

Serum bioactive lysophospholipids prevent TRAIL-induced apoptosis via PI3K/Akt-dependent cFLIP expression and Bad phosphorylation

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Abstract

Serum contains a variety of biomolecules, which play an important role in cell proliferation and survival. We sought to identify the serum factor responsible for mitigating tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and to investigate its molecular mechanism. TRAIL induced effective apoptosis without serum, whereas bovine serum decreased apoptosis by suppressing cytochrome *c* release and caspase activation. Indeed, albumin-bound lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) inhibited TRAIL-induced apoptosis by suppressing caspase activation and cytochrome *c* release. LPA increased phosphatidylinositol 3-kinase (PI3K)-dependent Akt activation, cellular FLICE-inhibitory protein (cFLIP) expression, and Bad phosphorylation, resulting in inhibition of caspase-8 activation and Bad translocation to mitochondria. The antiapoptotic effect of LPA was abrogated by PI3K inhibitor, transfection with dominant-negative Akt, and specific downregulation of cFLIP expression using siRNA and further increased by siRNA-mediated suppression of Bad expression. Moreover, sera from ovarian cancer patients showed more protective effect against TRAIL-induced apoptosis than those from healthy donors, and this protection was suppressed by PI3K inhibitor. Our results indicate that albumin-bound LPA and S1P prevent TRAIL-induced apoptosis by upregulation of cFLIP expression and in part by Bad phosphorylation, through the activation of PI3K/Akt pathway. *Cell Death and Differentiation* (2004) 11, 1287–1298.

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Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; BSA, bovine serum albumin; dBSA, delipidated BSA; LPA, lysophosphatidic acid; S1P, sphingosine-1-phosphate; PI3K, phosphatidylinositol 3-kinase; cFLIP, cellular FLICE-inhibitory protein; WT-Akt, wild-type Akt; DN-Akt, dominant-negative Akt; FBS, fetal bovine serum; DEVDase, caspase-3-like protease; IETDase, caspase-8-like protease; ActD, actinomycin D; DAPI, 4,6-diamidino-2-phenylindole; PTX, pertussis toxin

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL),¹ also known as Apo-2 ligand,² is a member of the TNF family of cytokines and is capable of inducing apoptosis in a wide variety of cancer cells in culture. The apoptosis-inducing receptors for TRAIL include the transmembrane type-I receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). These receptors are expressed on the surface of many types of tumor cells and transmit apoptotic signals via cytoplasmic death domains. However, TRAIL also binds to the nonapoptosis-inducing decoy receptors, DcR1 and DcR2, which are truncated TRAIL receptors in which the cytoplasmic regions containing death domains are deleted.

Recombinant human TRAIL protein preferentially induces apoptosis of tumor cells but not in most types of untransformed cells.³ However, not all tumors respond to TRAIL. This lack of response may be attributed either to an unfavorable ratio of death receptors to decoy receptors or to other intracellular resistance mechanisms.⁴ With respect to intracellular mechanisms, several intracellular antiapoptotic molecules including antiapoptotic Bcl-2 family proteins, IAPs, and cellular FLICE-inhibitory protein (cFLIP) inhibit the apoptotic signaling cascade,^{5–7} through the inhibition of mitochondrial cytochrome *c* release, apoptosome formation, and recruitment of procaspase-8 to the receptor death domain. Furthermore, extracellular peptide growth factors (platelet-activating factor and epidermal growth factor) and cytokines including interleukin-1 β have been shown to protect cells from TRAIL-induced apoptosis by activating a protective intracellular signal.^{8,9} These factors are usually present in serum, but most of these serum factors occur at concentrations far below those necessary to elicit their survival effects. However, recent studies showed that some cells including hepatocytes are sensitive to TRAIL-induced apoptosis *in vitro* without serum,¹⁰ but resistant with even low concentrations of serum.¹¹ These evidences suggest that other survival factors against TRAIL-induced apoptosis are present in serum, and these factors have not been characterized.

The lysophospholipid mediators, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), are potent

bioactive lipids, which are synthesized from phospholipids in platelets, fibroblasts, adipocytes, and tumor cells and then secreted into biological fluids. LPA and S1P occur normally in serum and bind with high affinity to albumin, while retaining their biological activities.¹² Although these molecules exist in serum at concentrations of 2–20 μM ,¹³ their serum levels become increased in tumor-bearing patients.¹⁴ LPA and S1P have been shown to have pleiotropic functions such as mitogenesis and angiogenesis. In addition, they promote cell survival through activation of specific G-protein-coupled transmembrane receptors encoded by endothelial differentiation genes (EDG). LPA is predominantly bound to EDG2, EDG4, and EDG7, while S1P specifically binds to EDG1, EDG3, EDG5, EDG6, and EDG8.¹⁵ Following G-protein activation, one of the resulting intracellular signaling events is the activation of the lipid kinase phosphatidylinositol 3-kinase (PI3K) and its downstream kinase Akt.¹⁶ Activated Akt phosphorylates Bad, procaspase-9, forkhead, CREB, and IKK,¹⁷ resulting in the suppression of the apoptotic signal cascade or expression of antiapoptotic genes.

We hypothesized that phospholipids could prevent TRAIL-induced tumor cell death by altering expression and activation of critical molecules in apoptotic signal transduction. We here identified albumin-bound LPA and S1P as two serum survival factors that protect cells from TRAIL-induced apoptosis. LPA activates the PI3K/Akt signaling pathway and subsequently increases cFLIP expression and Bad phosphorylation, which lead to the inhibition of caspase activation and mitochondrial cytochrome *c* release. These results indicate that albumin-bound LPA and S1P are lipid-based survival factors in serum, thus suggesting that control of the serum LPA and S1P levels may be important for *in vivo* TRAIL-mediated cancer therapy.

Results

Serum and albumin protect tumor cells from TRAIL-induced apoptosis

To examine the effects of serum on TRAIL-induced tumor cell apoptosis, we treated HeLa cells with TRAIL (100 ng/ml) in the presence or absence of various fetal bovine serum (FBS) concentrations and determined cell viability by crystal violet staining after 8 h. TRAIL induced apoptotic cell death in ~60% of cells in the absence of FBS, and this apoptosis was suppressed in a dose-dependent manner by the addition of FBS (Figure 1a). A similar result was observed when FBS was replaced by corresponding concentrations of calf serum (data not shown). To characterize a survival factor of serum, we removed low-molecular-weight components of serum by dialysis using a membrane with a 12 kDa molecular cutoff and examined the effect of dialyzed serum on apoptosis induced by TRAIL. Dialyzed serum exhibited the same protective effect as nondialyzed serum (Figure 1b). Albumin is a major component of serum and plays a role as a carrier of bioactive lipids.¹⁸ Therefore, we next examined the protective effects of bovine serum albumin (BSA) and delipidated BSA (dBSA) on TRAIL-induced apoptosis. BSA suppressed the apoptotic cell death, while dBSA failed to do so (Figure 1b). In addition, the serum albumins prepared from rat, horse, and human showed similar antiapoptotic effect (data not shown), indicating that the protective effects of albumin are not species specific. Similar protective effects were observed in the colon cancer cell line DLD-1 (Figure 1c). These results suggest that albumin-bound lipid component(s) may be responsible for prevention against TRAIL-induced apoptosis.

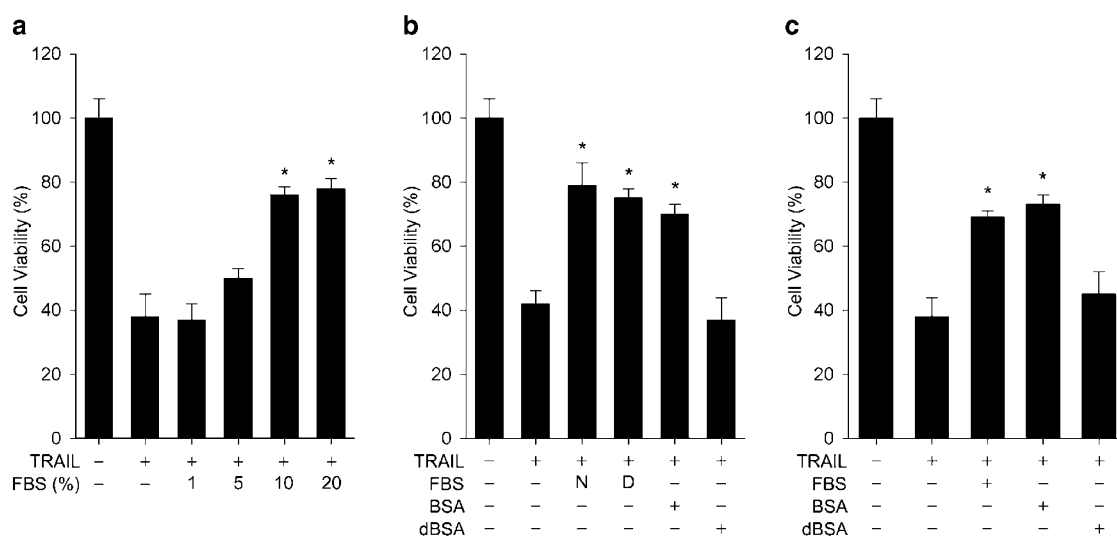


Figure 1 Effect of FBS and BSA on TRAIL-induced apoptosis. Cells plated in 12-well dishes at a density of 1×10^6 cells/ml were cultured for 8 h in 10% FBS containing DMEM and further cultured in the serum-free medium for another 6 h. (a) HeLa cells were treated with TRAIL (100 ng/ml) in the presence or absence of FBS. (b) HeLa cells were treated with TRAIL (100 ng/ml) in the presence of 10% nondialyzed (N) or dialyzed FBS (D), 5% BSA, or 5% dBSA. (c) DLD-1 cells were treated with TRAIL (100 ng/ml) in the presence of 10% FBS, 5% BSA, or 5% dBSA. After 8 h of these treatments, cell viability was measured by crystal violet staining. Data represent the mean \pm S.D. of more than three independent experiments. * $P < 0.01$ versus TRAIL alone

LPA and S1P are bioactive molecules responsible for serum antiapoptotic factors

Albumin transports multiple substances within the bloodstream to and from tissues, including phospholipids such as LPA and S1P.^{19,20} LPA and S1P are thought to be involved in many biological activities including mitogenesis and proliferation of cells.²¹ We thus determined whether LPA and S1P act as antiapoptotic factors that accounted for the cytoprotective activity of serum and albumin. HeLa cells were treated with TRAIL in the presence of free LPA (4 μ M) or S1P (0.4 μ M), and cell viability was measured after 8 h incubation. These free LPA and S1P effectively protected cells from TRAIL-induced apoptosis (Figure 2a). We next examined the preventive effect of dBSA-LPA or dBSA-S1P on TRAIL-induced apoptosis. dBSA-LPA prevented TRAIL-induced apoptosis in a dose-dependent manner, with maximal protection observed at 4 μ M. Additionally, 0.4 μ M of dBSA-S1P also prevented apoptotic cell death (Figure 2b). FBS, BSA, dBSA-LPA, and dBSA-S1P, but not dBSA alone, significantly inhibited TRAIL-induced DNA fragmentation characteristic of typical apoptosis (Figure 2c). Furthermore, HeLa cells undergoing TRAIL-mediated cell death displayed typical apoptotic features,

including chromatin condensation, nuclear pyknosis, and in some cases nuclear fragmentation (Figure 2d, ii, arrow). These morphological changes were decreased by cotreatment with FBS, BSA, dBSA-LPA, or dBSA-S1P, but not by dBSA alone (Figure 2d, iii–vii). These results indicate that LPA and S1P are likely to be important factors responsible for the survival effect of FBS.

LPA suppresses the activation of caspase-3 and -8 induced by TRAIL

Caspase activity and cytochrome *c* release are known to be one of the major events in apoptotic tumor cells.²² Treatment of HeLa cells with TRAIL increased caspase-8-like (IETDase) and caspase-3-like (DEVDase) activities more than eight-fold, and these increases were significantly inhibited by FBS and BSA (Figure 3a and b). As expected, dBSA-LPA and dBSA-S1P inhibited these enzyme activities; however, dBSA itself did not. All caspases are constitutively present as zymogens in cells and become proteolytically cleaved into their active forms when cells are stimulated with apoptogenic stimuli.²³ We next determined whether the inhibitory effect of

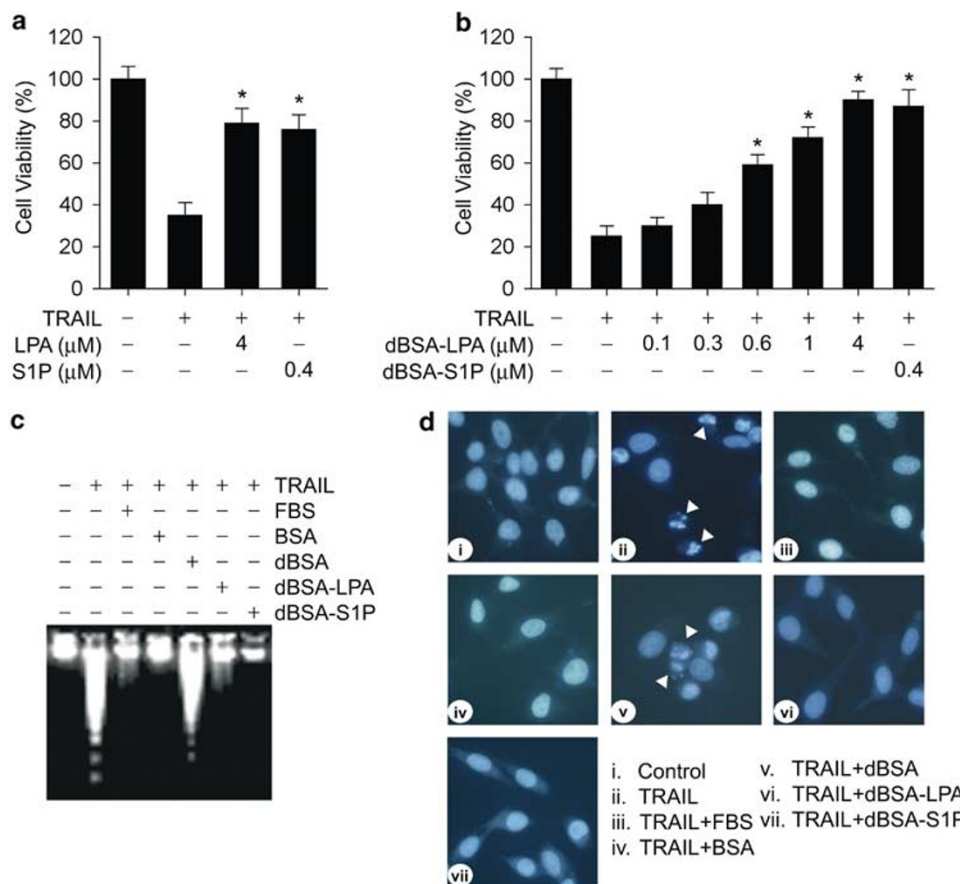


Figure 2 LPA and S1P prevent TRAIL-induced HeLa cell apoptosis. (a) HeLa cells were treated with TRAIL (100 ng/ml) in the presence of LPS or S1P for 8 h, and cell viability was measured by the crystal violet staining method. Data represent the mean \pm S.D. of four independent experiments. * $P < 0.01$ versus TRAIL alone. (b) Cells were treated TRAIL in the presence of dBSA-LPA or dBSA-S1P for 8 h, and cell viability was measured by the crystal violet staining method. Data represent the mean \pm S.D. of four independent experiments. * $P < 0.01$ versus TRAIL alone. Cells were treated with TRAIL (100 ng/ml) in the presence of 10% FBS, 5% BSA, 5% dBSA, 4 μ M dBSA-LPA, or 0.4 μ M dBSA-S1P. (c) After 5 h, DNA was isolated from cells treated as indicated, and DNA fragmentation was determined by agarose gel electrophoresis. (d) After 5 h, changes of nuclear morphology were assessed by DAPI staining

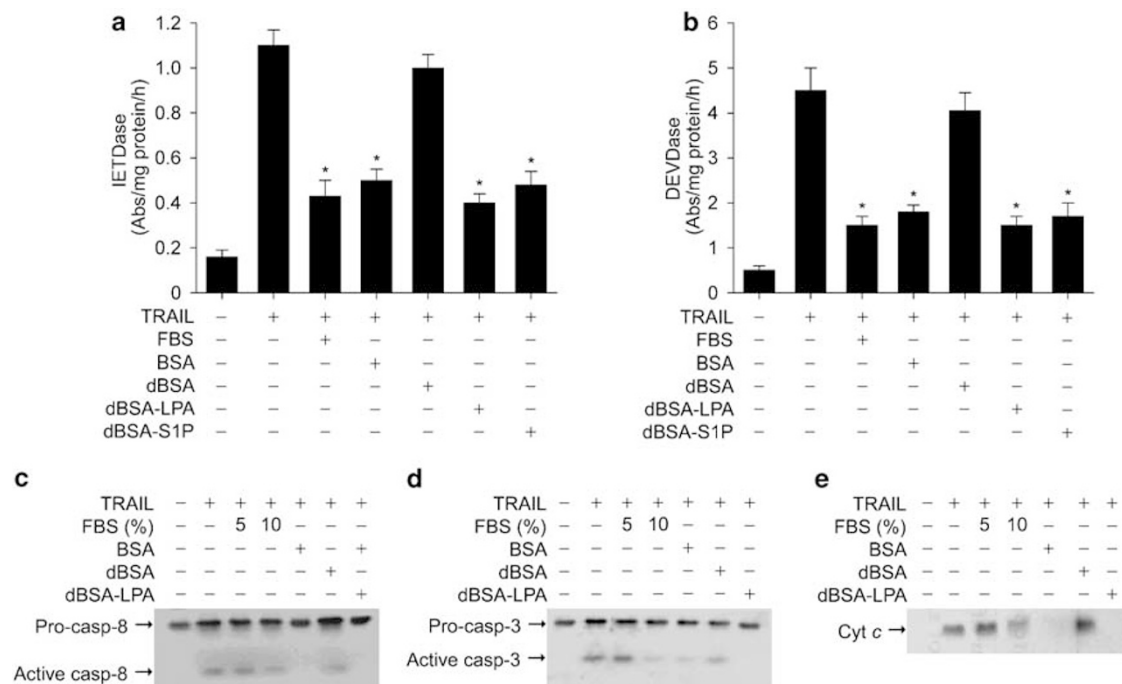


Figure 3 dBSA-LPA and dBSA-S1P suppress caspase-8 and -3 activities and cytochrome *c* release induced by TRAIL. HeLa cells were treated with TRAIL in the presence of 10% FBS, 5% BSA, 5% dBSA, 4 μ M dBSA-LPA, or 0.4 μ M dBSA-S1P. After 5 h, cells were harvested and lysed by three cycles of freeze and thaw. Activities of caspase-8 (a) and caspase-3 (b) were determined by colorimetric assays using the chromogenic substrates 240 μ M Ac-IETD-pNA (caspase-8-like activity) and 150 μ M Ac-DEVD-pNA (caspase-3-like activity), respectively. Data shown are the mean \pm S.D. of more than three independent experiments. * P < 0.01 versus TRAIL alone. (c, d) Cytosolic proteins were separated by SDS-PAGE and protein bands were detected by Western blot analyses using antibodies specific for caspase-8 and -3. (e) For cytochrome *c* release, cells were harvested after 4 h incubation and homogenized in an isotonic sucrose solution using a Dounce homogenizer. Cytosolic fractions were obtained after centrifugation at 12 000 \times *g* for 20 min at 4°C. Cytosolic cytochrome *c* was determined by Western blot analysis using an antibody for cytochrome *c*

dBSA-LPA on apoptotic cell death is associated with the suppression of caspase activation. Western blot analyses confirmed the appearance of active fragments of caspase-8 and -3 in the TRAIL-treated cells, and this activation was suppressed by cotreatment with FBS, BSA, or dBSA-LPA, but not by dBSA alone (Figure 3c and d). Mitochondrial cytochrome *c* release into the cytosol, which is an apoptotic signaling event that occurs between activation of caspase-8 and -3,²² was observed after treatment with TRAIL. However, redistribution of cytochrome *c* was suppressed by the addition of FBS, BSA, or dBSA-LPA, but not by dBSA (Figure 3e). These results suggest that LPA inhibits the activation of proximal caspase-8 and mitochondrial cytochrome *c* release during TRAIL-induced apoptotic signaling.

LPA activates PI3K-dependent Akt

Activation of the lipid kinase PI3K and its downstream effector Akt has been shown to suppress apoptosis and promote cell survival.²⁴⁻²⁶ To explore whether LPA may suppress apoptosis and apoptotic signaling via the activation of the PI3K/Akt pathway, we examined the functional involvement of PI3K/Akt pathway in LPA-mediated cell survival. The PI3K inhibitors Wortmannin and LY294002 markedly inhibited the antiapoptotic effect of dBSA-LPA (Figure 4a). Activation of Akt is known to be a downstream mediator of PI3K in the survival signal cascade.^{24,25} Since phosphorylation of Akt at Ser-473 is required for its full activation,²⁶ we examined the phosphory-

lation status of endogenous Akt using an antibody that specifically recognizes Akt phosphorylated at Ser-473. Incubation of cells with dBSA-LPA or FBS resulted in phosphorylation of endogenous Akt, and this activation was markedly inhibited by LY294002 or Wortmannin (Figure 4b). Because LPA can activate PI3K via the $\beta\gamma$ subunits of G_i in COS cells²⁷ and Schwann cells,²⁸ we examined whether this pathway is activated by LPA treatment in HeLa cells. Phosphorylation of Akt by LPS was inhibited by pretreatment of HeLa cells with pertussis toxin (PTX) (Figure 4c). These data indicate that the PTX-sensitive $G_{i/o}$ coupled to LPA-specific G-protein-coupled EDG2 may be involved in LPA-mediated activation of the PI3K/Akt pathway. As the activation of PI3K/Akt survival pathway has been shown to inhibit caspase activation/activity,^{25,29} we examined whether PI3K inhibitor regulates the suppressive effect of LPA on DEVDase activity. Treatment with LY294002 or Wortmannin reversed the suppressive effect of dBSA-LPA on caspase activity (Figure 4d). These results indicate that the antiapoptotic effects of LPA involve the activation of the PI3K/Akt pathway in HeLa cell apoptosis induced by TRAIL.

LPA increases Bad phosphorylation and cFLIP expression

Since activation of Akt inhibits apoptosis by phosphorylating Bad,²⁵ cells were incubated with dBSA-LPA in the presence or

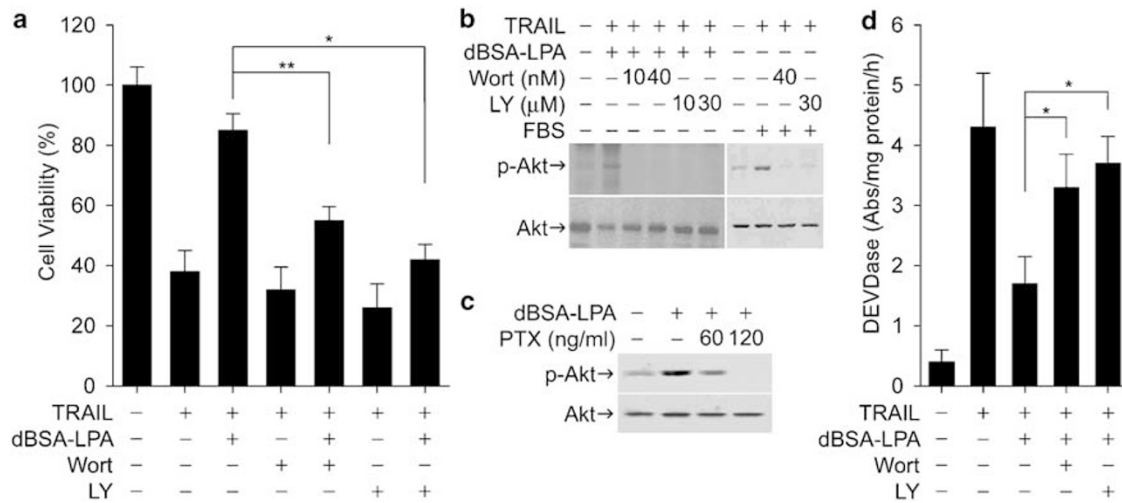


Figure 4 dBSA-LPA and FBS prevent TRAIL-induced HeLa cell apoptosis by PI3K-dependent Akt activation. HeLa cells were cotreated with TRAIL and FBS (10%) or dBSA-LPA (4 μ M) in the presence or absence of Wortmannin (Wort, 40 nM), LY294002 (LY, 30 μ M), or pertussis toxin (PTX, 60 and 120 ng/ml). (a) After 8 h, cell viability was determined by crystal violet staining. (b, c) After 40 min incubation, cells were collected and cell lysates were prepared by three cycles of freeze and thaw and centrifugation at 12 000 \times g for 20 min. Akt activation was determined by Western blot analyses using antibodies for phospho-Akt and Akt. (d) After 5 h, DEVDase activity was determined by colorimetric assay using the chromogenic substrate Ac-DEVD-pNA (150 μ M). Data shown in (a) and (d) are the mean \pm S.D. of more than three independent experiments. * P <0.01 and ** P <0.05

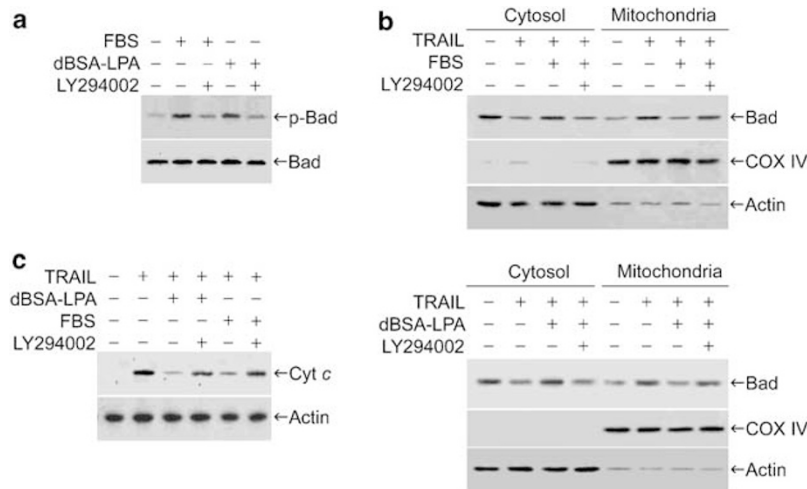


Figure 5 FBS and dBSA-LPA increase Bad phosphorylation and suppress cytochrome *c* release induced by TRAIL. HeLa cells were treated with TRAIL (100 ng/ml) and FBS (10%) or dBSA-LPA (4 μ M) in the presence or absence of LY294002 (30 μ M) for 90 min. (a) Cells were harvested and lysed by three cycles of freeze and thaw. Cytosolic fractions were obtained by centrifugation at 12 000 \times g for 20 min. Cytosolic proteins were separated by SDS-PAGE. Bad phosphorylation and Bad protein were determined by Western blot analyses using specific antibodies for phospho-Bad and Bad. (b) Cells were homogenized in a Dounce homogenizer with a loose pestle and fractionated into cytosol from the mitochondrial fraction. Localization of Bad was determined by Western blot analysis with anti-Bad antibody. Equally loaded proteins of the cytosolic fraction and the mitochondrial pellet were controlled using antibodies against actin and cytochrome *c* oxidase subunit IV (Cox IV), respectively. (c) For cytochrome *c* release, cells were harvested after 4 h incubation and homogenized in an isotonic sucrose solution using a Dounce homogenizer. Cytosolic fractions were obtained after centrifugation at 12 000 \times g for 20 min at 4°C. Cytosolic cytochrome *c* was determined by Western blot analysis using an antibody for cytochrome *c*

absence of LY294002 for 90 min, and Bad phosphorylation was determined using an antibody specific to phosphorylated Bad at Ser-136. FBS and dBSA-LPA increased Bad phosphorylation, and this phosphorylation was suppressed by LY294002 (Figure 5a). One possible mechanism by which Akt signaling suppresses apoptosis is associated with the inhibition of cytochrome *c* release by phosphorylating proapoptotic Bad and blocking its translocation into mitochondria from the cytosol.³⁰ Phosphorylation of Bad can couple survival signals

to extrinsic death pathway³¹ as well as intrinsic death machinery.³² We therefore examined the effects of FBS and dBSA-LPA on intracellular localization of Bad and cytochrome *c* release by Western blot analysis. TRAIL increased the translocation of cytosolic Bad to mitochondria compared with control, and this translocation was suppressed by treatment with FBS or dBSA-LPA. The suppressive effects of FBS and dBSA-LPA on cytochrome *c* redistribution were reversed by LY294002 (Figure 5b). Moreover, the inhibitory effects of FBS

and dBSA-LPA on TRAIL-induced cytochrome *c* release were reversed by treatment with LY294002 (Figure 5c). These results suggest that Bad phosphorylation by LPA-mediated PI3K/Akt activation prevents, in part, apoptotic cell death induced by TRAIL.

LPA increases cFLIP expression and inhibits caspase-8 activation

It has also been shown that Akt blocks apoptotic cell death by increasing cFLIP expression, which inhibits caspase-8 activation by interfering with the recruitment of procaspase-8 to Fas-associated protein with a death domain.^{6,33} We further examined the effect of dBSA-LPA on the expression level of cFLIP and caspase-8 activation. Incubation of cells with BSA and dBSA-LPA increased the protein levels of cFLIP as FBS did, and these increases were suppressed by LY294002 (Figure 6a). Furthermore, the transcription inhibitor actinomycin D (ActD) decreased the increased cFLIP level by dBSA-LPA (Figure 6b) and increased caspase-8 activation/activity (Figure 6c). Taken together, these results suggest that dBSA-LPA may prevent TRAIL-induced apoptosis by PI3K/Akt pathway-dependent increase in cFLIP expression and subsequent inhibition of caspase-8 activation.

Akt activation and cFLIP expression are required for the protective effects of serum and LPA against apoptosis

To further determine the role of Akt in antiapoptotic signaling elicited by serum and LPA, the protective effects of serum and dBSA-LPA on HeLa cells stably transfected with dominant-negative Akt (DN-Akt) were compared with their effects on control cells transfected with an empty vector (pcDNA3) or cells overexpressing wild-type Akt (WT-Akt) (Figure 7a). These transfectant cells were treated with TRAIL in the presence or absence of FBS or dBSA-LPA, and cell survival was determined after 8 h. When cells were cultured without

FBS or dBSA-LPA, TRAIL-induced apoptosis was partially decreased in WT-Akt-overexpressing cells, but slightly increased in DN-Akt-expressing cells, compared with control cells (Figure 7b). As expected, treatment with FBS or dBSA-LPA significantly blocked TRAIL-induced apoptosis in cells transfected with empty vector or WT-Akt, but not in DN-Akt-expressing cells, compared with cells cultured without them. The potential role of Akt in antiapoptotic effects of FBS and dBSA-LPA was further examined by measuring caspase-8-like IETDase activity in these transfectant cells. FBS and dBSA-LPA significantly reduced the increased IETDase activity by TRAIL both in control and WT-Akt-transfected cells, but not effectively in DN-Akt-expressing cells (Figure 7c). To further evaluate whether the increased cFLIP level affects the protective effect of serum or dBSA-LPA, we transfected HeLa cells with pcDNA3-cFLIP and examined the effect of cFLIP overexpression on apoptotic cell death. Cells transfected with pcDNA3-cFLIP increased the cellular cFLIP protein level, while empty vector-transfected cells expressed the same level of cFLIP as that of control cells, as determined by Western blot analysis (Figure 7d). cFLIP-overexpressing cells significantly suppressed TRAIL-induced apoptosis and IETDase activity compared with empty vector-transfected cells (Figure 7e and f). dBSA-LPA inhibited IETDase activity and apoptotic cell death in both cells. These results suggest that serum and LPA protect cells from TRAIL-induced apoptosis by activating Akt-dependent signal pathway and that albumin-bound LPA may be an important factor for antiapoptotic effect of serum.

siRNAs for cFLIP and Bad reversed serum-mediated protection against TRAIL-induced cell death

To test whether cFLIP expression and Bad phosphorylation are necessary targets for serum- and LPA-induced protection against TRAIL-induced apoptosis, an siRNA approach was employed. Transfection with cFLIP siRNA reduced cFLIP

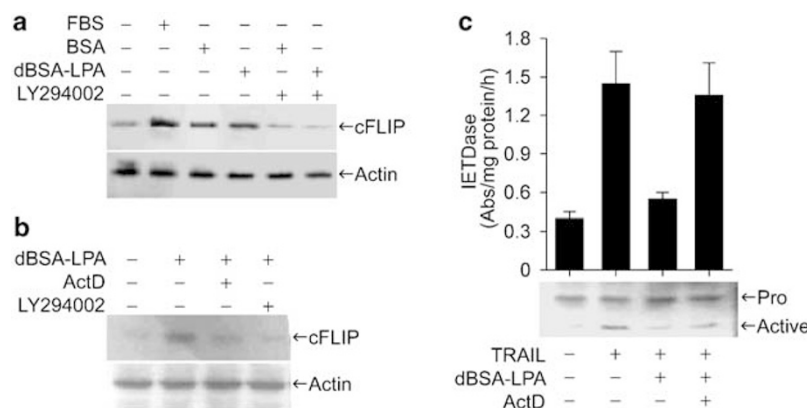


Figure 6 dBSA-LPA increases cFLIP expression and inhibits caspase-8 activation. (a) HeLa cells were treated with FBS (10%), BSA (5%), or dBSA-LPA (4 μ M) in the presence or absence of LY294002 (30 μ M). Cells were harvested and lysed by three cycles of freeze and thaw. Cytosolic fractions were obtained by centrifugation at $12\,000 \times g$ for 20 min. Cytosolic proteins were separated by SDS-PAGE. cFLIP was determined by Western blot analyses using a specific antibody for cFLIP. (b) Cells were treated with dBSA-LPA (4 μ M) in the presence or absence of ActD (80 ng/ml) and LY294002 (30 μ M) for 90 min. cFLIP expression was determined by Western blot analysis. (c) Cells were treated with TRAIL (100 ng/ml) in the presence of dBSA-LPA (4 μ M) or Act-D (80 ng/ml). After 5 h, cells were harvested and lysed by three cycles of freeze and thaw. Caspase-8 activity and activation were measured by colorimetric assay using Ac-IETD-pNA and Western blot analysis

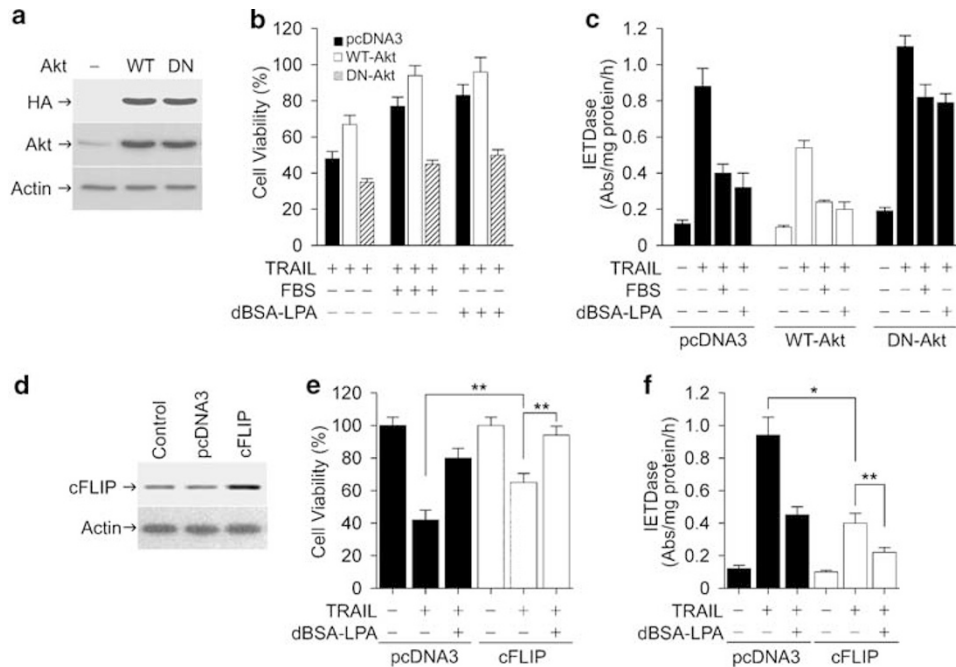


Figure 7 Akt activation and cFLIP expression are required for the protective effects of serum and LPA against apoptosis. HeLa cells were transfected with WT-Akt, DN-Akt, cFLIP, or empty vector (pcDNA3) by the lipofectamine method. The stable transfectant clones with high expression of the target genes were selected by culturing with G418 (800 μ g/ml). Cells were treated with TRAIL in the presence or absence of FBS (10%), dBSA-LPA (4 μ M), or ActD (80 ng/ml). Expression levels of Akt (a) and cFLIP (d) were determined by Western blot analysis. (b, e) Cell viability was determined by crystal violet staining. (c, f) IETDase activity was measured in the cytosolic fraction by colorimetric assay using Ac-IETD-pNA. Data shown are the mean \pm S.D. of more than three independent experiments. * $P < 0.01$ and ** $P < 0.05$

expression (Figure 8a). Downregulation of cFLIP expression was found to decrease significantly the protective effect of serum and LPA against TRAIL-induced apoptosis and further increase TRAIL-induced apoptosis in the absence of serum and dBSA-LPA (Figure 8b). In addition, transfection with Bad siRNA suppressed Bad expression and total Bad phosphorylation compared with the untransfected control (Figure 8c) and further prevented HeLa cells from TRAIL-induced apoptosis in the presence or absence of serum or dBSA-LPA (Figure 8d). These results indicate that cFLIP and Bad are both important targets of LPA survival signaling against TRAIL-induced apoptosis.

Sera from ovarian cancer patients show more preventive effect against TRAIL-induced cell death than those of healthy donors

Ovarian cancer cells constitutively synthesize and release significant amounts of LPA,³⁴ and LPS concentration is significantly higher in sera from ovarian cancer patients than those from healthy donors.³⁵ We prepared sera from 12 ovarian cancer patients and 12 healthy female donors. To examine the effect of the human sera on apoptotic cell death, we treated HeLa cells with TRAIL in the presence or absence of these sera and determined cell viability by clonogenic assay after 8 h (Figure 9). Sera from patients with ovarian cancer showed more protective effect against TRAIL-induced apoptosis than those from healthy donors, and this protection was

suppressed by the PI3K inhibitor Wortmannin. The addition of 1 μ M LPA to the healthy sera increased their protective effects, which was comparable to those of the cancer patient sera. These results suggest that some tumors may protect themselves from TRAIL-mediated apoptosis, probably by synthesizing bioactive lipid mediators such as LPA and S1P.

Discussion

This study was undertaken to identify serum factors inhibiting TRAIL-induced apoptosis. We found that serum and the major protein in bovine serum, BSA, but not dBSA, prevented TRAIL-induced apoptosis. This indicates that the antiapoptotic factor of serum is likely to be lipid component(s) bound to albumin, but not albumin itself. Indeed, we found that the bioactive lysophospholipids LPA and S1P, which occur normally in serum and bind with high affinity to albumin, suppressed HeLa cell apoptosis induced by TRAIL through the inhibition of activation of caspase-8 and -3, mitochondrial cytochrome *c* release, and DNA fragmentation. This protective effect was associated with PI3K/Akt-dependent increases in cFLIP expression and Bad phosphorylation, which are responsible for the suppression of caspase-8 activation and mitochondrial cytochrome *c* release, respectively. However, the antiapoptotic effect of LPA was abrogated by PI3K inhibitor, transfection with DN-Akt, and specific downregulation of cFLIP expression using siRNA. The protective effect of LPA was further increased by siRNA-mediated suppression of

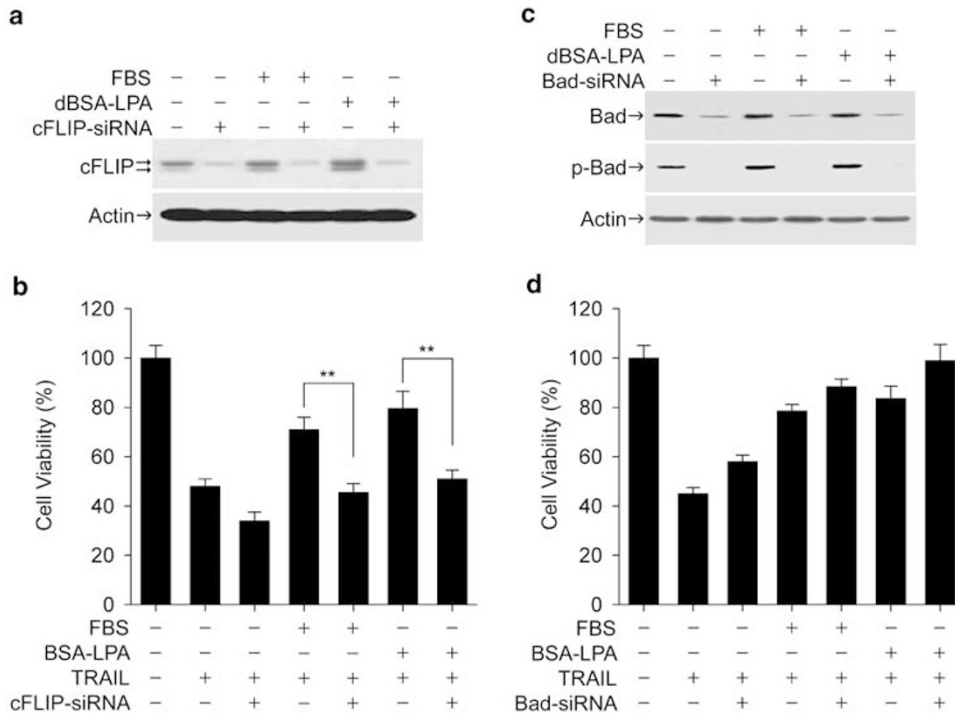


Figure 8 Knockdown of cFLIP or Bad by siRNA prevents TRAIL-induced cell death. HeLa cells were transfected with siRNAs of cFLIP or Bad for 12 h and recovered in fresh medium containing 10% FBS. After 24 h, cells were cultured in serum-free media for 6 h and further incubated in the presence or absence of 10% FBS for 6 h. Cells were harvested and lysed by three cycles of freeze and thaw. Cytosolic proteins were separated by SDS-PAGE. The protein levels of cFLIP (a) and Bad (c) were determined by Western blot analyses. Cells were transfected with the siRNAs of cFLIP (b) or Bad (d) for 12 h and recovered in fresh medium containing 10% FBS. The cells were further cultured in serum-free medium for another 6 h and then treated with TRAIL in the presence or absence of 10% FBS for 8 h. Cell viability was determined by crystal violet staining. Data shown are the mean \pm S.D. of more than three independent experiments. $**P < 0.05$

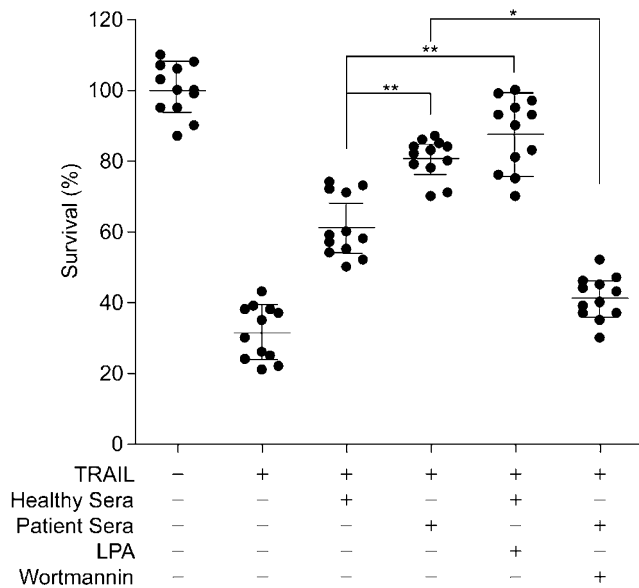


Figure 9 Sera from ovarian cancer patients prevent TRAIL-induced cell death. HeLa cells were treated with TRAIL in the presence or absence of sera from ovarian cancer patient and healthy donors, LPA (1 μ M), or Wortmannin (40 nM) for 8 h. The cells were washed with fresh medium, replated in 100-mm culture dishes, and then cultured in fresh medium containing 10% FBS for an additional 2-week period. The plates were stained with crystal violet, and the number of colonies was counted. Data shown are the mean \pm S.D. of more than three independent experiments. $*P < 0.01$ and $**P < 0.05$

Bad expression. These results suggest that LPA and S1P are extracellular nonpeptide serum survival factors that may inhibit tumor cell apoptosis induced by TRAIL through the activation of PI3K/Akt survival pathway. Indeed, our data demonstrated that the sera from patients with ovarian cancer, which constitutively synthesizes and releases significant amounts of LPA,³⁴ were more protective against TRAIL-induced apoptosis than those from healthy donors, and the protection was suppressed by PI3K inhibitor. Thus, the serum level of lysophospholipids can be an important factor for therapy of cancer patients with TRAIL.

TRAIL is known to induce apoptosis in a variety of tumor cells. Preclinical studies in rodents and nonhuman primates have demonstrated that repeated systemic administration of TRAIL induces antitumor activity without detectable toxic side effects.^{36,37} However, not all tumor cells are sensitive to TRAIL-induced apoptosis. This lack of sensitivity may be attributed either to an unfavorable ratio of death to decoy receptors or to expression of resistant genes.^{3,31} So far, the majority of normal human cells tested appear to be relatively TRAIL resistant; however, recent experiments have demonstrated that cultured human liver cells may be sensitive to TRAIL-induced apoptosis.¹⁰ This discrepancy may be due to different serum concentrations used.^{10,11} We demonstrated that TRAIL-mediated tumor cell apoptosis was inhibited by a dose-dependent manner by increasing serum concentrations (Figure 1a). The major component in serum that suppressed

TRAIL-induced apoptosis was found to be albumin. In addition, we found that albumins isolated from rat, horse, and human showed similar antiapoptotic effects, indicating that the source of albumin is not a critical factor for inducing an antiapoptotic signal cascade.

Albumin is a major serum protein that functions as a carrier with high affinity to lysophospholipids such as LPA and S1P within the bloodstream.^{12,19} Albumin serves two functions for bioactive LPA and S1P, which have pleiotropic functions such as mitogenesis and antiapoptosis.³⁸ First, albumin acts as a carrier to solubilize them, without affecting their biologic activities.¹² Second, albumin increases their physiological half-lives by protecting them from degradation by phospholipases.³⁹ Since normal serum contains about 5% albumin, 10% FBS used in this study is equal to a concentration of 0.5% albumin. Our results showed that the protective effect of 5% albumin was similar to that of 10% FBS, indicating that the effect of FBS is about 10-fold higher than that of BSA. It suggests that bioactive lipid components bound to BSA used in this study may be oxidized or lost during preparation or storage. Indeed, the level of malondialdehyde, as a lipid peroxidation product, was significantly higher in BSA (Sigma, $0.23 \pm 0.03 \mu\text{mol/mg protein}$) used in this experiment than freshly isolated BSA ($0.14 \pm 0.02 \mu\text{mol/mg protein}$). In addition, our results showed that BSA, but not dBSA, prevented apoptotic cell death (Figure 1b and c). These data suggest that albumin-bound lipid components are responsible for the antiapoptotic effect of albumin. Moreover, we found that dBSA-LPA and dBSA-S1P, but not dBSA alone, protected HeLa cells from apoptosis induced by TRAIL in serum-free medium (Figure 2b–d). These results suggest that albumin-bound bioactive LPA and S1P may account for the antiapoptotic activity of serum against TRAIL-induced apoptosis. However, involvement of other serum-surviving factors, such as growth factors and hormones, cannot be completely excluded.

LPA and S1P are produced in platelets, fibroblast, adipocytes, and tumor cells and secreted into biological fluids. LPA binds predominantly to the cell surface receptors EDG2, EDG3, and EDG7, while S1P is more specific to EDG1, EDG3, EDG5, and EDG8.¹⁵ They exert diverse effects such as platelet aggregation, cell differentiation, tumor invasion, angiogenesis, cell survival, and proliferation.^{21,40} By inhibiting apical caspase-8 activation and mitochondrial cytochrome *c* release, LPA and S1P are potent antiapoptotic serum factors against TRAIL-induced apoptosis in HeLa as well as DLD-1 cells (Figure 1b and c), but not in MCF7 and HOS cells (data not shown). HeLa cells express predominantly EDG2, EDG4, and EDG7 receptors that induce LPA-mediated intracellular signal pathway, while MCF cells express low levels of these receptors and do not effectively respond to LPA.¹⁶ This cell-line-specific response is likely linked to the degree of their receptor expression. Interestingly, serum LPA levels are significantly higher in cancer patients than in normal controls.⁴¹ We found that the sera from patients with ovarian cancer, which synthesizes significant amounts of LPA,^{34,41} showed more protective effects on TRAIL-induced cell death than those from healthy donors. Tumor cells capable of LPA production can increase their resistance to apoptosis induced by chemotherapeutic drugs^{42,43} and may also be

protected from TRAIL-induced apoptosis by these mechanisms. Therefore, in therapeutic treatments of cancer patients with TRAIL or other antitumor drugs, the serum level of LPA generated from tumors or inflammatory cells should be considered.

LPA and S1P have been demonstrated to bind specific G-protein-coupled EDG receptors,⁴⁴ which activate the intracellular signaling event of PI3K-dependent activation of Akt.^{16,45} We showed that HeLa cells cultured with dBSA-LPA increased the activation of PI3K/Akt survival pathway compared with untreated control cells. Our data also showed that PTX-sensitive $G_{i/o}$ coupled to LPA-specific G-protein-coupled EDG2 may be involved in LPA-mediated activation of PI3K/Akt pathway (Figure 4c). Signals transduced from activated Akt have been shown to be sufficient for the prevention of apoptosis or the promotion of cell survival through phosphorylation of several proteins including proapoptotic Bad²⁵ as well as through upregulation of cFLIP expression.³³ Bad heterodimerizes with Bcl-X_L or Bcl-2 and then promotes mitochondrial cytochrome *c* release and caspase-9 activation. Phosphorylated Bad binds to cytosolic protein 14-3-3, rather than to mitochondrial Bcl-2 or Bcl-X_L, preventing cytochrome *c* release.³⁰ Although Bad is known to regulate intrinsic death machinery,³² it has been demonstrated that Bad phosphorylation can prevent TNF- α -induced apoptosis.³¹ Furthermore, the upregulation of Bad expression enhanced TRAIL-induced apoptosis, and the suppression of Bad phosphorylation by siRNA of PKA1, a kinase that phosphorylates Bad in some systems, prevented TRAIL-induced apoptosis.⁴⁶ Consistent with these observations, our data showed that FBS and dBSA-LPA increased Bad phosphorylation, resulting in suppression of the translocation of cytosolic Bad to mitochondria and mitochondrial cytochrome *c* release (Figure 5). cFLIP is structurally similar to procaspase-8 in that it contains similar death effector domains and a caspase-like domain. However, cFLIP lacks the cysteine within the active site essential for the catalytic activity of caspase-8. Thus, cFLIP acts as a competitive inhibitor of caspase-8 in the formation of the death-inducing signaling complex, which subsequently blocks apical caspase-8 activation.⁴⁷ Our data showed that FBS and dBSA-LPA increased cellular cFLIP expression and thus suppressed caspase-8 activation/activity (Figure 6). Increase in cFLIP level by dBSA-LPA was blocked by the transcription inhibitor ActD and the PI3K inhibitor LY294002, indicating that the increased level of cFLIP by LPA was regulated at the transcriptional step by PI3K/Akt pathway. Furthermore, the important role of Akt and cFLIP in antiapoptotic signal pathway elicited by serum and dBSA-LPA was demonstrated using molecular approaches of stable transfection with Akt and cFLIP genes (Figure 7). Indeed, overexpression of DN-Akt abrogated the antiapoptotic effects of FBS and dBSA-LPA, and transfection with WT-Akt or cFLIP reduced TRAIL-induced apoptosis, indicating that Akt activation is one of the critical mediators for the antiapoptotic effect of LPA. Taken together, our results indicate that Akt-dependent cFLIP expression and Bad phosphorylation are common mediators of antiapoptotic signal pathway of serum and albumin-bound LPA. Our results further suggest that LPA is an important factor for antiapoptotic effect of serum.

It has recently been demonstrated that 21-base-pair double-stranded RNA (siRNA) is a potent mediator of the RNA interference effect, which specifically downregulates target gene expression in mammalian cells. We used this strategy to identify the functional roles of cFLIP and Bad in the antiapoptotic effect of serum on TRAIL-induced apoptosis. Our results indicate that specific knockdown of cFLIP by transfection with cFLIP siRNA effectively inhibited serum- and LPA-mediated protection against TRAIL-induced HeLa cell apoptosis (Figure 8b). Transfection with Bad siRNA prevented cells from TRAIL-induced apoptosis in the presence or absence of serum or LPA (Figure 8d). These results indicate the involvement of cFLIP upregulation and Bad phosphorylation in LPA-induced protection from TRAIL-induced apoptosis. However, cFLIP upregulation is a more crucial factor than Bad phosphorylation in the survival signal pathway against TRAIL-induced apoptosis.

We here present evidence that the albumin-bound LPA and S1P act as extracellular mediators responsible for regulation of major antiapoptotic factors that prevent tumor cells from apoptosis induced by TRAIL. Most known serum survival factors are polypeptide growth factors or hormones such as PDGF, EGF, IGF-1, and insulin. These serum survival factors, with the exception of PDGF and IGF-1, occur at concentrations far below those necessary to elicit survival effects. LPA and S1P showed maximal antiapoptotic effects at 4 and 0.4 μM , respectively (Figure 2a). These concentrations are pathophysiologically relevant, since their levels in serum range from 2 to 28 and 0.2 to 0.5 μM , respectively.^{48,49} Since LPA concentrations attaining 28 μM were reported in FBS,⁵⁰ medium containing 10% FBS used in this study was equal to 2.8 μM of LPA, which significantly protected cells from TRAIL-induced apoptosis (Figure 1). However, plasma levels of LPA from healthy individuals are relatively low, approximately 0.5 μM .^{41,51} However, its concentration was elevated to more than 4- to 60-fold in plasma samples from cancer patients but not all the patients, and this concentration is correlated with the degree of cancer malignancy.³⁵ Therefore, antiapoptotic concentration of LPA (1–4 μM) used in this study was lower than plasma levels ($\sim 8 \mu\text{M}$) in cancer patients.⁴¹ Here, we showed that sera from patients with ovarian cancer, which synthesizes significant amounts of LPA,³⁵ possessed more protective effect against TRAIL-induced apoptosis than those from healthy donors (Figure 9). These data suggest that altering production or metabolism of LPA and S1P may provide an additional strategy to develop cancer therapy with TRAIL or other anticancer drugs. However, it cannot completely exclude the possibility that other antiapoptotic phospholipid mediators including shingosylphosphorylcholine, which is present at the level of $\sim 0.1 \mu\text{M}$ in serum,⁵² may be involved in antiapoptotic effect of serum.

In conclusion, we demonstrated that a possible molecular mechanism for the antiapoptotic effects of the serum components LPA and S1P is to suppress TRAIL-induced caspase-8 activation and mitochondrial cytochrome *c* release by increases in cFLIP expression and Bad phosphorylation, which were critically dependent upon the activation of the PI3K/Akt signal pathway. These findings suggest that the suppression of lysophospholipid synthesis in tumors or immune cells represents a potential target to improve therapy

of cancer patients with TRAIL or other cancer therapeutic drugs.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), FBS, penicillin, streptomycin, and L-glutamate were purchased from Life Technologies (Gaithersburg, MD, USA). Dimethyl sulfoxide, actin antibody, Wortmannin, and LY294002 were purchased from Calbiochem (La Jolla, CA, USA). Cytochrome *c* antibody (556432) and caspase-8 antibody (66231A) were obtained from PharMingen (San Diego, CA, USA). Polyclonal caspase-3 antibody (H-277) and cFLIP antibody (H-202) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for phospho-specific Akt (Ser-473) was obtained from Transduction Laboratories (Lexington, KY, USA). Cytochrome *c* oxidase subunit IV (COX IV) was purchased from Molecular Probes (Eugene, OR, USA). Ac-DEVD-pNA and Ac-IETD-pNA were obtained from Alexis Corporation (San Diego, CA, USA). PTX was purchased from Calbiochem (La Jolla, CA, USA). BSA, dBSA (Fraction V), S1P, and LPA were purchased from Sigma (St. Louis, MO, USA). dBSA reconstituted with LPA (dBSA-LPA) or S1P (dBSA-S1P) was prepared by mixing 5% dBSA solution in calcium and magnesium-free phosphate-buffered saline (PBS) with LPA or S1P. The solutions were made fresh for each experiment. Blood samples were drawn from 12 ovarian cancer patients (aged 32–58 years) and 12 healthy female donors (aged 30–55 years). The blood was clotted at 4–8°C, and sera were subsequently prepared by centrifugation at 3000 $\times g$ for 40 min.

Cell culture

The human tumor cell lines, HeLa (cervical carcinoma), DLD-1 (colon cancer), HOS (prostate cancer), and MCF-7 (breast cancer), were purchased from American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 5 mM HEPES at 37°C in 95% air/5% CO₂. HeLa cells were transfected with empty vector (pcDNA3)₊ or vector containing WT-Akt, DN-Akt (K179M), or cFLIP using lipofectamine (Life Technologies Inc.). Clonal selection was conducted by culturing with G418 (800 $\mu\text{g}/\text{ml}$), followed by serial dilution of the cells in 96-well plates. Stable transfectant clones with high expression of the target genes were identified by Western blot analysis.

Cell viability

Cell viability was determined by the crystal violet staining method, as described previously.⁵³ In brief, cells plated in 12-well dishes at a density of 1×10^6 cells/ml were cultured for 8 h in 10% FBS containing DMEM overnight and further cultured in serum-free medium for another 6 h. Cells were treated with TRAIL (100 ng/ml) in the presence or absence of FBS, albumin, dBSA-LPA, or dBSA-S1P. After 8 h incubation, viable cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 10 min at room temperature. Plates were then washed four times with tap water. After drying, cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using a 96-well plate reader. Cell viability was calculated from relative dye intensity compared with untreated samples. For a long-term assay for cell viability, clonogenic assay was used. In brief, HeLa cells were treated with 100 ng/ml TRAIL in the presence or absence of human serum for 8 h. The cells were washed with fresh medium, replated in 100-mm culture dishes, and then cultured in fresh medium

containing 10% FBS for an additional 2-week period. The plates were stained with crystal violet, and the number of colonies was counted.

Western blot analysis

Cells were harvested, washed twice with ice-cold PBS, and resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml pepstatin, and 1 μ g/ml chymostatin). Cells were lysed by three cycles of freeze and thaw, and cytosolic fractions were obtained by centrifugation at $120\,000 \times g$ for 20 min. Cytosolic proteins (20 μ g) were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against caspase-3, caspase-8, cFLIP, p-Akt, and Akt, and protein bands were visualized by exposure to an X-ray film.

Transfection with siRNA

The target sequences of siRNAs for cFLIP and Bad are 5'-AAGTAAAGAACAAGACTTAACCTGTCTC-3' and 5'-AAGAAGG-GACTTCTCGCCCG-3', respectively. HeLa cells were transfected with these double-stranded siRNAs (40 nmol/ml) for 12 h by lipofectamine method according to the manufacturer's protocol (Life Technologies Inc.) and recovered in fresh media containing 10% FBS for 24 h. The cells were cultured in serum-free media for 6 h and then treated with TRAIL in the presence or absence of FBS or dBSA-LPA. The protein levels of cFLIP and Bad were analyzed by Western blot analysis, and cell viability was determined by crystal violet staining.

Detection of cytochrome *c* release

Cells were incubated with TRAIL (100 ng/ml) for the indicated times, harvested, and resuspended in ice-cold mitochondrial fractionation buffer (20 mM HEPES, 1 mM sodium EDTA, 10 mM KCl, 1 mM DTT, 10 μ M aprotinin, 10 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose). After homogenization using a Dounce homogenizer, cytosolic fractions were obtained by centrifugation at $12\,000 \times g$ for 20 min at 4°C. The cytosolic proteins (40 μ g) were loaded onto a 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed using a cytochrome *c* antibody to determine mitochondrial cytochrome *c* release.

DNA fragmentation assay

Cells were harvested using a plastic scraper and washed with PBS by centrifugation at maximum speed in a microcentrifuge for 30 s at 4°C. Cells were lysed in lysis solution (0.5% SDS, 10 mM EDTA, 0.5 mg/ml proteinase K, and 50 mM Tris-HCl, pH 8.0). DNA was extracted with a standard phenol-chloroform method and precipitated with cold ethanol. The pellets were dried and resuspended in 100 μ l of 20 mM Tris-HCl, pH 8.0. After digesting RNA with RNase (0.1 mg/ml) at 37°C for 1 h, samples (15 μ l) were electrophoresed through a 1.2% agarose gel in 450 mM Tris borate-EDTA buffer, pH 8.0. DNA was photographed under visualization with UV light.

4,6-Diamidino-2-phenylindole staining of nuclei

Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI). HeLa cells were grown on glass coverslips and treated with TRAIL in the absence or presence of serum

components for 5 h. Cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde for 30 min. Following washes with PBS, cells were incubated in 1 μ g/ml DAPI solution for 30 min in the dark. Coverslips were then washed with PBS and analyzed by fluorescence microscopy.

Determination of caspase activity and lipid peroxidation

The cell pellets were washed with ice-cold PBS and resuspended in 100 mM HEPES buffer, pH 7.4, containing a protease inhibitor mixture (5 mg/ml aprotinin and pepstatin, 10 mg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). The cell suspension was lysed by three freeze-thaw cycles, and the cytosolic fraction was obtained by centrifugation at $12\,000 \times g$ for 10 min at 4°C. Caspase activity was determined by measuring proteolytic cleavage of the chromogenic substrate Ac-DEVD-pNA (caspase-3-like activity) or Ac-IETD-pNA (caspase-8-like activity) as described previously.⁵⁴ The lipid peroxidation product malondialdehyde was measured as thiobarbituric acid-reactive substance as previously described.⁵⁵

Data analysis and statistics

The data are presented as the means \pm S.D. from more than three independent experiments. Statistical comparisons between groups were performed using the Student's *t*-test.

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