A), pretreatment with 250nM ASO reduced the TLR4 level on the surface of U937IFN cells. This expression level was similar to that of undifferentiated U937 cells. In contrast, transfection with control oligonucleotide (CTO) into U937IFN cells did not alter the

TLR4 level (Figure 4A). When U937IFN cells transfected with ASO were treated with LPS, cleavage of PARP was not detected in the cell lysate (Figure 4B). ASO-transfected U937IFN cells also did not exhibit any morphological changes when cells were exposed to LPS (Figure. 4C). In contrast, LPS induced limited digestion of PARP, nuclear condensation and cytoplasmic shrinkage in the CTO-transfected U937IFN cells (Figure 4B and 4C). Transfection with ASO into U937IFN cells did not prevent morphological changes induced by an anti-Fas antibody (Figure 4C).

Fig. 4A

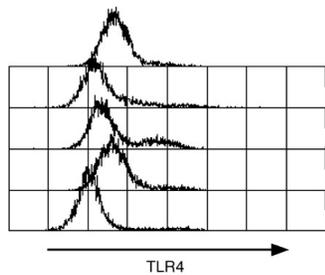


Fig.4B

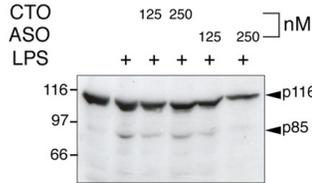


Fig. 4C

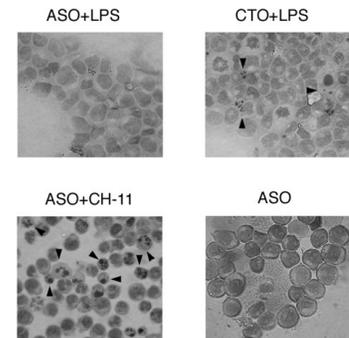


Figure.4. Effect of a TLR4 antisense oligonucleotide on LPS-induced U937IFN cells apoptosis. (A) Downregulation of TLR4 expression on the U937IFN cell surface by ASO transfection. U937IFN cells were transfected with ASO or CTO and probed with anti-TLR4 MoAb followed by incubation with FITC-conjugated anti-mouse IgG. Cell-surface TLR4 expression was analyzed with a FACScan flow cytometer and CELLQuest software. (B) Blockage of PARP cleavage in ASO-transfected U937IFN cells treated with LPS. Cells transfected with oligonucleotide (125 nM or 250 nM) were cultured with LPS (100ng/ml) for 7h, and then samples were prepared for SDS-PAGE. Cleavage of PARP was analyzed by immunoblotting. Molecular masses (in KDa) of standard marker proteins are indicated. (C) Light microscopic analysis of oligonucleotide transfected U937IFN cells. After induction of apoptosis, oligonucleotide transfected cells were fixed and stained with hematoxylin. Arrowheads indicate the apoptotic cells.

It has been reported that an anti-TLR4 monoclonal antibody (MoAb) blocks cytokine production in response to LPS [12]. This indicates that the anti-TLR4 MoAb antagonizes LPS-signaling. Thus, we examined whether the anti-TLR4 MoAb prevents LPS-mediated apoptosis. When the anti-TLR4 MoAb (3 μ g/ml) was added to the U937IFN cells culture prior to LPS, Ac-DEVD-MCA-hydrolysis activity of the cell lysate decreased to approximately 40% that of the cells treated with LPS (Figure 5A). Control

MoAb did not block activation of caspase. Addition of 1 μ g/ml and 3 μ g/ml of anti-TLR4 MoAb reduced caspase activity by 40% and 65%, respectively, in the lysate prepared from LPS-treated cells (Figure 5B). Immunoblot analysis showed that PARP hydrolysis induced by LPS was also inhibited by the anti-TLR4 MoAb (Figure 5C). However, the anti-TLR4 MoAb did not prevent Fas-mediated cleavage of PARP (Figure 5C), indicating that apoptosis induced by LPS was mediated by the LPS receptor.

Fig. 5A

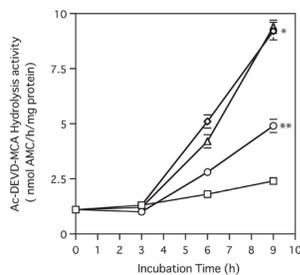


Fig. 5B

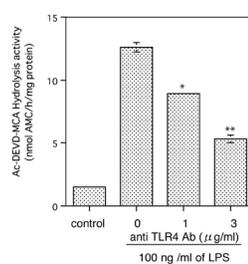


Fig. 5C

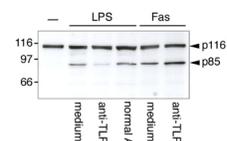


Figure. 5. Blockage of TLR4 function suppresses LPS-mediated U937IFN apoptosis. (A) Changes in LPS-induced caspase activity due to treatment with or without the anti-TLR4 antibody. U937IFN cells were stimulated with LPS (100ng/ml) or anti-Fas antibody (50ng/ml) in the presence of the anti-TLR4 MoAb, a control mouse antibody, or medium only. Cytoplasmic fractions were prepared at the indicated times (0 to 9h) and the caspase activity assay was performed. The symbols indicate medium (squares), LPS (diamonds), LPS plus anti-TLR4 MoAb (circles), and LPS plus control MoAb (triangles). *Values (diamond) significantly different from control condition (square). (P<0.001). **Values (circle) significantly different from U937IFN cells (diamond). (P<0.05). (B) Dose-dependent suppression of LPS-induced caspase activity by the anti-TLR4 MoAb. Caspase activity was induced by LPS (100ng/ml) in the presence of different amounts of the anti-TLR4 MoAb and was assayed. *Values significantly different from U937IFN cells. (P<0.05). **Values significantly different from U937IFN cells. (P<0.001). (C) Blockage of PARP cleavage by the neutralization of TLR4. U937IFN cells were exposed to LPS (100ng/ml) or anti-Fas antibody CH-11 (50ng/ml) in the presence of anti-TLR4 or control MoAbs. Cleavage of PARP was analyzed by immunoblotting analysis. Molecular masses (in KDa) of the standard marker proteins are indicated.

The death ligand and death receptor are not activated during LPS-induced apoptosis.

It is conceivable that LPS induces apoptosis through the autocrine production of tumor necrosis factor- α (TNF- α). To investigate this question, we first decided the amount of TNF- α (100units/ml) that was sufficiently able to induce apoptosis, and then determined the requisite concentration (100–300 ng/ml) of anti-TNF- α antibody to neutralize TNF- α (data not shown). In the presence of an anti-TNF- α antibody, LPS induced cleavage of PARP (Figure 6A left). When we carried out a similar experiment utilizing a neutralizing anti-Fas antibody, PARP cleavage was not blocked (Figure 6A right). The cytoplasmic adaptor molecule FADD (Fas-associated death domain containing protein) binds to the death domain of the death receptor and transmits apoptotic signals. The recombinant carboxy terminal portion of FADD functions as a loss of function molecule that prevents receptor-mediated apoptosis [13]. To prevent death receptor signals, we

tried to transport this FADD mutant into cells. The HIV TAT sequence, corresponding to amino acids 49 to 57 of the TAT protein, is known to facilitate the delivery of proteins into mammalian cells in a concentration-dependent manner [14–16]. A fusion protein combining a FADD mutant (FADD D/N) and the TAT sequence was prepared and transfected into U937IFN cells. The amount of intracellular FADD D/N increased in a concentration-dependent manner (Figure 6B bottom). Limited digestion of PARP induced by the anti-Fas antibody was prevented at FADD D/N concentrations of 1 and 3 μ M (Figure 6B top). However, LPS induced cleavage of PARP even though cells were transfected with 3 μ M of the recombinant protein (Figure 6B middle). When cells were stained with hematoxylin after exposure to LPS, the FADD D/N transfected cells showed nuclear fragmentation induced by LPS but not by an anti-Fas antibody (Figure 6C). These results suggest that LPS induces apoptosis independent of the activities of death ligands and death receptors.

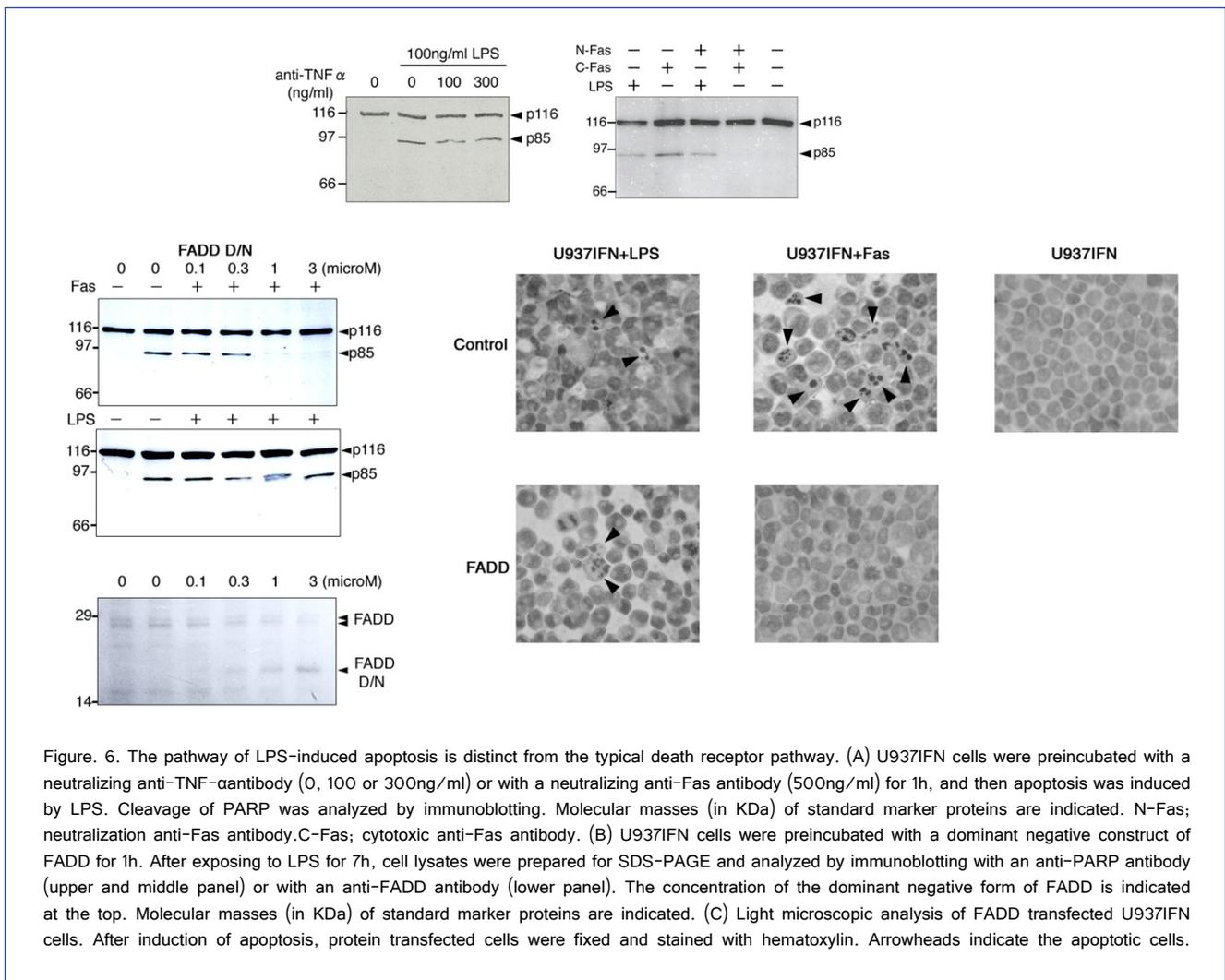


Figure 6. The pathway of LPS-induced apoptosis is distinct from the typical death receptor pathway. (A) U937IFN cells were preincubated with a neutralizing anti-TNF- α antibody (0, 100 or 300ng/ml) or with a neutralizing anti-Fas antibody (500ng/ml) for 1h, and then apoptosis was induced by LPS. Cleavage of PARP was analyzed by immunoblotting. Molecular masses (in KDa) of standard marker proteins are indicated. N-Fas; neutralization anti-Fas antibody. C-Fas; cytotoxic anti-Fas antibody. (B) U937IFN cells were preincubated with a dominant negative construct of FADD for 1h. After exposing to LPS for 7h, cell lysates were prepared for SDS-PAGE and analyzed by immunoblotting with an anti-PARP antibody (upper and middle panel) or with an anti-FADD antibody (lower panel). The concentration of the dominant negative form of FADD is indicated at the top. Molecular masses (in KDa) of standard marker proteins are indicated. (C) Light microscopic analysis of FADD transfected U937IFN cells. After induction of apoptosis, protein transfected cells were fixed and stained with hematoxylin. Arrowheads indicate the apoptotic cells.

Discussion

In this study, we investigated the relationship between differentiation of U937 cells and their changing susceptibility to apoptosis in response to LPS. Our most important finding was that IFN induced expression of the LPS receptor on U937 cells, and the increase in the amount of the LPS receptor was closely related to its increased susceptibility to LPS-induced apoptosis. This represents the first evidence that differentiation in response to IFN increases the susceptibility of U937 cells susceptibility to LPS-mediated apoptosis.

It has been reported that IFN induces the increased cell-surface expression of death receptors and their ligands, such as Fas and Fas ligand [17], and that LPS promotes TNF- α production [18,19]. Thus, IFN and LPS might theoretically also mediate apoptosis by employing the pathways triggered by Fas or by the TNF receptor. In U937IFN cells, however, antibodies against Fas or TNF- α had no effect on LPS-induced apoptosis. Furthermore, in the case of death receptor-mediated apoptosis, the adaptor molecule FADD plays an important role in the formation of DISC and the activation of caspase-8, to initiate the cascade of apoptosis [13,20]. Our data showed that LPS induced apoptosis in U937IFN cells, even when the cells were pretreated with a FADD loss of function mutant that prevents Fas-mediated apoptosis (Figure 6B and 6C). These results suggest that the mechanism of LPS-triggered apoptosis is distinct from the typical death receptor-mediated apoptosis.

U937 cells alter the susceptibility to apoptosis caused by the Fas ligand and by TNF- α when cells are pretreated with IFN or RA [1]. This change of apoptotic susceptibility, which accompanies by differentiation, is caused by changes in the expression level of Fas. IFN induces expression of Fas [3,4], whereas RA reduces the expression of this death receptor [2]. In this study, following induction of cell differentiation the U937 cells also alter their susceptibility to LPS-induced apoptosis. To elucidate the reason why U937IFN cells become susceptible to LPS-induced apoptosis, we focused on the expression level of the LPS receptor, which consists of TLR4 and MD-2. IFN treatment enhances expression of TLR4 and MD-2 in U937 cells (Figure 3). The increased expression of TLR4 and MD-2 that we observed in response to IFN is consistent with previous studies that show IFN stimulates TLR4 and MD-2 expression in human mononuclear phagocytes and HL-60 monocytic cells [21-23]. It has been reported that IRF (interferon regulatory factors) activate the promoter region of TLR4 [24,25]. A similar transcription mechanism may be involved in enhancing the expression of the LPS receptor complex in U937IFN cells.

Because IFN induced a high expression level of the LPS receptor as well as enhanced susceptibility to apoptosis, we investigated why increased sensitivity to LPS-induced apoptosis required either pretreatment with IFN or expression of the LPS receptor. The TLR4 gene was transfected into undifferentiated U937 cells, but this did not result in establishment of transfectants. Therefore, the reduction of TLR4 expression by transfection of antisense oligonucleotides was carried out in U937IFN cells. U937IFN cells with low levels of TLR4 expression resist LPS-induced apoptosis (Figure 4). Furthermore, when these cells were cultured with an anti-TLR4 MoAb, the caspase activity caused by LPS was reduced in a concentration-dependent manner (Figure 5B). Our results showed that the neutralization antibody binds to TLR4 and restricts its activity, indicating that active TLR4 is reduced by this antibody, similar to the effect of an ASO. Consequently, we concluded that increase of LPS receptor levels on cell surfaces induced by IFN is necessary for increased susceptibility to LPS-induced apoptosis.

Indeed, U937IFN cells acquired susceptibility to LPS-induced apoptosis (Figure 1 and 2). Although Fas transmits death signals, the LPS receptor transmits primarily cell activation signals, rather than death signals. Therefore, analysis of LPS-induced apoptosis is performed in the presence of mRNA or protein synthesis inhibitors [26-28]. However, U937IFN cells showed apoptosis without these inhibitors. This result indicates that the fate of U937IFN cells switches from survival to death. These are two possible interpretations of this observation. The first explanation is that cells can only respond to a limited number of signals from the LPS receptor. When numerous signals stimulate the cells, the intracellular cascade automatically shifts to an apoptotic pathway. A second explanation is that proapoptotic factors induced by IFN divert signals from cell survival pathways to apoptotic pathways. Both interpretations are consistent with the result that the increased levels of LPS receptors in response to IFN transmit apoptotic signals, and that reduction or neutralization of the LPS receptor results in resistance against LPS-induced apoptosis.

In conclusion, U937 cells pretreated with IFN showed increased susceptibility to LPS-induced apoptosis. IFN stimulated expression of TLR4 and MD-2. These results suggest that the expression level of the LPS receptor is closely related to cellular susceptibility to LPS-induced apoptosis. From this analysis of a monocyte/macrophage differentiation model using U937 cells, we conclude that LPS-induced monocyte/macrophage apoptotic responses may play an important role in the regulation of innate immune responses.

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