TNF-α-Induced Sphingosine 1-Phosphate Inhibits Apoptosis Through a Phosphatidylinositol 3-Kinase/Akt Pathway in Human Hepatocytes

Yosuke Osawa, Yoshiko Banno, Masahito Nagaki, David A. Brenner, Takafumi Naiki, Yoshinori Nozawa, Shigeru Nakashima and Hisataka Moriwaki

*J Immunol* 2001; 167:173-180;

doi: 10.4049/jimmunol.167.1.173

http://www.jimmunol.org/content/167/1/173

**References**

This article cites 50 articles, 32 of which you can access for free at:

http://www.jimmunol.org/content/167/1/173.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
TNF-α-Induced Sphingosine 1-Phosphate Inhibits Apoptosis Through a Phosphatidylinositol 3-Kinase/Akt Pathway in Human Hepatocytes

Yosuke Osawa,* Yoshiko Banno,† Masahito Nagaki,* David A. Brenner,‡ Takafumi Naiki,* Yoshinori Nozawa,§ Shigeru Nakashima,‡ and Hisataka Moriwaki2*

Human hepatocytes usually are resistant to TNF-α cytotoxicity. In mouse or rat hepatocytes, repression of NF-κB activation is sufficient to induce TNF-α-mediated apoptosis. However, in both HuH-7 human hepatoma cells and Hc human normal hepatocytes, when infected with an adenovirus expressing a mutated form of IκBα (Ad5IκBα), which almost completely blocks NF-κB activation, >80% of the cells survived 24 h after TNF-α stimulation. Here, we report that TNF-α activates other antiapoptotic factors, such as sphingosine kinase (SphK), phosphatidylinositol 3-kinase (PI3K), and Akt kinase. Pretreatment of cells with N,N-dimethylsphingosine (DMS), an inhibitor of SphK, or LY 294002, an inhibitor of PI3K that acts upstream of Akt, increased the number of apoptotic cells induced by TNF-α in Ad5IκBα-infected HuH-7 and Hc cells. TNF-α-induced activations of PI3K and Akt were inhibited by DMS. In contrast, exogenous sphingosine 1-phosphate, a product of SphK, was found to activate Akt and partially rescued the cells from TNF-α-induced apoptosis. Although Akt has been reported to activate NF-κB, DMS and LY 294002 failed to prevent TNF-α-induced NF-κB activation, suggesting that the antiapoptotic effects of SphK and Akt are independent of NF-κB. Furthermore, apoptosis mediated by Fas ligand (FasL) involving Akt activation also was potentiated by DMS pretreatment in Hc cells. Sphingosine 1-phosphate administration partially protected cells from FasL-mediated apoptosis. These results indicate that not only NF-κB but also SphK and PI3K/Akt are involved in the signaling pathway(s) for protection of human hepatocytes from the apoptotic action of TNF-α and probably FasL.


1 Abbreviations used in this paper: IAP, inhibitor of apoptosis protein; SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; Edg, endothelial differentiation gene; PI3K, phosphatidylinositol 3-kinase; TRAIL, TNF-related apoptosis-inducing ligand; FasL, Fas ligand; DMS, N,N-dimethylsphingosine; DHPS, dihydro-3-hydroxysphingosine; PTX, pertussis toxin; PARP, poly(ADP-ribose) polymerase; HPTLC, high-performance TLC; Ad5, adenovirus 5; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PKC, protein kinase C.

Copyright © 2001 by The American Association of Immunologists

Received for publication December 5, 2000. Accepted for publication April 26, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (10670462) and by the Research Group of Intractable Liver Diseases sponsored by the Ministry of Health and Welfare of Japan.

2 Address correspondence and reprint requests to Dr. Hisataka Moriwaki, First Department of Internal Medicine, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500-8705, Japan. E-mail address: hmori@cc.gifu-u.ac.jp

*First Department of Internal Medicine and † Department of Biochemistry, Gifu University School of Medicine, Gifu, Japan; Departments of Medicine, Biochemistry, and Biophysics, University of North Carolina, Chapel Hill, NC 27599; and ‡ Gifu International Institute of Biotechnology, Institute of Applied Biochemistry, Gifu, Japan

Human hepatocytes are usually resistant to TNF-α cytokotoxicity. In mouse or rat hepatocytes, repression of NF-κB activation is sufficient to induce TNF-α-mediated apoptosis. However, in both HuH-7 human hepatoma cells and Hc human normal hepatocytes, when infected with an adenovirus expressing a mutated form of IκBα (Ad5IκBα), which almost completely blocks NF-κB activation, >80% of the cells survived 24 h after TNF-α stimulation. Here, we report that TNF-α activates other antiapoptotic factors, such as sphingosine kinase (SphK), phosphatidylinositol 3-kinase (PI3K), and Akt kinase. Pretreatment of cells with N,N-dimethylsphingosine (DMS), an inhibitor of SphK, or LY 294002, an inhibitor of PI3K that acts upstream of Akt, increased the number of apoptotic cells induced by TNF-α in Ad5IκBα-infected HuH-7 and Hc cells. TNF-α-induced activations of PI3K and Akt were inhibited by DMS. In contrast, exogenous sphingosine 1-phosphate, a product of SphK, was found to activate Akt and partially rescued the cells from TNF-α-induced apoptosis. Although Akt has been reported to activate NF-κB, DMS and LY 294002 failed to prevent TNF-α-induced NF-κB activation, suggesting that the antiapoptotic effects of SphK and Akt are independent of NF-κB. Furthermore, apoptosis mediated by Fas ligand (FasL) involving Akt activation also was potentiated by DMS pretreatment in Hc cells. Sphingosine 1-phosphate administration partially protected cells from FasL-mediated apoptosis. These results indicate that not only NF-κB but also SphK and PI3K/Akt are involved in the signaling pathway(s) for protection of human hepatocytes from the apoptotic action of TNF-α and probably FasL.
but independent of the NF-κB pathway. These results indicate that TNF-α simultaneously but independently activates at least these two survival signaling pathways. Human hepatocytes can be sensitized to TNF-α-mediated apoptosis by blocking these pathways.

Materials and Methods

Materials

Huh-7 cells, a human hepatoma cell line, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Hc cells (normal human hepatocytes) were purchased from the Applied Cell Biology Research Institute (Kirkland, WA). Cell culture media for Huh-7 (RMPI 1640) and Hc cells (CS-C complete) were obtained from Life Technologies (Rockville, MD) and from Cell Systems (Kirkland, WA), respectively. Recombinant human TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) were obtained from Genzyme (Cambridge, MA). Fas ligand (FasL) was obtained from Upstate Biotechnology (Lake Placid, NY). N,N-dimethylsphingosine (DMS), d-threo-dihydrosphingosine (DHS), sphingosine, and SIP were obtained from Matreya (Pleasant Gap, PA). Pertussis toxin (PTX), PD 98059, and GF 109203X were obtained from Calbiochem-Novabiochem (La Jolla, CA). LY 294002 was obtained from Alexis (San Diego, CA). Hoechst 33258 (bisbenzimide) staining dye was obtained from Wako (Osaka, Japan). Anti-phosphotyrosine Ab (PY20) was obtained from the Peptide Institute (Osaka, Japan). [3-32P]ATP was obtained from ICN Biomedicals (Costa Mesa, CA). [14C]Serine was obtained from Amersham-Pharmacia Biotech (Buckinghamshire, England). Ricin A-chain was obtained from the National Cancer Institute (Bethesda, MD) and from Cell Systems (Kirkland, WA), respectively. Recombinant TNF-α, human TNF-β, TNF-β (ecto-enzymatic), and TNF-β (ecto-enzymatic) were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and from Cell Systems (Kirkland, WA), respectively. Recombinant TNF-β (ecto-enzymatic) was obtained from the Peptide Institute (Osaka, Japan). [125I]Sialyl Lewis X, 20–100 ng/ml TRAIL, or 20–100 ng/ml FasL, the cells were incubated with icole-cholic acid, 0.3 mM PMSF, and 30 mM NaCl, 10 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% deoxyribonuclease, cells were sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5 mM sodium orthovanadate, 1 mM sodium molybdate, and 0.5 mM 4-deoxypyridoxine). After ultracentrifugation at 100,000 × g for 30 min, the supernatant was measured by incubation with 20 μM sphingosine-BSA complex and [γ-32P]ATP (1 Ci-assay) for 30 min at 37°C in reaction buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 0.5 mM EDTA, 20% glycerol, 1.2 mM DTT, and 0.5 mM 4-deoxypyridoxine). The reaction was stopped by adding HCl to obtain a 0.1 M final concentration. Radiolabeled lipids were separated on HPTLC plates in the solvent system described above. After autoradiography, the spot corresponding to SIP was scraped off the plate and the radioactivity was measured. The Spk activity was normalized based on the total protein. Protein concentrations were assayed by using the Bradford protein assay reagent with BSA as a standard.

Western blot analysis

Cytosolic proteins were used for the Western blot analysis of Akt and phospho-Akt, and the total cellular protein extracts were used for PARP detection. For isolation of cytosolic proteins, cells were sonicated in lysis buffer (10 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.1 mM sodium molybdate, and 0.5 mM 4-deoxypyridoxine). After ultracentrifugation at 100,000 × g for 30 min, the supernatant was used for the cytosolic fraction. For the preparation of total cell proteins, cells were sonicated in RIPA buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 0.3 mM PMSF, and 30 μg/ml (3-3,trans-carboxyoxirane-2-carbonyl)-1-leucyl-agaritine). The proteins were separated by SDS-PAGE and were electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were probed with the Abs against Akt, phospho-Akt, and PARP, and then incubated with the anti-rabbit IgG HRP-coupled secondary Ab. Detection was performed with an ECL system.

Measurement of P38 activity

PI3K activity was measured as described previously (25). Briefly, Hc cells were washed with ice-cold PBS and sonicated in lysis buffer (10 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.1 mM sodium molybdate, and 0.5 mM 4-deoxypyridoxine). After ultracentrifugation at 100,000 × g for 30 min, the supernatant was measured by incubation with 20 μM sphingosine-BSA complex and [γ-32P]ATP (1 Ci-assay) for 30 min at 37°C in reaction buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 0.5 mM EDTA, 20% glycerol, 1.2 mM DTT, and 0.5 mM 4-deoxypyridoxine). The reaction was stopped by adding HCl to obtain a 0.1 M final concentration. Radiolabeled lipids were separated on HPTLC plates in the solvent system described above. After autoradiography, the spot corresponding to SIP was scraped off the plate and the radioactivity was measured. The Spk activity was normalized based on the total protein. Protein concentrations were assayed by using the Bradford protein assay reagent with BSA as a standard.
methanol/25% ammonia/water (43:38:5.7, v/v; Ref. 25). After autoradiography, the radioactive phosphatidylinositol 3-phosphate spot was scraped off the plate and the radioactivity was measured.

Results

Inhibition of NF-κB activation and apoptotic cell death in TNF-α-treated human hepatocytes infected with Ad51kB

TNF-α induced NF-κB activation within 5 min in both human hepatoma Huh-7 cells (Fig. 1A) and normal human hepatocyte Hc cells (Fig. 1B). The activated NF-κB complex in Huh-7 cells was mainly composed of p50-p65 heterodimers, whereas Hc cells contained p50-p50 homodimers as well as heterodimers, as determined by supershifts. NF-κB activation by TNF-α was almost abolished when Huh-7 and Hc cells were infected with Ad51kB but not with control adenovirus Ad5GFP (Fig. 1). Because of missense mutations at phosphorylation sites, where serines 32 and 36 are replaced with alanines, the mutant IκB irreversibly binds to NF-κB and prevents its activation (6).

Normal mouse or rat hepatocytes are usually resistant to the cytotoxicity of TNF-α. However, infection with Ad51kB sensitizes hepatocytes to TNF-α-mediated apoptosis (6, 13, 26). To assess whether the NF-κB inactivation sensitizes human hepatocytes to apoptosis by TNF-α, Huh-7 and Hc cells were infected with Ad51kB. The adenovirus infection or TNF-α alone did not cause apoptotic changes (data not shown). TNF-α treatment induced only 10–20% apoptosis in Huh-7 and Hc cells infected with Ad51kB at 24 h, as inferred by Hoechst 33258 staining (Table I). The sensitizing effect by Ad51kB was much less in human hepatocytes than in the mouse and rat hepatocytes observed in previous reports (6, 26).

Enhancement of TNF-α-induced apoptosis by inhibition of SphK or PI3K

The above results indicate that survival signal(s) other than NF-κB may be activated by TNF-α treatment of human hepatocytes. Previous studies demonstrated that SphK (14), Akt (18, 19), and extracellular signal-regulated kinase (ERK) (27), which have been proposed to mediate antiapoptotic actions in several cell types, were activated by TNF-α. To gain further insight into the mechanisms of self-protection by TNF-α in human hepatocytes, the roles of SphK, PI3K, and ERK were examined in the presence of their specific or selective inhibitors. Pretreatment of Ad51kB-infected Huh-7 cells with SphK inhibitors (DMS and DHS) greatly enhanced TNF-α-induced apoptosis (Fig. 2A). The extent of apoptotic cell death was 71.4% and 50.1% at 24 h in the presence of 10 μM DMS or 10 μM DHS, respectively. In contrast, death rate was 12.0% in the absence of DMS and DHS. The PI3K inhibitor LY 294002 (25 μM) also brought about a fourfold increase in cell death. In contrast, PD 98059 (100 μM), a commonly used inhibitor of mitogen-activated protein kinase/ERK kinase (MEK) 1, had no effect on TNF-α-induced apoptosis in Ad51kB-infected cells (Fig. 2B).%

Table 1. Inhibition of NF-κB activation by Ad51kB-sensitized hepatocytes to TNF-α-induced apoptosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Apoptotic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Huh-7</td>
</tr>
<tr>
<td>TNF</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Ad51kB + TNF</td>
<td>12.0 ± 1.0*</td>
</tr>
<tr>
<td>Ad5GFP + TNF</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Huh-7 cells and Hc cells uninfected or infected with Ad51kB or Ad5GFP were treated with 20 ng/ml TNF-α for 24 h. Typical apoptotic cells stained with Hoechst 33258 were counted among >1000 cells and percent cell apoptosis was determined. Data are means ± SD from three independent experiments, each performed in triplicate.

FIGURE 1. TNF-α induced NF-κB activation and its inhibition by Ad51kB. Nuclear extracts were prepared from Huh-7 cells (A) and Hc cells (B) at the time indicated after exposure to 20 ng/ml TNF-α (center). To examine the effect of Ad51kB, uninfected control cells (C) or cells infected with Ad5GFP or Ad51kB were incubated for 15 min with TNF-α (right). The samples were incubated with radiolabeled probe, and gel shift assays were performed as described in Materials and Methods. For the supershift assays, the reaction mixture was incubated with anti-p50 NF-κB or anti-p65 NF-κB Abs (Ab; left). The positions of the p50/p65 NF-κB heterodimer and p50 homodimers are marked as NF-κB and (p50)2, respectively, comp. A sample of cells treated for 15 min with TNF-α was incubated with a 50-fold excess of unlabeled oligonucleotide. The results shown are representative of at least two independent experiments.

FIGURE 2. Enhancement by SphK inhibitors (DMS and DHS) and PI3K inhibitor (LY 294002) of TNF-α-induced apoptosis in human hepatocytes infected with Ad51kB. Huh-7 cells (A) and Hc cells (B) infected with Ad51kB were pretreated with 10 μM DMS, 10 μM DHS, 25 μM LY 294002 (LY), or 100 μM PD 98059 (PD) for 1 h and then exposed to 20 ng/ml TNF-α for 24 h. Typical apoptotic cells stained with Hoechst 33258 were counted among >1000 cells, and the percentage of cell apoptosis was determined. Data are means ± SD from three independent experiments, each performed in triplicate. C. Effects of DMS or LY294002 on TNF-α-induced NF-κB activation in both Ad51kB-uninfected (left) and -infected (right) Hc cells. Nuclear extracts were isolated from Ad51kB-infected or -uninfected Hc cells that were untreated or treated for 15 min with TNF-α alone or with DMS + TNF-α or LY 294002 + TNF-α. Gel shift assays were performed as described in Fig. 1. comp, Presence of a 50-fold excess of unlabeled oligonucleotide. The results shown are representative of at least two independent experiments. C. Control.
2A), although activation of ERK1/2 was abrogated, as assessed by phosphorylation with anti-phospho-ERK1/2 Abs (data not shown). Apoptotic death of Hc cells infected with Ad5IxB also was augmented by 10 μM DMS and 25 μM LY 294002 (Fig. 2B). However, NF-κB activation induced by TNF-α was not prevented by either DMS or LY 294002 (Fig. 2C), indicating that NF-κB activation was independent of SphK and PI3K/Akt. These data suggest that SphK and PI3K play important roles in mediating survival signaling in an NF-κB-independent manner. Interestingly, DMS (10 μM) pretreatment sensitized both Huh-7 and Hc cells to TNF-α-induced apoptosis without Ad5IxB infection (Table II). In contrast, LY 294002 itself did not sensitize to TNF-α-mediated apoptosis without simultaneous Ad5IxB infection (Table II).

DNA fragmentation and caspase activation in TNF-α-induced apoptosis

Previous studies have demonstrated that TNF-α causes caspase activation and PARP cleavage in rat hepatocyte RALA255-10G cells infected with Ad5IxB (6, 28). To assess whether caspase activation occurs, cleavage of PARP, a substrate for caspasas, was examined by Western blot analysis. PARP cleavage occurred within 24 h after TNF-α treatment (Fig. 3A). Moreover, a broad-spectrum caspase inhibitor, z-VAD-FMK, completely prevented cell death and PARP cleavage (Fig. 3B). This finding indicates that apoptosis induced by TNF-α is dependent on caspase activation. Apoptotic cell death and its enhancement by DMS and LY 294002 also were confirmed by fragmentation of chromosomal DNA (Fig. 3C).

SphK activation and S1P formation induced by TNF-α

To further explore the role of SphK, SphK activity and the amount of S1P produced were measured in Hc cells stimulated with TNF-α. The TNF-α treatment caused a rapid increase in SphK activity, which reached a maximum within 10 min (Fig. 4A1). Consistent with SphK activation, the S1P level elevated rapidly, peaking at 10 min after TNF-α treatment (Fig. 4B1). DMS inhibited TNF-α-induced SphK activation (Fig. 4A2), which in turn led to reduced S1P generation (Fig. 4B2) in a concentration-dependent manner in Hc cells.

Activation of the PI3K/Akt pathway by TNF-α through SphK activation

The enhancement of TNF-α-induced apoptosis by LY 294002 suggests the involvement of PI3K in human hepatocyte resistance to TNF-α-mediated apoptosis. In fact, TNF-α effected a 3.5-fold increase in the activation of PI3K in Hc cells 10 min after the administration of TNF-α (Fig. 5A). At 25 μM, LY 294002 completely abolished the TNF-α-induced activation of PI3K. Akt, a pivotal factor in cell survival (18–21) and downstream effector of PI3K (29), has been reported to be activated by TNF-α (16–19).

<table>
<thead>
<tr>
<th>Table II. Sensitization to TNF-α cytotoxicity and enhancement of TNF/Ad5IxB-induced apoptosis by DMS and LY 294002*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>TNF</td>
</tr>
<tr>
<td>Ad5IxB + TNF</td>
</tr>
<tr>
<td>Ad5GFP + TNF</td>
</tr>
</tbody>
</table>

* Huh-7 cells and Hc cells uninfected or infected with Ad5IxB or Ad5GFP were treated with 20 ng/ml TNF-α for 24 h following preincubation with 10 μM DMS or 25 μM LY 294002 for 1 h. Typical apoptotic cells stained with Hoechst 33258 were counted among >1000 cells, and the percentage of cell apoptosis was determined. Data are means ± SD from three independent experiments, each performed in triplicate.
absence of DMS using Student’s t means). Moreover, exogenous S1P (1C) only partially inhibited (Fig. 7). DMS could not inhibit the PI3K activation caused by S1P. At 1 in protecting human hepatocytes against apoptosis by TNF-α. These data indicate that the SphK/S1P pathway play a critical role in protecting human hepatocytes against apoptosis by TNF-α. Therefore, PKC did not mediate TNF-α-induced Akt activation. These findings indicate that TNF-α activates the PI3K/Akt pathway through SphK activation.

Accumulating evidence indicates that S1P formed by SphK acts not only as an autocrine and/or paracrine ligand via the Edg receptor(s), but also as an intracellular second messenger (31). Accordingly, the effect of exogenous S1P on the PI3K/Akt pathway was examined. At 1 µM, S1P caused PI3K activation, but it was inhibited by LY 294002 (25 µM) (Fig. 7A). In contrast, 30 µM DMS could not inhibit the PI3K activation caused by S1P. At 1 µM, S1P was noted to activate Akt as early as 2 min after administration (Fig. 7B). The involvement of Edg receptor(s) in the activation of Akt was examined in Hc cells pretreated with 100 ng/ml PMA (data not shown). Therefore, PKC did not mediate TNF-α-induced Akt activation. These findings indicate that TNF-α activates the PI3K/Akt pathway way through SphK activation.

Enhancement of FasL-induced apoptosis by DMS and its partial reversal by S1P

To further investigate whether the survival signal via SphK/S1P pathway modulates hepatocyte apoptosis mediated by other death receptors, the effects of TRAIL and FasL were examined in Hc cells. Activation of NF-κB and Akt were discernible by stimulation with FasL at 100 ng/ml (Fig. 8, A and B). In contrast, TRAIL (20–200 ng/ml) failed to activate these survival factors (Fig. 8, A and B). FasL (100 ng/ml) alone could induce apoptosis of Hc cells, as we previously reported in in vivo mouse hepatic failure model (5). Pretreatment of Ad5IκB and/or DMS potentiated FasL-induced apoptosis (Table IV), and the effects of these factors were additive. Exogenous S1P (1 µM) significantly protected Hc cells from apoptosis induced by FasL in the presence of DMS (Table IV). In contrast, 20 ng/ml TRAIL failed to induce apoptosis of Hc cells treated with 20 ng/ml TNF-α for 10 min in the absence or presence of 25 µM LY 294002 (LY). PI3K activities were determined as described in Materials and Methods. B. Hc cells were treated with 20 ng/ml TNF-α for the indicated periods of time or for 15 min in the absence or presence of 25 µM LY 294002. Extracted proteins were subjected to SDS-PAGE, and immunoblotting was performed with anti-phosphorylated Akt and Akt Abs. The results shown are representative of at least two independent experiments.

Enhancement of FasL-induced apoptosis by DMS and its partial reversal by S1P

To further investigate whether the survival signal via SphK/S1P pathway modulates hepatocyte apoptosis mediated by other death receptors, the effects of TRAIL and FasL were examined in Hc cells. Activation of NF-κB and Akt were discernible by stimulation with FasL at 100 ng/ml (Fig. 8, A and B). In contrast, TRAIL (20–200 ng/ml) failed to activate these survival factors (Fig. 8, A and B). FasL (100 ng/ml) alone could induce apoptosis of Hc cells, as we previously reported in in vivo mouse hepatic failure model (5). Pretreatment of Ad5IκB and/or DMS potentiated FasL-induced apoptosis (Table IV), and the effects of these factors were additive. Exogenous S1P (1 µM) significantly protected Hc cells from apoptosis induced by FasL in the presence of DMS (Table IV). In contrast, 20 ng/ml TRAIL failed to induce apoptosis of Hc cells treated with 20 ng/ml TNF-α for 10 min in the absence or presence of 25 µM LY 294002 (LY). PI3K activities were determined as described in Materials and Methods. B. Hc cells were treated with 20 ng/ml TNF-α for the indicated periods of time or for 15 min in the absence or presence of 25 µM LY 294002. Extracted proteins were subjected to SDS-PAGE, and immunoblotting was performed with anti-phosphorylated Akt and Akt Abs. The results shown are representative of at least two independent experiments.
cells, even when cells were treated with both Ad5IκB and DMS (data not shown).

Discussion

TNF-α is a potent mediator of hepatotoxicity in vivo and in cultured cells (5, 6, 11, 12). However, TNF-α alone cannot induce apoptosis in normal hepatocytes (5, 6). A sensitization step is required for the induction of apoptosis, because TNF-α also activates antiapoptotic signal pathway(s). Blockage of TNF-α-induced activation of the transcriptional factor NF-κB, which induces the expression of protective genes, is sufficient to induce apoptosis in cultured rat hepatocyte RALA255-10G cells (6) and in rat hepatocytes after partial hepatectomy (13). TNF-α treatment killed 88% of the primary cultured mouse hepatocytes (26) and 50% of the RALA255-10G rat hepatocytes (6) infected with Ad5IκB. However, in human hepatocyte cell lines (Huh-7 and Hc cells), inhibition of NF-κB by Ad5IκB was insufficient to induce massive cell death by TNF-α treatment (Table I). These results led us to consider the involvement of additional survival signaling factor(s) other than NF-κB. Therefore, we have attempted to reveal the mechanism(s) of resistance to TNF-α-mediated apoptosis in human hepatocytes. The results obtained in the present study indicate that TNF-α induces not only NF-κB activation but also S1P generation via SphK, which activates survival signals such as the PI3K/Akt pathway and protects human hepatocytes from TNF-α-induced apoptosis independently of NF-κB.

In several types of cells, such as HEK293 and COS7 cells, TNF-α induces ceramide formation by the activation of sphingomyelinase (32, 33). Ceramide is further hydrolyzed by ceramidase to sphingosine, which subsequently is converted to S1P by SphK. Ceramide is thought to be a second messenger involved in the apoptotic process (34, 35). Thus, the balance between intracellular concentrations of ceramide and S1P may be a critical factor in the determination of cell fate (36, 37). Therefore, perhaps the SphK inhibitor DMS enhances the ceramide level by sphingosine accumulation and leads to TNF-α-mediated apoptosis. However, ceramide accumulation was undetectable at least within the first 30 min after TNF-α treatment, and DMS did not alter the ceramide level in TNF-α-treated Hc cells (data not shown). Ceramide appears to induce hepatocyte apoptosis via a caspase-independent pathway (38). In contrast, the apoptosis enhanced by DMS was

Table III. Protective effect of S1P on TNF-α-induced apoptosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Huh-7</th>
<th>Hc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>S1P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS + TNF</td>
<td>29.6 ± 3.9</td>
<td>19.4 ± 2.1**</td>
</tr>
<tr>
<td>Ad5IκB + DMS + TNF</td>
<td>71.4 ± 5.0</td>
<td>33.6 ± 8.2**</td>
</tr>
<tr>
<td></td>
<td>42.1 ± 3.3</td>
<td>61.4 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>30.0 ± 3.2*</td>
<td>38.9 ± 1.4***</td>
</tr>
</tbody>
</table>

*Huh-7 cells and Hc cells infected with or without Ad5IκB were preincubated with 10 μM DMS for 1 h and then treated with 20 ng/ml TNF-α for 24 h in the absence or presence of 1 μM S1P. Typical apoptotic cells stained with Hoechst 33258 were counted among >1000 cells and percent cell apoptosis was determined. Data are means ± SD from three independent experiments, each performed in triplicate. *p < 0.05; **p < 0.01 vs in the absence of S1P by Student’s t test.
dependent on caspase activation (Fig. 3). Therefore, the potentiation of apoptosis by DMS is not attributable to ceramide accumulation, and the activation of SphK is one of the critical steps for the antiapoptotic action. However, the regulatory mechanisms of SphK activation and S1P formation remain unclear.

TNF-α activated PI3K and Akt in HeLa cells. Activation of Akt via PI3K has been considered to protect cells from apoptosis induced by TNF-α (18–21). In Ad5IxB-infected hepatocytes, the PI3K inhibitor LY 294002 potentiated the cytotoxicity of TNF-α. PI3K is activated by TNF-α though interaction with an adapter protein, namely, Grb2 (39) or Ras (40). In our system, Akt activation by TNF-α was blocked by DMS and exogenous S1P-activated Akt. Activation of SphK and S1P formation peaked at 10 min, thus preceding Akt activation (detectable at 15 min) after TNF-α treatment. S1P activates c-Src tyrosine kinases and promotes Grb2-PI3K complex formation (41). These findings suggest that S1P formation induced activation of Akt in TNF-α-treated human hepatocytes. S1P is reported to act as a ligand for the Edg receptor(s) and also as an intracellular second messenger (31, 42). For example, microinjected S1P induced DNA synthesis in Swiss 3T3 cells (43) and overproduced intracellular S1P in NIH3T3 fibroblasts and HEK293 cells by overexpression of SphK, which promoted cell growth and survival (42). In our system, exogenous S1P underwent Akt activation, but it was completely inhibited by PTX pretreatment. In contrast, PTX caused partial inhibition of the Akt activation because of treatment with TNF-α. Administration of exogenous S1P resulted in a partial rescue from death induced by TNF-α plus DMS. Thus, S1P produced by TNF-α treatment activates Akt intracellularly and also functions as an extracellular ligand for Edg receptor(s) in human hepatocytes. DMS also enhanced apoptotic death of HeLa cells induced by another death receptor agonist, FasL, which caused Akt activation. However, exogenous S1P prevented apoptosis induced by FasL plus DMS. These results indicate that the survival signaling via the SphK/S1P pathway also may operate in Fas-mediated apoptosis of human hepatocytes.

Mechanisms for the antiapoptotic effects of Akt activation have been reported previously. In some types of cells, NF-κB is a potential target for the PI3K/Akt pathway (16, 17, 21). However, in human endothelial cells, antiapoptotic action of Akt was independent of NF-κB activation (19). Similarly, in TNF-α-stimulated HeLa cells, wortmannin, a PI3K inhibitor, did not inhibit NF-κB activation (18). In our system, NF-κB activation was not prevented by LY 294002. Moreover, S1P did not induce NF-κB activation in HuH-7 cells (data not shown). These results suggest that survival mechanism(s) other than NF-κB exists downstream of PI3K/Akt. Pastorino et al. (18) reported that TNF-α induced phosphorylation of BAD through PI3K/Akt pathway and that phosphorylated BAD lost its ability to bind to Bcl-XL, which is known to act on mitochondria to block the apoptotic signaling cascade (44). TNF-α was reported to induce apoptosis in hepatocytes via mitochondrial permeability transition (26). In contrast, overexpression of Bcl-XL prevented the liver injury caused by TNF-α plus α-galactosamine (45). These findings lead us to speculate that Akt protects hepatocytes from TNF-α cytotoxicity through inhibition of apoptotic mitochondrial events.

The DMS-induced enhancement of apoptosis was not affected by the addition of LY 294002, and DMS inhibited TNF-α-induced activation of PI3K and Akt. Therefore, the effect of DMS is mediated by the inhibition of PI3K/Akt pathway through blockage of S1P formation. DMS pretreatment was sufficient to sensitize cells to TNF-α cytotoxicity without prior infection with Ad5IxB, whereas LY 294002 alone was unable to enhance apoptosis by TNF-α (Table II). LY 294002 exerted an enhancing effect on apoptosis induced by TNF-α in the presence of Ad5IxB. These data suggest that blockage of the PI3K pathway is insufficient to sensitize human hepatocytes to TNF-α-induced apoptosis and that the sensitizing effect of DMS alone on TNF-α-induced apoptosis may be attributable to mechanism(s) other than PI3K/Akt inhibition. In addition to Akt, S1P stimulates many signaling pathways, such as ERK, cAMP-dependent kinase, and focal adhesion kinase (31). Indeed, ERK was activated by S1P stimulation in HeLa cells (data not shown). However, PD 98059, a selective inhibitor of MEK acting upstream of ERK, had no enhancing effect on apoptosis induced by TNF-α in Ad5IxB-infected hepatocytes. Therefore, the MEK/ERK cascade does not participate in the S1P-mediated survival signaling pathway in human hepatocytes. cAMP appears to inhibit apoptosis of primary rat hepatocytes by cAMP-dependent kinase activation (46, 47), and activation of focal adhesion kinase is an antiapoptotic effect in HL-60 cells (48). Therefore, it is reasonable to speculate that these survival signals also are involved in sensitizing effect by DMS. Their involvements in S1P-mediated antiapoptotic action in human hepatocytes should be examined to draw a complete picture of TNF-α signaling. An alternate interpretation, as Pitson et al. (49) reported, is that DMS inhibited the basal (housekeeping) SphK activity in unstimulated cells. DMS may have other, nonspecific effects in addition to SphK inhibition (29, 50). These effects of DMS also may be involved in sensitizing

### Table IV. Protective effect of S1P on FasL-induced apoptosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Apoptotic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17.2 ± 0.2</td>
</tr>
<tr>
<td>Ad5IxB</td>
<td>29.0 ± 0.6</td>
</tr>
<tr>
<td>DMS</td>
<td>52.9 ± 2.5</td>
</tr>
<tr>
<td>Ad5IxB + DMS</td>
<td>62.8 ± 1.2</td>
</tr>
</tbody>
</table>

* Hc cells infected with or without Ad5IxB were preincubated with 10 μM DMS for 1 h and then treated with 100 ng/ml FasL for 24 h in the absence or presence of 1 μM S1P. Typical apoptotic cells stained with Hoechst 33258 were counted among >1000 cells and percent cell apoptosis was determined. Data are means ±SD from three independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01 vs in the absence of S1P by Student’s t test.

![FIGURE 9. Hypothetical mechanisms for potentiation of TNF-α-mediated apoptosis by SphK inhibition in human hepatocytes.](http://www.jimmunol.org/)
action of DMS. Although pathway(s) other than the PI3K/Akt cannot be excluded at the present stage, we would like to propose a new hypothesis that SphK-mediated formation of S1P plays an important role in the antiapoptotic signaling transduction mediated by TNF-α.

In summary, we have shown here that TNF-α activates the PI3K/Akt pathway via SphK activation and S1P formation in human hepatocytes, and that this pathway regulates apoptosis mediated by TNFR and Fas. This protective effect appears to be independent of NF-κB. The hypothetical signaling pathways were schematically summarized in Fig. 9. Therefore, regulation of S1P levels may present a new therapeutic approach for liver diseases.

Acknowledgments

We thank Dr. Hisanori Kojima for providing adenovirus and Dr. Yasuhiro Yamada for help with gel shift assay.

References


