

# Lipopolysaccharide induces the expression of cellular inhibitor of apoptosis protein-2 in human macrophages

Xuefan Cui <sup>a</sup>, Tadaatsu Imaizumi <sup>a,\*</sup>, Hidemi Yoshida <sup>a</sup>, Kunikazu Tanji <sup>b</sup>,  
Tomoh Matsumiya <sup>c</sup>, Kei Satoh <sup>a</sup>

<sup>a</sup> Department of Vascular Biology, Institute of Brain Science, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori-ken 036-8562, Japan

<sup>b</sup> Department of Molecular Biology, Institute of Brain Science, Hirosaki University School of Medicine, Hirosaki, Aomori-ken 036-8562, Japan

<sup>c</sup> Department of Dentistry and Oral Surgery, Hirosaki University School of Medicine, Hirosaki, Aomori-ken 036-8562, Japan

Received 17 July 2000; received in revised form 5 October 2000; accepted 11 October 2000

## Abstract

Apoptosis is an important process in normal animal development as well as in diseases, and inhibitor of apoptosis protein (IAP) is one of the important factors that regulate apoptotic cell death. We found that lipopolysaccharide (LPS) enhances the expression of mRNA and protein of cellular IAP-2 (cIAP2) in human monoblastic U937 cells differentiated by phorbol ester pretreatment. cIAP2 mRNA was not detected in undifferentiated U937 cells. mRNAs of cIAP1 and X-chromosome-linked IAP (XIAP) were expressed constitutively and not affected by LPS in both undifferentiated and differentiated cells. LPS stimulated the expression of cIAP2 mRNA and protein in time- and concentration-dependent manners. LPS enhanced the expression of cIAP2 mRNA and protein in human monocyte-derived macrophages, which was associated with the inhibition of the caspase-3 activation, i.e., decrease in active p17 fragment of caspase-3 with simultaneous accumulation of precursor p20 fragment. We conclude that LPS may inhibit apoptosis of macrophages, at least in part, through the induction of cIAP2. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Cellular inhibitor of apoptosis protein-2; Lipopolysaccharide; Macrophage; U937 cell; Caspase

## 1. Introduction

Apoptosis plays an important role not only in animal development and tissue homeostasis but also in the pathogenesis of a variety of disorders such as cancer, autoimmune diseases, infectious diseases and neurodegenerative diseases [1,2]. There are a number of mechanisms for the regulation of apoptosis, and the members of the inhibitor of apoptosis protein (IAP) family effectively suppress cell death induced by various stimuli [3–5]. Human IAPs were first described as proteins, homologous to the baculoviral IAP family, induced by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [6]. The human IAP family includes cIAP1, cIAP2, XIAP, neuronal IAP (NIAP), and survivin [4–8]. There are two motifs characteristic to these IAPs: they have up to three amino acid repeats of approx. 65 residues in length, called baculovirus IAP repeat (BIR) at the N-terminus [9], and

except for NIAP and survivin, all other known IAPs contain a RING finger domain at the C-terminal end [10]. Most of these IAPs inhibit apoptosis by suppressing caspases, and the BIR is responsible for this inhibition [11]. Caspase-3 is a downstream effector molecule in the caspase cascade [12], and cIAPs directly inhibit this cell death protease [13].

Activation of macrophages by cytokine stimulation or a mild microbial infection is shown to inhibit cell death due to apoptosis [14], and control of macrophage cell death may have a potential effect on the immune responses. In fact, loss of macrophages by apoptosis may lead to the suppression of immune functions and increased susceptibility to microbial pathogens [15]. LPS has a variety of effects on macrophages and it has been shown to abolish the apoptosis of U937 cells induced by TNF- $\alpha$  [16]. U937 is a monoblastic leukemia cell line committed to monocyte differentiation, and treatment with agents such as TNF- $\alpha$ , 1,25-dihydroxycholecalciferol, or phorbol 12-myristate 13-acetate (PMA) induces the appearance of the characteristics consistent with mature macrophages [17]. We, herein, described the expression of IAPs in U937 cells in re-

\* Corresponding author. Fax: +81-172-39-5135;  
E-mail: timaizum@cc.hirosaki-u.ac.jp

sponse to stimulation with LPS, and the effect of PMA-induced cell differentiation on IAP expression was also examined. The results obtained with U937 cells were confirmed with human monocyte-derived macrophages.

## 2. Materials and methods

### 2.1. Cells

U937 cells were cultured using RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. To induce differentiation, the cells were treated with 0.1 mM PMA dissolved in dimethyl sulfoxide (DMSO) for 48 h, then the cells were cultured in PMA-free medium for a further 24 h and used for experiments. The final concentration of DMSO in medium was 0.1% and the control cells were treated with vehicle only.

Peripheral venous blood was drawn from healthy volunteers after an informed consent, then monocytes were isolated using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) [5]. The cells were cultured using RPMI 1640 supplemented with 10% FBS to differentiate into macrophages. Macrophages cultured for 3 days were used for the experiments.

### 2.2. Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from the cells using an RNeasy total RNA isolation kit (Qiagen, Hilden, Germany). Single-strand cDNA for a PCR template was synthesized from 1 µg of total RNA using primer oligo (dT)<sub>12–18</sub> and M-Mulv reverse transcriptase (Gibco, Rockville, MD, USA). Specific primers were designed from cDNA

sequences for cIAP1, cIAP2, XIAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and each cDNA was amplified by PCR with *Taq* DNA polymerase (Qiagen). The sequences of the sense (F) and antisense (R) primers were as follows: cIAP1-F, 5'-CCTGTGGTTAAATCTGCCTTG-3'; cIAP1-R, 5'-CAATTCGGCACCATAACTCTG-3'; cIAP2-F, 5'-AAGTTCCATCCCCTGTCCAA-TG-3'; cIAP2-R, 5'-CAAGTAGATGAGGGTAACTGGC-3'; XIAP-F, 5'-CTTGCATACTGTCTTTCTGAGC-3'; XIAP-R, 5'-ACACCATATACCCGAGGAAC-3'; GAPDH-F, 5'-CCACCCATGGCAAATTCCATGGGCA-3'; and GAPDH-R, 5'-TCTAGACGGCAGGTCAGG-TCCACC-3'.

The conditions for the reaction were 1×(94°C, 2 min); 32×(94°C, 1 min; 55°C, 1 min; 72°C, 1 min); and 1×(72°C, 10 min). The products were analyzed by electrophoresis on a 1.2% agarose gel containing ethidium bromide. The expected size of the PCR products for cIAP1, cIAP2, XIAP, and GAPDH were 1067 bp, 550 bp, 818 bp and 696 bp, respectively.

### 2.3. Northern blot analysis

Poly(A)<sup>+</sup>mRNA was purified from total RNA using Oligotex-dT30(Super) (Takara, Shiga, Japan) and subjected to electrophoresis on a 1% agarose gel containing formaldehyde. The RNA was blotted to a positively charged nylon membrane by capillary transfer and probed with the digoxigenin (DIG)-labeled antisense RNA for cIAP2 or β-actin. DIG-labeled probe for cIAP2 was synthesized, using a DIG-labeling kit (Roche, Mannheim, Germany), from the linearized full length cIAP2 cDNA in pcDNA3 (a gift from Dr. Ryosuke Takahashi, Riken Brain Science Institute). Hybridization was performed at 68°C for 16 h using a NorthernMax kit (Ambion, Austin,

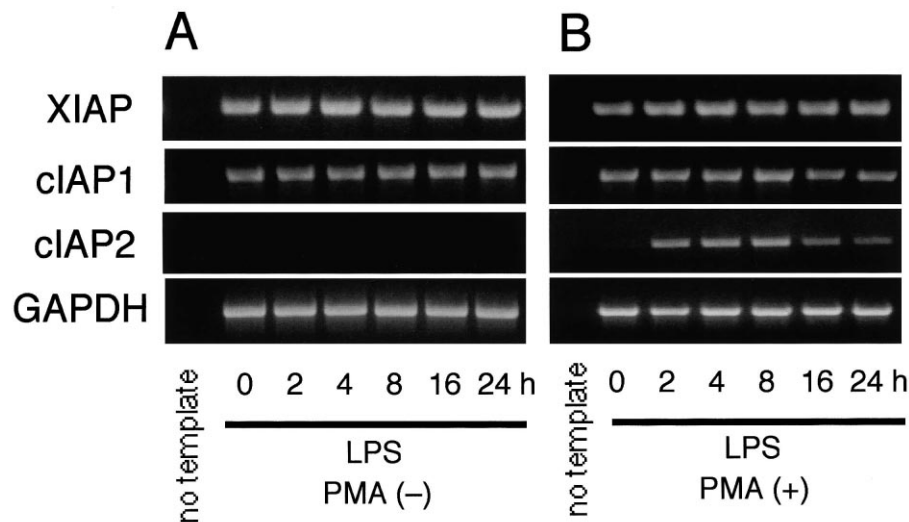


Fig. 1. Time course of the LPS-induced upregulation of cIAP2 mRNA in U937 cells differentiated with PMA. The cells were treated with (A) DMSO, vehicle for PMA, or (B) PMA, for 48 h and stimulated with 100 ng/ml LPS for up to 24 h. mRNAs for cIAP2, cIAP1, XIAP and GAPDH were semi-quantified by RT-PCR.

TX, USA), and the detection using a DIG detection kit (Roche).

#### 2.4. Western blot analysis

U937 cells or macrophages were lysed by incubating with cell lysis buffer, 20 mM phosphate-buffered saline (pH 7.4) containing 1% NP-40, 0.5% sodium deoxycholate, 0.15% SDS and 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Cell lysates were separated by electrophoresis on a gradient polyacrylamide gel (4–20%) containing 0.1% SDS. Proteins were subsequently transferred to a PVDF membrane (Millipore Japan, Tokyo, Japan), and the membrane was incubated for 2 h at 37°C with SuperBlock blocking buffer (Pierce, Rockford, IL, USA). The membrane was incubated for 24 h at 4°C with 1.5 mg/ml of a rabbit anti-cIAP2 polyclonal antibody (R&D Systems, Minneapolis, MN, USA) or 1 mg/ml of an anti-XIAP monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) or 1:2000 of a polyclonal rabbit anti-caspase-3 antiserum (PharMingen, San Diego, CA, USA). After washing six times, the membrane was incubated with 1/25 000 dilution of anti-rabbit IgG for cIAP2 or anti-mouse IgG labeled with horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD, USA). Detection was performed by incubating with a chemiluminescence substrate (SuperSignal West Pico, Pierce) and the membrane was exposed to a Kodak BioMax film (Kodak, Rochester, NY, USA).

### 3. Results

The results of RT-PCR analyses on the expression of cIAP2 mRNA in U937 cells are shown in Fig. 1. cIAP2 mRNA was not induced by LPS in U937 cells without

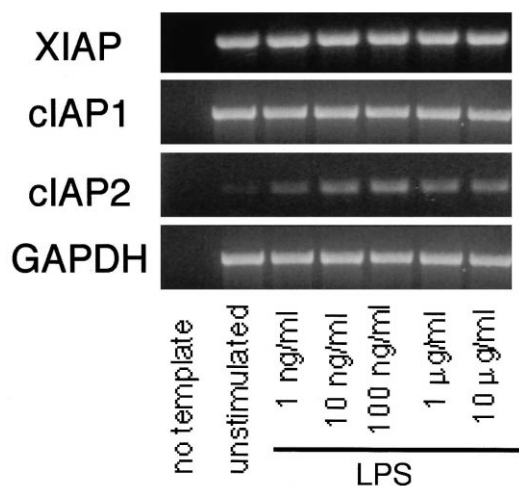


Fig. 2. Concentration-dependent induction, by LPS, of cIAP2 mRNA in differentiated U937 cells. The cells were pretreated with PMA and stimulated for 8 h with 1 ng–10 µg/ml LPS. mRNAs for IAPs and GAPDH were analyzed by RT-PCR.

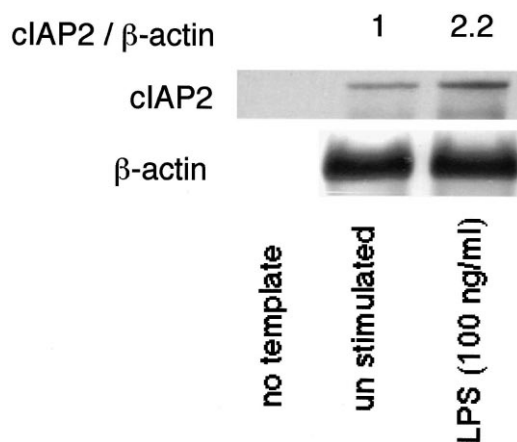


Fig. 3. Northern blotting of cIAP2 mRNA in U937 cells. The PMA-pretreated cells were stimulated for 8 h with 100 ng/ml LPS. Poly-(A)<sup>+</sup>RNA was isolated and subjected to electrophoresis. The RNA was blotted to a nylon membrane and probed with DIG-labeled antisense RNA for cIAP2 or β-actin.

PMA pretreatment (Fig. 1A); however, pretreatment of the cells with PMA for 48 h resulted in the induction of cIAP2 mRNA in response to LPS stimulation (Fig. 1B). This was unique to cIAP2, and U937 cells constitutively expressed mRNAs for cIAP1 and XIAP, which were not affected by PMA-induced differentiation or by LPS stimulation. The LPS-induced stimulation of cIAP2 expression in differentiated U937 cells was time-dependent and reached its maximal level after 4 h. This stimulation was also found to be concentration-dependent and maximal expression was observed with 100 ng/ml LPS (Fig. 2).

Fig. 3 shows the result of Northern blot analysis. The LPS-induced enhancement of cIAP2 expression in differentiated U937 cells was confirmed by Northern blotting. LPS enhanced the intensity of the cIAP2 band relative to that of β-actin by about 120%.

The expression of cIAP proteins was analyzed by Western blotting as shown in Fig. 4. Time-dependent produc-

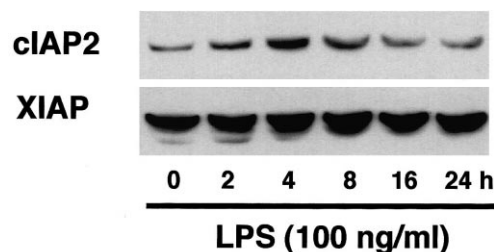


Fig. 4. Western blot analysis of LPS-induced cIAP2 protein expression in PMA-pretreated U937 cells. The cells were stimulated with 100 ng/ml LPS for up to 24 h and cell lysates were subjected to gradient SDS-PAGE. Proteins were transferred to a PVDF membrane, and the membrane was incubated sequentially with an anti-cIAP2, HRP-labeled anti-rabbit IgG and a chemiluminescent substrate. The membrane was exposed to an X-ray film. The membrane was also subjected to Western blot analysis for XIAP using an anti-XIAP monoclonal antibody and HRP-labeled anti-mouse IgG.

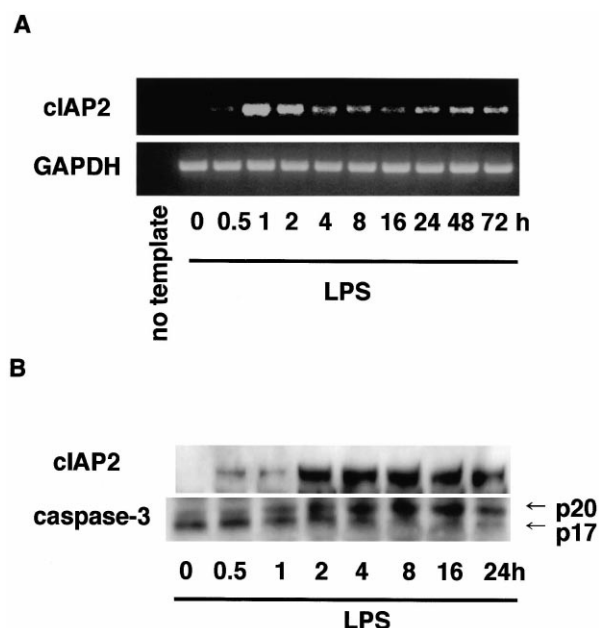


Fig. 5. LPS-induced changes in cIAP2 expression and caspase-3 activation of normal human macrophages. Monocyte-derived macrophages from healthy volunteers were stimulated with 100 ng/ml LPS for up to 72 h. (A) The levels of mRNA for cIAP2 were analyzed by RT-PCR. (B) Western blot analysis of LPS-induced expression of cIAP2 protein (upper panel). Western blot analysis of LPS-induced changes in caspase-3 activation (lower panel). Note that cIAP2 expression is associated with decreased p17 and increased p20.

tion of cIAP2 protein in U937 cells agreed with that of mRNA expression and reached its maximal level 4 h after stimulation with LPS, while XIAP protein levels remained unchanged. The LPS-induced expression of cIAP2 protein was also observed only in the cells pretreated with PMA.

LPS-induced expression of cIAP2 in normal human macrophages is summarized in Fig. 5. LPS enhanced the expression of cIAP2 mRNA (Fig. 5A) and protein (Fig. 5B) in normal human macrophages in culture. The result of Western blot analysis of caspase-3 is also shown in Fig. 5B. Stimulation of macrophages with LPS reduced the amount of p17 subunit of caspase-3 with a concomitant increase in p20 subunit, and this coincided with the induction of cIAP2.

#### 4. Discussion

LPS is a glycolipid component of the outer membrane of Gram-negative bacteria and has a variety of immunomodulating activities. It induces activation of various types of cells including monocytes/macrophages. LPS is known to induce apoptosis in endothelial cells [18,19] and hepatocytes [20], while it has a protective effect against apoptosis in macrophages and neutrophils [16,21,22]. LPS suppresses the apoptosis of U937 cells in-

duced by TNF- $\alpha$ , taxol or okadaic acid, but not by reactive oxygen species [16]. There are several potential molecular mechanisms for the antiapoptotic activity of LPS: activation of nuclear factor (NF)- $\kappa$ B [16,23], c-Jun N-terminal kinase [24] and p38 mitogen-activated protein kinase [16,25]. Induction of IAPs is mediated by NF- $\kappa$ B [26,27] and IAPs also depend on NF- $\kappa$ B activation to exert their antiapoptotic effect [26,28,29]. In the present study, we found the induction of cIAP2 mRNA by LPS in U937 cells differentiated with PMA. Although U937 cells express cIAP1 and XIAP, these are detected constitutively in both differentiated and undifferentiated U937 cells, and their expression was not altered by stimulation with LPS. We also found that LPS induced the expression of cIAP2 in human monocytes. LPS-induced cIAP2 expression was also confirmed with normal human macrophages.

Caspase-3 is the terminal effector protease in apoptosis induced by the caspase cascade [12]. Activation of caspase-3 proceeds through two steps: the first step is cleavage, by another caspase, of pro-caspase-3 (p32) into a p20 large subunit and a p12 small subunit, and the second is autocatalytic breakdown of p20 into p17, which is the active component [8,13]. XIAP and cIAPs are known to inhibit the autocatalytic generation of the p17 active component [8,13], and we also confirmed a decrease in p17 along with the LPS-induced expression of cIAP2 in normal macrophages.

These results may suggest that cIAP2 mediates, at least in part, LPS-induced antiapoptosis in macrophages. In fact, a recent study [30] demonstrated that IAPs are degraded, by the ubiquitin-proteasome system, during apoptotic cell death, suggesting the central role of these factors in the regulation of apoptosis. Besides LPS is shown to protect macrophages from apoptosis induced by microbial infection [21], and the induction of cIAP2 by LPS may provide a mechanism to maintain the immune functions of macrophages. Human cIAP2 is most abundantly expressed in the spleen and thymus [6], and this fact may also support an important role of this protein in the maintenance of the immune system-derived macrophages. In addition, caspase-3 activity to autocatalysis of the p20 subunit to the p17 subunit was inhibited in those macrophages according to the expression of cIAP2 protein.

In conclusion, LPS induces cIAP2 both in U937 cells differentiated with PMA and in normal human macrophages, and this may suggest that LPS protects macrophages from apoptotic cell death and thereby supports the immune function.

#### Acknowledgements

We thank Dr. Ryosuke Takahashi of the Riken Brain Science Institute for providing cIAP2 cDNA and Ms. Kumiko Munakata for technical assistance.

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