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Cytotoxic activity and probable apoptotic effect of Sph2, a sphigomyelinase hemolysin from *Leptospira interrogans* strain Lai

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Our previous work confirmed that Sph2/LA1029 was a sphigomyelinase-like hemolyisn of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Lai. Characteristics of both hemolytic and cytotoxic activities of Sph2 were reported in this paper. Sph2 was a heat-labile neutral hemolysin and had similar hemolytic behavior as the typical sphingomyelinase C of *Staphylococcus aureus* upon sheep erythrocytes. The cytotoxic activity of Sph2 was shown in mammalian cells such as BALB/C mouse lymphocytes and macrophages, as well as human L-02 liver cells. Transmission electron microscopic observation showed that the Sph2 treated BALB/C mouse lymphocytes were swollen and ruptured with membrane breakage. They also demonstrated condensed chromatin as a high-density area. Cytoskeleton changes were observed *via* fluorescence confocal microscope in Sph2 treated BALB/C mouse lymphocytes and macrophages, where both cytokine IL-1β and IL-6 were induced. In addition, typical apoptotic morphological features were observed in Sph2 treated L-02 cells *via* transmission electron microscope and the percentage of apoptotic cells did increase after the Sph2 treatment detected by flow cytometry. Therefore, Sph2 was likely an apoptosis-inducing factor of human L-02 liver cells. [BMB reports 2008; 41(2): 119-125]

INTRODUCTION

Leptospirosis (Weils syndrome) is a worldwide zoonosis caused by pathogenic species of *Leptospira*, particularly, *Leptospira interrogans*. Leptospirosis is manifested with jaundice and renal failure along with prominent respiratory symptoms.

With respect to the cytotoxicity of virulence factors produced by pathogenic leptospirea, glycolipoprotein fraction from *L. interrogans* was shown to be cytotoxic to mouse fibroblast cultures (1) and administration of lipopolysaccharide from *L. interrogans* induced apoptosis of lymphocytes in mice (1). On the other hand, the potential cytotoxic property of hemolysins, an important group of virulence factors widely produced by *L. interrogans*, is yet to be elucidated.

Genomic sequencing and annotation identified 10 genes encoding putative hemolysins in the large circular chromosome (CI, GB: AE010300) of *L. interrogans* strain Lai (2). All these genes were cloned and expressed in *Escherichia coli* except LA3540 and LA0177 (3). They were classified into two groups, sphingomyelinase and non-sphingomyelinase hemolysins based on whether they can hydrolyze sphingomyelin (3). Sph2/LA1029 had the strongest hemolytic activity among the four sphingomyelinase-like hemolysins (the other three: Sph1/LA1027, Sph3/LA4004 and Sph4/LA3050) and thus, was considered the representative sphingomyelinase-like hemolysin of strain Lai (3). Sph2 was secreted into the environment however, when cultivated in Korthof medium, the level of secretion of the virulent strain Lai was significantly higher ($P < 0.01$) than that of the avirulent conterpart strain IPAV (3). Therefore, it implied an important role in the pathogenesis of leptospirosis. In this study, both hemolytic and cytotoxic activites of Sph2 were tested in order to shed lights on its potential virulence effect on the pathogenesis of strain Lai.

RESULTS

Purification of Sph2 expressed in recombinant *Escherichia coli*

The Sph2 was successfully expressed in *E. coli* cells harboring the pET28b-LA1029 as a soluble N terminal His-tag fusion protein and then purified by affinity chromatography with the Ni-NTA His-Bind resin (Materials and Methods). The *in vivo* expressed protein in crude cell extracts, as well as the purified Sph2 were detected by SDS-PAGE with coomassie brilliant stain and the fusion protein was confirmed by western-blot assay em-

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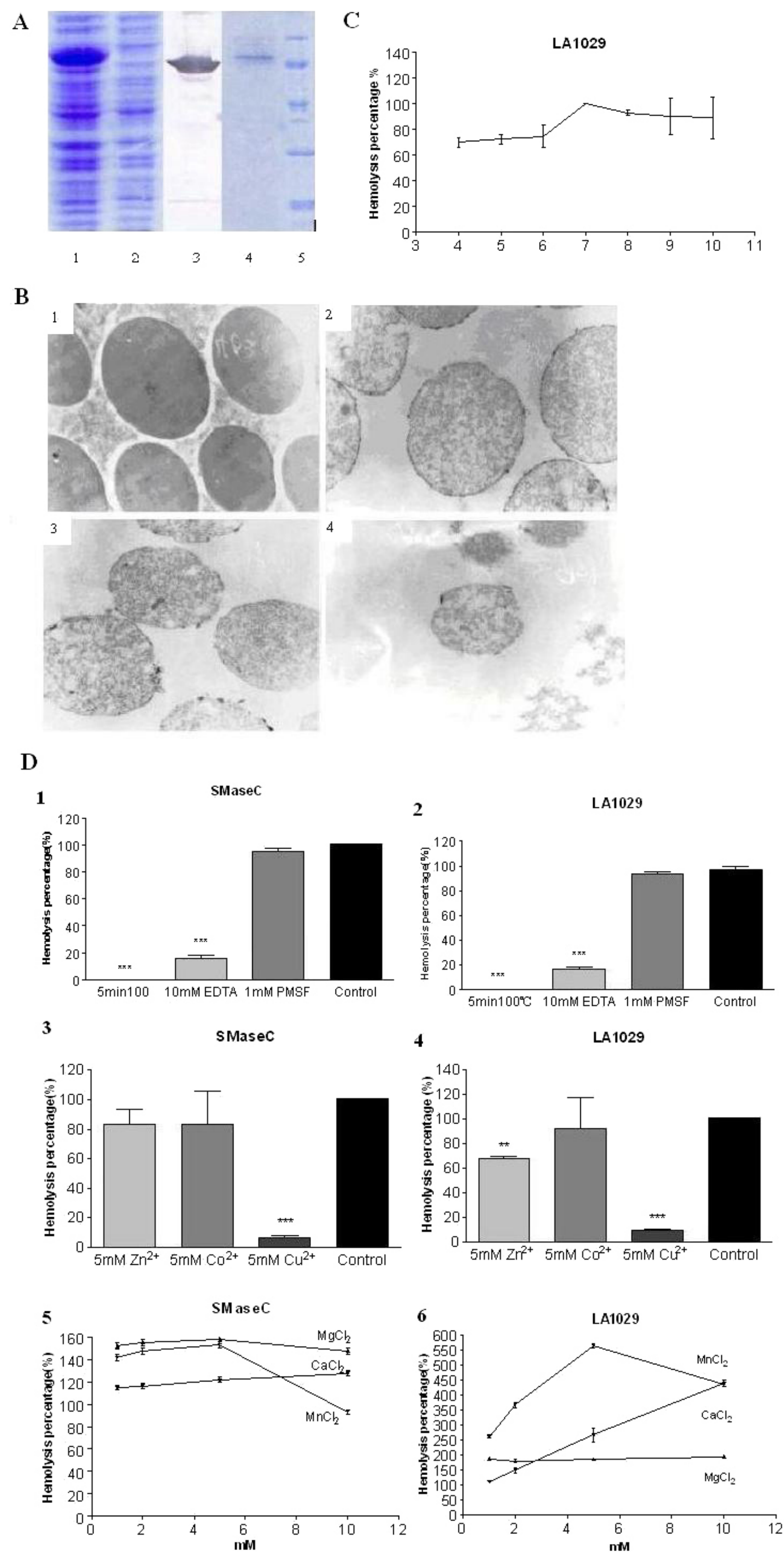

Fig. 1. Characterization of Sph2 expressed in recombinant *E. coli*. Hemolytic activity of Sph2 was examined on sheep erythrocytes *via* either spectrophotometrical determination of percentage of cell lysis or transmission electron microscopic observation as described in *Materials and Methods*. Panel A. Expression and purification of recombinant Sph2. All samples were from cell extracts of recombinant *E. coli* harboring different plasmids: Lane 1. *E. coli* harboring pET28b-LA1029; Lane 2. *E. coli* harboring pET28b; Lane 3. Western-blot result of *E. coli* harboring pET28b-LA1029; Lane 4. Purified recombinant LA1029; Lane 5. Protein marker (97.4, 66.2, 43.0, 31.0, 20.1, and 14.4 kDa). Panel B. Sheep erythrocytes changes observed *via* transmission electron microscope. Sub-panel 1: Negative control treated sheep erythrocytes. Sub-panels 2, 3 and 4: Sph2 treated sheep erythrocytes. Magnification: 10,000 ×. Panel C. Hemolytic activity of Sph2 under a serious of pH conditions. Levels of significance [***($P < 0.001$), **($P < 0.01$), *($P < 0.05$)] were evaluated using one-way ANOVA (Tukey's test) by prism 3.0. Panel D. Effect of PMSF and divalent cations upon hemolytic activity of Sph2.

ploying the mouse anti-His-tag monoclonal antibody (Fig. 1A).

Sph2 is a heat-labile neutral sphingomyelinase-like hemolysin that has strong hemolytic activity towards sheep erythrocytes

Previous sheep erythrocyte hemolysis assay on sheep blood agar plates indicated that Sph2/LA1029 was the strongest sphingomyelinase-like hemolysin among the four candidates encoded by *L. interrogans* strain Lai (3). In this study, cytoplasmic features of the hemolysis on sheep erythrocytes caused by Sph2 treatment were observed *via* transmission electron microscope. Cytoplasm of the negative control erythrocytes was electron-dense and their membrane was complete and integrated (Fig. 1B-1). However, for the Sph2 treated erythrocytes, the cytoplasm was electron-faint and their membrane was broken and occasionally disappeared leaving only the cytoplasm observed (Fig. 1B2-4).

The above-mentioned property of Sph2 offered an assay system to access its hemolytic activity and its response to environmental conditions. The optimal pH condition for Sph2 hemolytic activity is within the range of 7-10 (Fig. 1C), characteristic of a neutral hemolysin. The hemolytic behavior of Sph2 was quite similar to Sphingomyelinase C, a typical sphigomyelinase from *Staphylococcus aureus* (Fig. 1D1-6) and the hemolytic activities of both enzymes were completely lost after 5 min boiling, indicative of heat-labile. Addition of 1 mM PMSF showed almost no influence to their hemolytic activities while 10 mM EDTA may reduce the activities to one fifth of their original (Fig. 1D1-2). Although the hemolytic activities of both

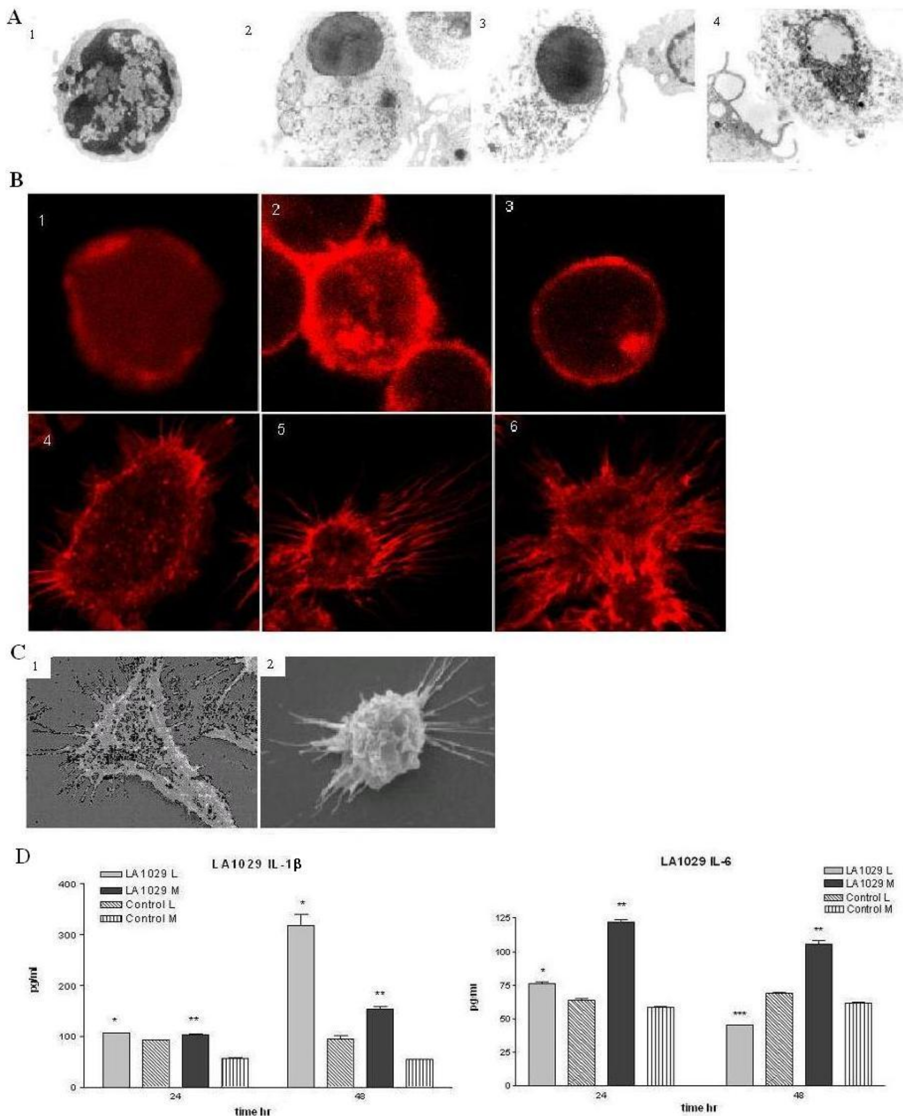

Fig. 2. Cellular morphologic and cytokine changes of Sph2 treated BALB/C mouse lymphocytes and macrophages. The experiments were performed as described in the *Materials and Methods*. Panel A. Cellular interior changes of Sph2 treated BALB/C mouse lymphocytes observed *via* transmission electron microscope. Sub-panel 1: The negative control treated mouse lymphocytes. Sub-panels 2, 3 and 4: Sph2 treated mouse lymphocytes. Magnification, 10,000 ×. Panel B. Cellular cytoskeleton changes of Sph2 treated BALB/C mouse lymphocytes and macrophages *via* fluorescence confocal microscope. Sub-panel 1: The negative control treated mouse lymphocytes. Sub-panels 2 and 3: Sph2 treated lymphocytes. Sub-panel 4: The negative control macrophages. Sub-panels 5 and 6: Sph2 treated macrophages. Panel C. Cellular surface changes of Sph2 treated BALB/C mouse macrophages *via* scanning electron microscope. Sub-panel 1: The control mouse macrophages. Sub-panel 2: Sph2 treated macrophages. Magnification, 5,000 ×. Panel D. Cytokine changes of Sph2 treated BALB/C mouse lymphocytes (L) and macrophages (M). Experiments were performed as described in *Materials and Methods*. Levels of significance [***($P < 0.001$), **($P < 0.01$), *($P < 0.05$)] were evaluated using one-way ANOVA (Tukey's test) by prism 3.0.

enzymes were completely inhibited by 5 mM Cu^{2+} or restrained to about 70% of the original in the presence of 5 mM Zn^{2+} or 5 mM Co^{2+} (Fig. 1D3-4), there was a little difference between Sph2 and SmaseC in response to Mg^{2+}, Mn^{2+} and Ca^{2+}. None of Mg^{2+} (1-10 mM), Mn^{2+} (1-10 mM) or Ca^{2+} (1-10 mM) demonstrated any stimulation effects upon the hemolytic activity of SmaseC (Fig. 1D-5). While in the case of Sph2, Mn^{2+} significantly stimulated the hemolysis of Sph2 at 4-6 mM as the maximal and Ca^{2+} demonstrated a linear stimulation effect over the whole concentration range of 1-10 mM (Fig. 1D-6).

The cytotoxic activity of Sph2 was shown by cellular morphologic changes of its treated BALB/C mouse lymphocytes and macrophages along with IL-1β/IL-6 induction

Transmission electron microscope observation showed that the negative control lymphocytes of BALB/C mouse, treated by the column-eluted cell crude extracts from *E. coli* harboring the pET28b vector, had well-defined clear outlines for whole cells and cellular organelles with clear short microvilli and smooth endoplasmic reticulum (Fig. 2A-1). Morphological changes of the cells were observed after 24 hours' exposure to Sph2. These cells were swollen and ruptured with breakage of their membranes. The short microvilli were disappeared and chromatin was condensed strongly, forming a high-density area (Fig. 2A2-4). Addition of rabbit anti-Sph2 antibody (1 : 1,024 dilution in PBS) to the lymphocytes assay system protected around 70% of the cells from the morphological change. Increase the titer of the antibody to about 10 times (1 : 128 dilution in PBS), the protection was almost complete (data not shown).

The reassembly of the F-actin of the cytoskeleton of BALB/C mouse lymphocytes and macrophages caused by Sph2 treatment was observed *via* fluorescence confocal microscopy (Fig. 2B). The cellular surface changes of BALB/C mouse macrophages caused by Sph2 treatment were observed *via* scanning electron microscopy. The pseudopod of the Sph2 treated macrophages was obviously longer than that of the controls (Fig. 2C).

The production of IL-1β and IL-6 from lymphocytes and

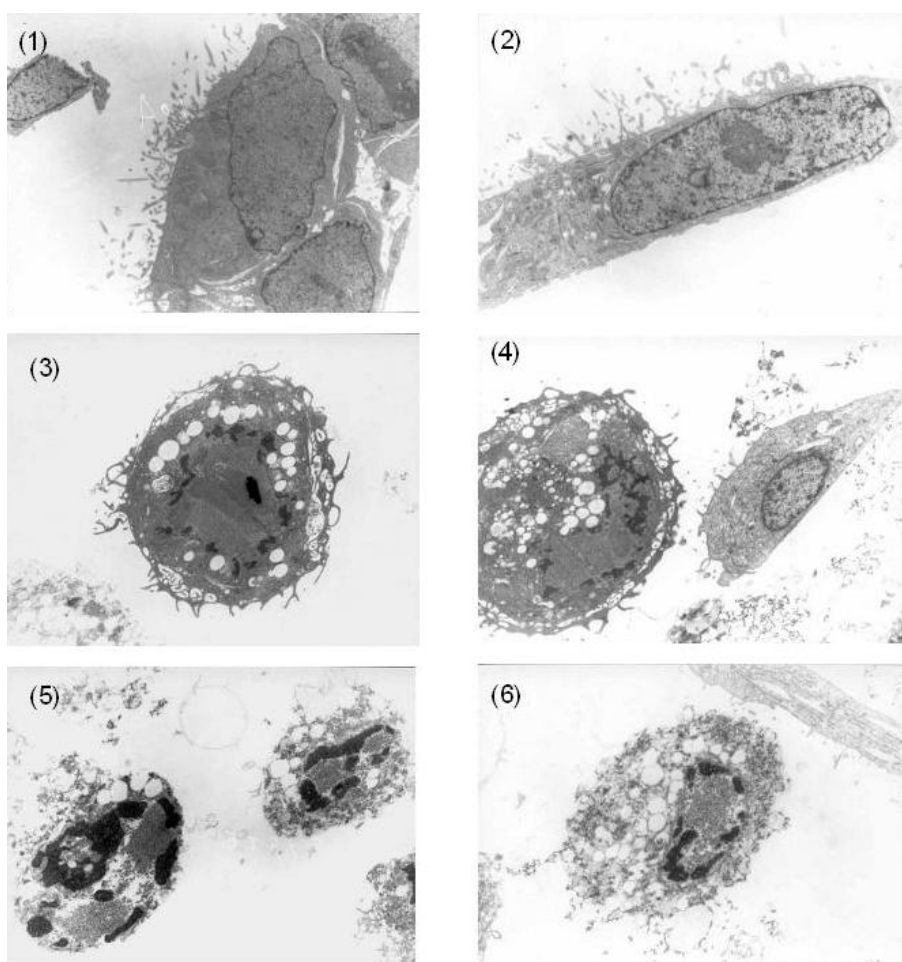

Fig. 3. Cellular interior changes of Sph2 treated human liver L-02 cells observed *via* transmission electron microscopy. Experiments were performed as described in *Materials and Methods* but only the data for 24 hr treatment are shown. Panels 1 and 2: The negative control treated human L-02 cells. Panels 3, 4, 5 and 6: Sph2 treated human L-02 cells. Magnification for Panel 1 is 3,500 ×; for Panel 2 is 4,000 × and for Panels 3-6 is 3,000 ×.

macrophages were stimulated remarkably by 24 hours' and 48 hours' Sph2 treatment (Fig. 2D). It is a biochemical/molecular biological indication of the cytotoxic activity of Sph2, which may also infer the effect of apoptosis.

Typical apoptotic morphological features were observed in hemolysin Sph2 treated human liver L-02 cells

Liver is one of the major infected organs for leptospirosis. Therefore, the cytotoxicity properties of the Sph2 were further tested on human liver L-02 cells (Fig. 3). The human L-02 cells treated with the column-eluted cell crude extracts from *E. coli* harboring the pET28b vector were used as a negative control. These control cells were generally in a good condition with a well-defined and clear outlines for both whole cells and cellular organelles, in addition to abundant short microvilli and smooth endoplasmic reticulum. After 24 hours' Sph2 treatment, the human L-02 cells were damaged obviously. Typical morphological features of apoptosis such as shrinkage of cell, margination of nuclear chromatin and altered cytoplasm with swollen mitochondria were all observed (Fig. 3). Apoptosis of human L-02 liver cells were confirmed *via* flow cytometry, which demonstrated that human liver L-02 cells underwent obvious apoptosis after treatment with Sph2 for 48 hr under the condition defined by the experiment (Table 1).

DISCUSSION

Hemolysins were classified into three categories based on the mechanism of action upon target cell membranes: enzymes (*e.g.*, sphigomyelinase), pore formation, and surfactant. Sphingomyelinase C from *L. interrogans* serovar Pomona can hydrolyze sphingomyelin of the target cell membranes causing cytolysis (4). Surfactant hemolysins such as the delta-toxin of *S. aureus* (5) are highly hydrophobic and can solubilize the target cell membranes. The pore-forming protein involves the binding of the toxin to the target cell membrane to form an ion-passing complex (6).

Previous work categorized the hemolysins from *L. interrogans* into two groups, the sphigomyelinase-like and the non-sphigomyelinase-like hemolysins (3). Sphigomyelin is one of the major components of cell membrane, *e.g.*, erythrocytes usually contain approximately 50% sphingomyelin in their membrane, while it may rise up to 60% in the case of sheep (7). Hydrolysis of sphigomyelin displayed the damage of cell membranes, and because sphingomyelin hydrolysis to ceramide with subsequent stimulation of a ceramide-linked kinase was inferred to be involved in a signal transduction pathway in HIV-1-infected HL-60 cells (8). The morphological changes of the Sph2 treated cells might relate to some apoptotic mechanisms yet to be studied.

We demonstrated that hemolysin Sph2 is a cytotoxin of *L. interrogans*. BALB/C mouse lymphocytes and macrophages were damaged by Sph2 treatment. Morphological changes of lymphocytes and macrophages were observed after exposure to Sph2 for 24 hr. Cells were swollen and ruptured with breakage of the membrane. Chromatin condensed strongly and formed a high-density area. Fluorescence intensity of Sph2 treated lymphocytes was weakened markedly. The reassembly of the F-actin of cytoskeleton showed the activation of the macrophages. Meanwhile, cytokine IL-1β and IL-6 were induced from Sph2 treated BALB/C mouse lymphocytes and macrophages. IL-1β is a potent immuno-modulator, which mediates a wide range of immune and inflammatory responses including the activation of B and T-cells. Staphylococcal α-toxin stimulates the secretion of IL-1β and tumor necrosis factor-α in human monocytes (9). IL-6 is a key cytokine mediator of the acute phase response to injury and infection (10).

Sph2 treated human liver L-02 cells displayed morphological features of apoptosis such as shrinkage of cell and nucleus, margination of nuclear chromatin, but only the breakage of membrane was observed in stead of the production of "apoptotic bodies" with intact membrane. Because the production of "apoptotic bodies" is considered the most important characteristic feature of apoptosis along with condensation of nuclear chromatin initiated from the nuclear periphery (11), we measured the percentage of apoptotic human liver L-02 cells induced by Sph2 *via* flow cytometry (Table 1). Under our experiment conditions (10 $\mu g.ml^{-1}$ Sph2), significant portions of cell apoptosis was observed but only after 48 hours' incubation, not at 24 hour. Previous *in vivo* assay for *L. interrogans* serogroup icterohaemorrhagiae infected guinea pig demonstrated that the peak of hepatocyte apoptosis in the liver was observed 48 hr post inoculation, which was the time when virulent leptospirea were present at low abundance (≤10 bacteria per microscopic field) and the apoptosis of hepatocytes seemed to be an early event in the infection process during leptospirosis (12). Because our *in vitro* study employed purified Sph2 instead of living bacteria and the production of Sph2 as well as other hemolysins from *L. interrogans* is yet to be analyzed, it is hard to quantitatively correlate these two results. However, concerning the evidence of hepatocytes apoptosis during the course of leptospirosis, our observation indicated that Sph2 is likely to be involved in this process.

One may imagine that the effect of hemolysin treatment is

Table 1. Percentage of apoptotic human liver L-02 cells treated with Sph2 over time (24 hr vs 48 hr)

Assays[a]	Treatment	24 hr (% apoptosis)	48 hr (% apoptosis)
1	Control[b]	0.90	1.31
	Sph2	0.00	14.73
2	Control[b]	21.07	25.04
	Sph2	20.95	71.40

[a]Experiments were performed as illustrated in *Materials and Methods*. Results of two independent assays were listed in the table.
[b]The column-eluted cell crude extracts of *E. coli* harboring the pET28b vector was used as the negative control.

cytoplasmic membrane lysis and thus, it would be difficult to observe intact membrane systems after hemolysin treatment. Our study of Sph2, particularly its cytotoxicity detected under *in vitro* conditions, has opened an area of research, not only for the virulence function of hemolysin, but also a probable novel definition of apoptosis even at its regulatory/signal transudation levels. Concerning the study of possible hepatocytic apoptosis *in vivo*, Sph2 might contribute to the typical symptom of leptospirosis beyond the liver damage based jaundice but also being related to the possible apoptosis *via* its own induction pathway.

MATERIALS AND METHODS

Expression and purification of Sph2

The cultured *E. coli* cells harboring the *sph2* expressing clone, pET28b-LA1029 (3) were harvested by centrifuging at 10,000 × *g* and resuspended in Tris-HCl buffer (20 mmol/L Tris HCl, pH 7.9) and lysed by sonication. The soluble supernatant (cell crude extracts, hereafter) was applied to Ni-NTA His-Bind resin and the N-terminal His-tag fusion Sph2 protein was eluted *via* a gradient of imidazole from 10 mmol/L to 1000 mmol/L in the elution buffer (20 mmol/L Tris-HCl pH 7.9, 0.5 mol/L NaCl, 10% glycerin). The gradient-eluted fractions as well as the final purified Sph2 was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie brilliant stain and followed by western-blot confirmation employing the mouse anti-his tag monoclonal antibody.

Hemolytic activity assay

Hemolytic activity of Sph2 was examined by adding Sph2 (10 $\mu g.ml^{-1}$) to a 10% (w/v) sheep erythrocyte suspension in PBS, pH 7.4, followed by incubation at 37°C for 30 min. Then, the hemolysis systems were centrifuged at 3,000 × *g* and the supernatant was measured spectrophotometrically at 420 nm and the percentage of hemolysis was calculated base on the A_{420nm} readings. SmaseC (0.5 U, from *S. aureus*, purchased from Sigma) was used as the positive control. The column-eluted cell crude extracts from *E. coli* harboring the pET28b vector was used as the negative control. To determine the optimal pH, assays were carried out under a serious of pH conditions ranging from 4 to 10. For testing the effects of divalent cations, assays were carried out in the presence of 1 mM PMSF, divalent cations (1-10 mM Mg^{2+}, 1-10 mM Mn^{2+}, 1-10 mM Ca^{2+}, 5 mM Co^{2+}, 5 mM Cu^{2+}, and 5 mM Zn^{2+}) or cation chelator (10 mM EDTA). These were individually added to the reaction mixture.

Transmission electron microscopic observation of Sph2 treated sheep erythrocytes

Sheep erythrocytes suspended in PBS (10% w/v) were incubated with *E. coli* cell crude extracts habouring recombinant hemolysin Sph2 (200 $\mu g.ml^{-1}$) at 37°C for 30 min. The sheep erythrocytes were centrifuged at 2,000 × *g* for 5 min, and prefixed with 2% (w/v) glutaraldehyde containing 0.1 M PBS at 4°C for 1 hr. The cell crude extracts of *E. coli* harboring only pET28b (200 $\mu g.ml^{-1}$) was used as the negative control. Subsequent steps were carried out as described (13). The samples were examined under an H-500 Transmission Electron Microscope (HITACHI, Holand).

Preparation of BALB/C mouse lymphocytes and macrophages and cytotoxic activity assays

Spleen was removed from BALB/C mouse (18-20 g) and crushed thoroughly. Ficol-Hypaque was used to separate and obtain lymphocytes. BALB/C mice were intraperitoneally injected with 1 ml 2.5% (w/v) thioglycolate and executed after 4 days. Macrophages obtained from abdominal cavity were cultured in culture plates for 1 hr and the suspended cells were removed. Those adherent cells were macrophages. Lymphocytes and macrophages were cultured in RPMI 1640 medium with 10% (w/w) fetal calf serum. To start the test, fresh RPMI 1640 medium with 2% (w/w) fetal calf serum was added to the cultured cells (5.0 × 10^6) and then incubated with Sph2 (10 $\mu g.ml^{-1}$) at 37°C for 24 or 48 hr. The Sph2 specific cytotoxic activity was confirmed by addition of rabbit anti-Sph2 antibody (prepared by our lab, 1 : 1024 dilution in PBS and 1 : 128 dilution in PBS) to protect the cells from morphological changes. The column-eluted cell crude extracts of *E. coli* haboruring the pET28b vector alone was used as the negative control.

Observation of Sph2 treated BALB/C mouse cells *via* transmission electron microscope, scanning electron microscope and fluorescence confocal microscope

Sample preparation for transmission electron microscopy was the same as described (13). BALB/C mouse lymphocytes incubated with Sph2 (10 $\mu g.ml^{-1}$) at 37°C for 24 hr were used to observe cellular interior changes *via* transmission electron microscopy.

Sample preparation for fluorescence confocal microscope: samples were washed three times in 0.01 M PBS and fixed with fresh-made 0.1% (w/v) sodium borohydrate for 15 min, treated with 2% (w/v) Triton X-100 for 15 min and washed again. Samples were blocked with philloidin for 45 min and washed by 0.01 M PBS. The images were observed by fluorescence confocal microscope. BALB/C mouse lymphocytes and macrophages incubated with Sph2 (10 $\mu g.ml^{-1}$) at 37°C for 24 hr were used to observe the cytoskeleton changes *via* fluorescence confocal microscopy.

Sample preparation for scanning electron microscopy: Sph2 (10 $\mu g.ml^{-1}$) treated macrophages were fixed with 2% (w/v) glutaraldehyde containing 0.1 M PBS at 4°C for 1 hr. Cells were dehydrated with alcohol in a series of concentration gradient and finally 100% acetone. The dried samples were sputtered with gold and examined under QUANTA-200 scanning electron microscope (PHILIP, USA). BALB/C mouse macrophages incubated with Sph2 (10 $\mu g.ml^{-1}$) at 37°C for 24 hr were used to observe cellular surface changes *via* scanning

electron microscopy.

Cytokine assay of Sph2 treated BALB/C mouse lymphocytes and macrophages

Lymphocytes and macrophages were incubated with Sph2 (10 $\mu g.ml^{-1}$) for 24 or 48 hr at 37^oC in a CO_2 incubator. IL-1β and IL-6 in the culture supernatants were detected with IL-1β and IL-6 ELISA Kits (Shanghai Senxiong Co., China) following the manufacturer's instruction.

Observation of human liver L-02 cells *via* transmission electron microscope

Human liver L-02 cells were cultured in RPMI 1640 medium with 10% (w/w) fetal calf serum. To start the test, fresh RPMI 1640 medium with 2% (w/w) fetal calf serum was added to the cultured cells and then incubated with Sph2 (10 $\mu g.ml^{-1}$) at 37^oC for 24 or 48 hr. The Sph2 treated human liver L-02 cells were then observed by transmission electron microscope (HITACHI, Japan). The column-eluted cell crude extracts of *E. coli* harboring the pET28b vector was used as the negative control.

Flow cytometry assay of Sph2 treated human liver L-02 cells

Sph2 treated human L-02 liver cells as described in section 2.7 were prefixed with 70% (w/v) ethanol at 4^oC for 2 hr, stained by propidium iodide (PI), and examined by flow cytometry. The experiment was repeated twice.

Statistical data treatment

Mean values and standard deviations (SD) of recorded parameters were calculated by prism 3.0 (n = 3). Levels of significance were evaluated using one-way ANOVA (Tukey's test) by prism 3.0.

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