Non-apoptotic Signaling Pathways Activated by Soluble Fas Ligand in Serum-starved Human Fibroblasts: Mitogen-activated Protein Kinases and NF-κB-dependent Gene Expression*

Jin-Hyung Ahn‡§, Sun-Mi Park‡§#, Ho-Sung Cho§, Myung-Shik Lee†, Jong-Bok Yoon#, Jan Vilcek**, and Tae H. Lee§#

From the §Department of Biology and #Protein Network Research Center, Yonsei University, Seoul 120-749, Korea, †Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea, and **Department of Microbiology, New York University Medical Center, New York, NY10016, USA

To whom correspondence should be sent:

Dr. Tae H. Lee

Tel.: 82-2-2123-4084; Fax: 82-2-312-2242

E-mail: thlee@yonsei.ac.kr

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RUNNING TITLE: Non-apoptotic signaling pathways activated by soluble Fas ligand in human fibroblasts
SUMMARY
Many Fas-expressing cells do not undergo cell death upon Fas stimulation. In the normal human diploid cell line GM6112, the addition of soluble Fas ligand (sFasL) leads to morphological signs of cell death in less than 1% cells. Treatment of serum-starved GM6112 fibroblasts with sFasL resulted in a rapid and transient phosphorylation of ERK1/2 without a significant increase in JNK and p38 activities. Unless co-treated with the protein synthesis inhibitor anisomycin, sFasL did not show gene-inducing activity in cells maintained in complete medium. However, when cells were serum-starved for 4 days, treatment with sFasL alone induced IL-6 gene expression and less strongly IL-8 gene expression. Sensitization of the gene-inducing activity by serum starvation correlated with NF-κB activation by sFasL. Furthermore, we found that the expression of FADD and caspase-8 was significantly reduced in serum-starved cells, while the level of cFLIP remained unchanged. Transfection of GM6112 cells with the antisense caspase-8 expression construct sensitized cells toward sFasL-induced NF-κB-dependent reporter activation. Our results support the notion that a change in the ratio of cFLIP and caspase-8 may be responsible for turning on the Fas-activated NF-κB pathway, which otherwise is supplanted by the death-inducing pathway.
INTRODUCTION

Fas (Apo-1/CD95) is recognized as the principal cell surface receptor triggering apoptotic signals in a variety of cells. Stimulation of Fas leads to the recruitment of Fas-associated death domain protein (FADD) to its cytoplasmic death domain. FADD then recruits procaspase-8, and subsequent autoproteolytic activation of procaspase-8 within the receptor signaling complex initiates the apoptosis program of the cell (1, 2). However, many Fas-expressing cells do not always undergo apoptosis upon Fas stimulation, indicating that susceptibility to Fas-mediated death does not invariably correlate with its expression in any given cell type. There is evidence that in addition to inducing cell death, in at least some cell lines Fas can mediate other activation signals, including increased proliferation in human T cells (3) and fibroblasts (4), induction of IL-8 synthesis in human colon carcinoma cells (5), and activation of the transcription factor NF-κB (6-8).

Furthermore, the soluble form of Fas ligand (sFasL) shed from activated T cells can act directly as a chemoattractant for neutrophils (9). Collectively, these observations indicate that Fas-mediated signaling is not limited to inducing cell death. However, the molecular events triggering Fas-mediated signals distinct from apoptosis induction have not been clearly delineated.

FADD is not only an essential signaling mediator for Fas-transduced apoptosis, but it also can be involved in signals that promote proliferation of TCR-activated T cells, as shown by the absence of activation-induced T proliferation in FADD knockout mice (10) or transgenic mice expressing dominant negative FADD (11, 12). Therefore, Fas-mediated signaling pathways not associated with apoptosis induction can be branched out from FADD. It has been suggested that cFLIP (Casper/CASH/I-FLICE/CLARP) controls Fas signaling pathways downstream of FADD (13-17). cFLIP is a caspase-8-like molecule that lacks catalytic activity and has been known to act as a caspase-8 inhibitor by competing for the binding to FADD (18, 19). Under conditions in which CD3-activated proliferation of T cells is augmented by Fas co-stimulation, cFLIP is shown to be recruited to the Fas receptor complex by interacting with FADD. The FADD-associated cFLIP then
interacts with tumor necrosis factor (TNF)-associated factor 1 and 2 (TRAF1 and 2), leading to the activation of NF-κB and extracellular signal regulated kinase 1 and 2 (ERK1/2) (20). This observation suggests that the switch from the Fas-induced apoptotic pathway to a non-apoptotic signaling pathway may be determined by the equilibrium between caspase-8 and its repressor cFLIP and their competition for binding to FADD. Changes in the dynamics of the molecules involved in the decision making step may be affected by additional signals such as TCR stimulation in this specific setting. In fact, the function of Fas in a given cell type is known to be influenced by a cellular factor(s) whose expression or activation is affected by other stimuli. This idea is supported by the fact that many cells resistant to Fas-induced apoptosis become sensitive in the presence of metabolic inhibitors such as cycloheximide (CHX) and actinomycin D. In more physiological conditions, sensitivity to Fas-mediated apoptosis was shown to be influenced by certain combinations of inflammatory cytokines such as TNF, interferon-γ or interleukin-1 (21). In addition, the ability of Fas to activate NF-κB and subsequent gene induction can be observed under conditions in which de novo protein synthesis is blocked or the apoptotic pathway is inhibited by treatment with caspase inhibitors (22).

In the present study, we used the human diploid fibroblast cell line GM6112 to characterize Fas-mediated signals not associated with apoptosis induction and to define conditions that favor the generation of Fas-mediated non-apoptotic signaling pathways. We show that upon sFasL treatment, GM6112 cells responded by the activation of ERK1/2 in the absence of c-Jun N-terminal kinase (JNK) and p38 activation. Serum starvation sensitized fibroblasts to Fas-mediated NF-κB activation, which correlated with the down-regulation of FADD and caspase-8 expression levels without a change in cFLIP expression. Our findings support the notion that a change in the ratio of cFLIP and caspase-8 may be responsible for the divergence of Fas signaling from the apoptotic pathway to a non-apoptotic pathway.
EXPERIMENTAL PROCEDURES

Cell culture and Reagents

Culture media were purchased from Life Technologies Inc. Normal human diploid fibroblasts GM6112 were purchased from Human Genetic Mutant Cell Repository, Camden, NJ 08103. GM6112 cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Human embryonic kidney cell line HEK293 was maintained in DMEM supplemented with 10% FCS.

Recombinant soluble FasL (sFasL) used in this study was prepared from culture supernatant of CHO-K1-sFasL cells grown in serum-free medium (CHO-S-SFM II; Life Technology). The biological activity of sFasL has been previously described (23, 24). In most experiments cells were treated with medium containing 10 % (vol/vol) of the sFasL preparation, corresponding to a final sFasL concentration of 1-2 µg/ml. Yeast-derived recombinant human TNF-α was kindly supplied by Dr. H. H. Chung, Biotech Research Institute, LG Chem., Korea. Phorbol-12-myristate-13-acetate (PMA), cycloheximide and anisomycin were purchased from Sigma. PD098059 was purchased from Calbiochem. Rabbit polyclonal α-PY(ab-1) antibody reactive to phospho-tyrosine residues was obtained from Dr. J. Schlessinger at NYU Medical Center. The rabbit anti-phospho-ERK and anti-phospho-p38 MAPK antibodies were purchased from New England Biolabs. The anti-Fas monoclonal antibody CH11 and antibodies to cFLIP and caspase-8 were purchased from Pharmingen. Antibodies to FADD and α-tubulin were from Transduction Laboratories and Sigma, respectively.

A cytomegalovirus (CMV) promoter-driven luciferase reporter plasmid, pCMV-luciferase (25) was used for indirect assessment of cell viability.

Antisense caspase-8 and FADD RNA expression constructs
It was reported that the green fluorescent protein (GFP)-fused antisense RNA showed an improved efficiency over conventional antisense RNA, because RNAs that lack an open reading frame (ORF) are labile (26). Thus, we constructed mammalian expression plasmids for the expression of antisense FADD and caspase-8 RNAs by inserting the respective antisense cDNAs downstream of the GFP coding sequence. The C-terminal GFP-fusion plasmid pEGFP-C2 (Clontech) was used for this construction, in which the HindIII-XhoI fragment coding for the entire open reading frame of FADD and the BglII-XhoI DNA fragment coding for procaspase-8 were cloned into the XhoI/HindIII and SalI/BamHI-digested pEGFP-C2 plasmids, respectively.

Western blot analysis

For immunoblot analysis of cellular MAP kinases, cells were washed with cold PBS, scraped and resuspended in a buffer consisting of 1% Nonidet P-40, 50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 3 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 100 mM sodium fluoride. Cell lysates were centrifuged and the supernatants were electrophoresed through 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Biorad). The blots were blocked in Tris-buffered saline/Tween-20 supplemented with 5% skim milk for 1 h, incubated with 1:1,000 diluted primary rabbit anti-phospho-ERK or p38 MAPK antibody for 1 h, and then with 1:5,000 diluted secondary antibody of horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were then treated with enhanced chemiluminescence reagents (Amersham), and the phosphorylated proteins were detected by autoradiography. For Western blot analysis of other proteins, cells were lysed in a buffer containing 1% SDS and boiled for 15 min to reduce viscosity of the lysates. After centrifugation, clear lysates were processed as described above.

In vitro JNK kinase assay

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GST-c-Jun(1-79) fusion protein was purified from bacterial lysates using GST-Sepharose beads (Pharmacia Biotech). Following stimulation, GM6112 cells were lysed in the lysis buffer described in the previous section. The lysates were mixed with 20 µl of GST-c-Jun bound to GST-Sepharose beads. The mixture was rotated at 4°C for 1 h and pelleted by centrifugation at 10,000 rpm for 3 min. The pelleted beads were washed two times with the lysis buffer and once with kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl₂, 20 mM β-glycerol phosphate, 0.2 mM sodium orthovanadate, 10 mM sodium fluoride and 0.2 mM DTT) and then resuspended in 50 µl of kinase buffer containing 50 µM ATP and 10 µCi of [γ-³²P]ATP (3,000 Ci/mmol, Amersham). After 30 min incubation at 30°C, the reaction was terminated by adding 2x Laemmli sample buffer and boiling for 3 min. Samples were resolved on 12% SDS-PAGE gel and subjected to autoradiography.

Transfection and Luciferase Assays

Transfection was carried out by the CaPO₄-DNA precipitation method using Hepes or N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) buffer as described elsewhere (27). Details of the transfection protocols used in GM6112 and HEK293 cells are described in the legend of Figure 6. Transfectants were lysed in 0.15 ml of lysis buffer (Promega) and centrifuged at 10,000 x g for 5 min to remove cell debris. The resulting clear lysates were assayed for luciferase and β-galactosidase activity, and the values of the luciferase assay were normalized with respect to the values of the β-galactosidase assay for relative comparison of each transfection.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)

GM6112 cells were treated with sFasL at the indicated times. Nuclear extracts were prepared as described by Dignam et al. (28), quantitated by the Bradford assay (Bio-Rad) and stored at −70 °C. The oligonucleotide probes for the EMSA corresponded to the NF-κB binding sites in the c-IAP2
promoter (sense, 5'-ATGGAAATCCCCGA-3' and antisense, 5'-TCGGGGATTTCCAT-3') (27). Two oligonucleotides complementary to each other were annealed to generate a double stranded probe. End-labeling was accomplished by treatment with T4 polynucleotide kinase in the presence of [γ-32P]ATP. Approximately 1 ng of the labeled probe was mixed with 2.4 µg of nuclear protein in a total of 20 µl of the binding buffer (20 mM Hepes, pH 7.9, 60 mM KCl, 1 mM MgCl₂, 20 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol) containing 2 µg of poly(dI·dC). After incubation for 20 min, the reaction mixture was separated on a 6% nondenaturing polyacrylamide gel with 0.5x TBE buffer (40 mM Tris borate, 1 mM EDTA, pH 8.0). The gel was vacuum dried and subjected to autoradiography. For competition experiments, a 100- or 300-fold molar excess of the unlabeled double stranded probe was added prior to the addition of the labeled probe.
RESULTS

Fas stimulation leads to ERK1 and ERK2 activation in human fibroblasts without significant increase in JNK and p38 MAPK activities

The serum-free culture supernatant of CHO-K1 cells secreting recombinant soluble FasL (sFasL) was used as an agent for triggering Fas expressed on a human diploid fibroblast cell line, GM6112. The apoptosis-inducing activity of the sFasL preparation was previously confirmed in T cells (23, 24). In experiments to test Fas sensitivity of GM6112 cells, we observed that even after 48 h treatment with medium containing 10% (vol/vol) of sFasL preparation (a concentration that killed >50% Jurkat T cell after treatment for 16 h), less than 1% of cells showed morphological signs of rounding and shrinking. However, combined treatment with sFasL and CHX (5 µg/ml) led to extensive cell death, indicating that Fas expressed on GM6112 cells was active in delivering a death signal. When the cells were serum-starved by maintaining confluent cultures in low serum-containing medium (0.25% FCS) for 48 h, the number of cells undergoing apoptotic cell death increased significantly after sFasL exposure for 16 h (over 10% of cells showing signs of rounding and shrinking), indicating that serum starvation sensitizes cells to Fas-activated apoptosis (data not shown).

We determined whether sFasL treatment of serum-starved GM6112 cells leads to an increase in tyrosine phosphorylation of cellular proteins. Lysates from the serum-starved cultures treated with sFasL for various periods were analyzed by immunoblotting with a rabbit polyclonal α-PY(ab-1) antibody specific for phospho-tyrosine residues. As can be seen in Fig. 1A, the α-PY(ab-1) antibody identified a transient increased phosphorylation of 42- and 44-kDa proteins, whose characteristic molecular sizes correspond to ERK2 and ERK1, respectively. An increase in the phosphorylation of the 42- and 44-kDa proteins peaked after 15 min incubation of cells with sFasL and decreased to the basal level after 60 min incubation. The magnitude of the increased phosphorylation detected after sFasL treatment for 15 min was comparable to that seen when the
fibroblasts were exposed to TNF (10 ng/ml) for the same time. In addition, concomitantly with the increased phosphorylation of the 42- and 44-kDa proteins after sFasL treatment, a tyrosine-phosphorylated band at the position of approximately 200-kDa was also detected (marked by asterisk in Fig. 1A). The activation of the high molecular weight protein was sFasL-specific because TNF treatment did not lead to phosphorylation of this protein.

ERK1/2 activation after exposure of GM6112 cells to sFasL, anti-Fas antibody CH11 and TNF for 15 min was confirmed by Western analysis using an ERK antibody recognizing phosphorylated tyrosine 204 of p42 and p44 MAPK (Fig. 1B). When GM6112 cells were preincubated with 50 µM of PD098059 for 2 h, an activation inhibitor of ERK1/2, the ERK1/2 phosphorylation by sFasL, CH11, or TNF was completely inhibited, indicating that signals leading to ERK1/2 activation are MEK1- and 2-dependent.

Next, we assayed for sFasL-induced p38 and JNK activation, since previous studies have shown a role of these kinases in the regulation of Fas-mediated apoptosis (29-31). When the same lysates were examined for p38 MAPK activation by Western analysis with the anti-phospho-p38 MAPK antibody specific for phosphorylated tyrosine 182 of p38 MAPK, no phosphorylated p38 MAPK was detected in sFasL-treated lysates (middle panel in Fig. 1A). JNK activity in the lysates was determined by phosphorylation of GST-c-Jun (1-79). Fig. 1A (bottom panel) shows that stimulation with sFasL did not result in JNK activation, while TNF treatment led to a strong activation of JNK.

**Activation of ERK1/2 by sFasL is phorbol ester-sensitive protein kinase C (PKC)-independent**

To analyze early signaling events coupled to ERK1/2 activation by sFasL, we examined the contribution of the phorbol ester-sensitive PKC isotypes to the sFasL-mediated ERK1/2 activation. Confluent cultures of GM6112 cells were treated with a high concentration (200 ng/ml) of PMA for 24 hr in order to desensitize PMA-sensitive PKC, and followed by stimulation with sFasL, anti-Fas antibody CH11, TNF or a low dose of PMA for 15 min. As shown in Fig. 2, a brief stimulation of
naïve GM6112 cells with a low concentration (10 ng/ml) of PMA caused ERK1/2 activation. In cells pretreated with the high dose of PMA, further treatment of PMA did not lead to ERK1/2 activation, indicating desensitization of the PKC pathway. However, PKC desensitization did not influence the ability of sFasL, CH11 and TNF to activate ERK1/2. Therefore, PMA-sensitive PKC isoforms are not involved in the activation of ERK1/2 by sFasL. It is interesting to note that TNF, which is mitogenic for serum-starved GM6112 cells (data not shown), also activated ERK1/2 in a PMA-sensitive PKC-independent manner. These results suggest that sFasL and TNF may use a common pathway leading to ERK activation and that ERK activation by sFasL is not linked to the apoptosis signaling pathways.

*sFasL treatment induces IL-6 and IL-8 gene expression in serum-starved fibroblasts*

Fas-mediated activation of non-apoptotic signaling in GM6112 cells was further explored by examining gene induction by sFasL. At first, we examined whether sFasL induces IL-8 gene expression in cells maintained in complete medium containing 10% FCS. Northern blot analysis revealed that sFasL treatment alone did not lead to an increase in the level of IL-8 mRNA in cells maintained in complete medium, whereas TNF or the protein synthesis inhibitor anisomycin (Anis) (1 µg/ml) alone induced IL-8 gene expression (Fig. 3A, *left panel*). Simultaneous treatment with sFasL and Anis for 3 h showed a stronger induction than Anis alone. As the treatment time increased, IL-8 mRNA level diminished because of the cytotoxic effect of the combined treatment with sFasL and Anis on GM6112 cells, as judged by a concomitant reduction in the level of the reference mRNA (*pHe7*). A synergistic induction by sFasL and Anis was also seen with low concentrations of Anis (10 or 50 ng/ml) which were incapable of IL-8 gene induction by themselves (Fig. 3A, *right panel*).

When confluent GM6112 cultures were serum-starved in medium containing 0.25% FCS for 4 days, the addition of sFasL alone clearly induced gene activation. As shown in Fig. 3B, sFasL alone
induced both IL-6 and IL-8 gene expression in serum-starved cells at levels similar to those induced by TNF. In the presence of a suboptimal concentration (50 ng/ml) of Anis, levels of IL-6 and IL-8 mRNA expression were further enhanced. These results indicate that serum starvation sensitizes cells toward Fas-mediated gene induction. Other experiments showed that IL-6 and IL-8 gene induction in GM6112 fibroblasts by sFasL alone was observed when cells were maintained in 0.25% FCS-containing medium for at least 3 days.

Sensitization of the gene inducing activity of sFasL by serum-starvation correlates with its NF-κB inducing activity

Induction of IL-6 gene expression by various stimuli is primarily dependent on NF-κB activation (32). Since our data demonstrated that in serum-starved cells, IL-6 gene can be activated by sFasL alone, we examined whether NF-κB is activated by sFasL under such conditions. We performed electrophoretic mobility shift assays (EMSA) with nuclear extracts of sFasL-treated cells that were either serum-starved or maintained in medium containing 10% FCS. As a probe, we used a NF-κB binding element from the promoter of the cellular inhibitor of apoptosis protein 2 (c-IAP2) gene (27), because preliminary experiments consistently demonstrated higher NF-κB binding affinity than with the NF-κB site derived from the promoter of IL-6. As shown in Fig. 4 (left panel), 30 min sFasL treatment of GM6112 cells serum-starved for 4 days resulted in the appearance of a shifted band, which decreased after 1 h and disappeared after 2 h treatment. However, no band was visible in EMSA with a nuclear extract from sFasL-treated cells that were not serum-starved. Combined treatment with sFasL and CHX (5 μg/ml) led to a stronger induction of NF-κB DNA binding activity in serum-starved cells than in cells maintained in complete medium, showing a correlation with the observed gene induction in Fig. 3.
Since sFasL induced IL-6 gene expression when cells were serum-starved for at least 3 days, we examined the kinetics of the sFasL-induced NF-κB DNA binding activity in GM6112 cells maintained in serum-starved condition for various periods. As shown in Fig. 4 (right panel), sFasL-induced NF-κB binding activity gradually increased as cells were serum-starved for 1, 2 or 4 days. Competition assays using 100-fold or 300-fold excess of unlabeled c-IAP2 NF-κB or IL-6 NF-κB oligonucleotides, respectively, resulted in the disappearance of radioisotope-labeled bands, confirming the binding specificity of the nuclear proteins induced by sFasL.

*Serum-starvation leads to the reduction of FADD and caspase-8 level in fibroblasts, while cFLIP level remains unchanged*

To search for the mechanism that may be responsible for sensitizing NF-κB-dependent gene activation in the serum-starved fibroblasts, we examined if there were any changes in the expression level of proteins which are part of the Fas signaling such as Fas, FADD, caspase-8 and cFLIP. As shown in Fig. 5, a significant reduction of FADD and caspase-8 expression levels but no change in cFLIP level was seen in cells that were serum-starved by maintaining confluent cultures in 0.25% FCS-containing medium for 4 days. Thus, FADD and caspase-8 expression may be prone to degradation under low metabolic conditions, relative to other proteins tested. When the expression level of p21, which is known to be up-regulated by growth arrest (33) was examined, no significant increase was found after serum starvation (data not shown). In addition, cells arrested at G_{i}/S transition by hydroxyurea (HU) treatment for 1 day did not show a significant change in the expression levels of FADD, caspase-8 and cFLIP. Rather, the level of Fas expression was enhanced. Since IL-6 gene induction by sFasL was not observed in either HU-treated cells or cells starved for 2 days in low serum-containing medium (data not shown), a simple cell cycle arrest, either at G_{i} or G_{o} stage, may not be sufficient to sensitize cells to Fas-mediated NF-κB activation.
Down-regulation of caspase-8 level by expressing antisense caspase-8 RNA sensitizes cells to sFasL-induced NF-κB activation

To establish whether a causal relationship exists between the reduction of FADD and caspase-8 expression levels in serum-starved fibroblasts and the sensitization to NF-κB activation by sFasL, we down-regulated endogenous FADD or caspase-8 levels by the expression of their antisense RNAs, and examined sFasL-induced NF-κB-dependent reporter gene activation. As a reporter plasmid, we used an NF-κB-inducible luciferase construct comprising four copies of the IL-6 NF-κB binding site (pIL-6-kB-luc). As shown in Fig. 6A, in GM6112 cells transfected with various doses of antisense FADD plasmid, sFasL-induced reporter gene activation could not be observed. However, considerable NF-κB reporter gene activation by sFasL (3.5-fold induction) was detected in cells transfected with a dose of 2.5 μg of antisense caspase-8 plasmid. As the amount of transfected DNA increased, the luciferase activities decreased, suggesting that a high level expression of antisense caspase-8 RNA was inhibitory to the sFasL-induced NF-κB activation. To determine whether the increased NF-κB-dependent luciferase activation seen in cells transfected with the antisense caspase-8 expression plasmid was simply due to increased cell survival, we performed similar transfection experiments using a constitutively active cytomegalovirus (CMV) promoter-driven luciferase reporter plasmid instead of pIL-6-κB-luciferase. Treatment of GM6112 cells with sFasL resulted in a 12% reduction of luciferase activity, reflecting a weak susceptibility of GM6112 cells to Fas-mediated killing (Fig. 6B). Co-transfection of cells with four different doses of the antisense caspase-8 expression plasmid did not have a significant effect on the levels of CMV promoter-driven luciferase activity, indicating that the increased NF-κB-dependent luciferase activity of cells transfected with the antisense caspase-8 expression vector is not due to a better survival of such cells after stimulation with sFasL.
An increase in NF-κB activation by down-regulation of caspase-8 was also observed in HEK293 cells transfected with a Fas expression plasmid (Fig. 6C). Even without sFasL treatment, cotransfection of Fas and the low dose of 0.2 µg of antisense caspase-8 plasmids enhanced NF-κB-dependent luciferase activity up to 3.3-fold. Upon treatment of this transfectant with sFasL for 8 h, the luciferase activity increased further up to 8.2-fold. Similar to GM6112 cells, the luciferase induction by sFasL decreased with increasing amounts of the transfected antisense DNAs. In addition, HEK293 cells transfected with the antisense FADD expression plasmid also showed the sensitized NF-κB induction after treatment of sFasL, which diminished with a high dose of DNA transfection. Therefore, our results suggest that the expression level of caspase-8 or FADD controls sensitivity towards sFasL-induced NF-κB activation.
DISCUSSION

Fas ligation on fibroblasts does not always lead to cell death (4). To characterize the molecular events involved in Fas-mediated signals that may not be associated with inducing cell death, we examined the effects of Fas stimulation on the activation of MAPK family proteins as well as cytokine gene induction in the human diploid fibroblast cell line GM6112, which is only weakly sensitive to Fas-mediated apoptosis but undergoes extensive cell death in the presence of sFasL and CHX. We demonstrate that the two MAPK isoforms, ERK1 and ERK2, were rapidly activated following stimulation with sFasL without a significant increase in JNK and p38 MAPK activities. Studies using the human Jurkat T cell line or in vitro-activated human peripheral blood lymphocytes have shown that the JNK and p38 pathway is activated in Fas-mediated apoptosis (28-30) and that Fas-mediated apoptosis in Jurkat T cells can be suppressed by ERK1/2 activation (34), suggesting that ERK1/2 can promote cell survival and inhibit apoptosis. It was also reported that Fas stimulation resulted in both ERK and JNK activation in the Fas-sensitive SHEP neuroblastoma cell line, and both ERK and JNK activation were implicated in Fas-induced apoptosis of these cells (35). Shinobara et al. (36) have recently demonstrated that, like GM6112 cells, human glioma cells respond to Fas stimulation by activating ERK in the absence of JNK and p38 activation. Unlike in GM6112 cells, Fas stimulation of the glioma cells was shown to be mitogenic. ERK activation without the activation of JNK and p38 was also observed in the normal diploid fibroblast line LF-1 which is highly sensitive to Fas killing (data not shown). Therefore, the preferential activation of the ERK pathway appears to be unrelated to susceptibility to Fas-mediated apoptosis and may be a distinct characteristic of Fas signaling in fibroblasts.

Fas-mediated non-apoptotic signaling in fibroblasts was further documented by our demonstration that sFasL was able to induce IL-6 and IL-8 gene expression in serum-starved cultures. IL-6 induction by sFasL in serum-starved cultures was comparable to that induced by TNF and correlated with the activation of NF-κB because we could detect NF-κB DNA binding activity.
in the nuclear extracts of serum-starved fibroblasts treated with sFasL, but not in the extracts of sFasL-treated cells maintained in serum-containing medium (Fig. 4). The ability of Fas to activate NF-κB has been demonstrated in some cell lines (6-8) and in cortical neuroblast cultures derived from the developing mouse brain (37). Moreover, as recently demonstrated by Wajant et al. (22), treatment of Fas-overexpressing HeLa cells with agonistic Fas antibody led to the induction of some genes whose activation is under the control of NF-κB. In our present experiments, NF-κB-dependent gene induction by sFasL was observed only in serum-starved GM6112 cells or when de novo protein synthesis was blocked by CHX, suggesting that one or more CHX-sensitive labile factors that also become depleted during serum starvation inhibit Fas-mediated NF-κB-dependent gene induction. In addition, treatment of CHX also sensitizes toward Fas-mediated apoptosis, as many cells resistant to Fas-mediated cell death become highly sensitive to Fas killing in the presence of CHX (1, 22). Whether the same CHX-sensitive labile factor acts as an inhibitor for Fas-activated pathway leading to both cell death and NF-κB activation remains to be determined.

A previous report that overexpression of FADD not only induces cell death but also activates NF-κB (38) suggests that these two distinct Fas effects are likely to be regulated at the level of FADD. Upon Fas stimulation, a death-inducing signaling complex (DISC) comprising FADD and procaspase-8 is formed, and within DISC the recruited procaspase-8 is oligomerized and autocleaved to a mature enzyme. This active caspase-8 then provides the initiating signal in the caspase cascade. A known inhibitor of the Fas-activated death pathway, cFLIP has been suggested to control Fas-induced cell death by competing with caspase-8 for FADD (18, 19). Several reports showed that up-regulation of cFLIP confers resistance to Fas-susceptible cells, and conversely down-regulation of cFLIP enhances Fas-mediated cell death (13, 39-41). Therefore, cFLIP was proposed to act as a prime candidate for the short-lived regulatory factor whose expression is rapidly down-regulated by CHX treatment (22, 42). Furthermore, cFLIP was shown to act as a
molecule for diverting signals from Fas-induced apoptosis to NF-κB and ERK activation pathways during TCR-activated proliferation of T cell by Fas costimulation (20).

Since the serum-starved GM6112 cells responded to Fas stimulation by activating ERK1/2 and NF-κB, we expected a change in cFLIP expression level in serum-starved cells. Western analysis of GM6112 cell lysates revealed that serum starvation did not cause a change in the level of cFLIP, but instead substantially reduced the level of FADD and caspase-8 (Fig. 5). This decreased level of caspase-8 and FADD relative to cFLIP could cause much of the remaining FADD to become complexed with cFLIP. This, in turn, would decrease the amount of FADD available to form FADD-caspase-8 complexes and thereby reduce the recruitment of caspase-8 to DISC, which may be responsible for the diversion of Fas-activated pathways toward NF-κB activation. In agreement with this notion, increased NF-κB binding activity as well as IL-6 or IL-8 gene induction by sFasL could be observed after maintaining the GM6112 cultures in medium containing 0.25% serum for at least 4 days, the time point when a significant reduction of FADD and caspase-8 expression was observed. In addition, reducing caspase-8 level by transfecting an antisense caspase-8 expression construct into GM6112 and Fas-coexpressing HEK293 cells resulted in sensitization to sFasL-induced NF-κB reporter gene activation (Fig. 6). This sensitized NF-κB induction was observed when these cells were transfected with low amounts of antisense DNA. Transfection with a high dose of the antisense caspase-8 construct in both GM6112 and HEK293 cells, as well as the antisense FADD construct in HEK293 cells, caused an inhibition of Fas-mediated NF-κB activation. Thus, it is conceivable that fine-tuning in the intracellular level of FADD or caspase-8 expression is required for the proper generation of NF-κB signaling. However, the exact nature of interactions involving FADD, caspase-8 and cFLIP within the DISC, which is responsible for diverting Fas-mediated signaling to NF-κB activation, requires further investigation.
In conclusion, our data suggest a mode of sensitizing cells toward Fas-mediated non-apoptotic signaling pathways, especially NF-κB activation, through changes in the balance of molecules involved in the decision making step brought about by external stimuli or conditions. Serum starvation of fibroblasts down-regulates the level of caspase-8 and FADD expression without affecting the level of cFLIP, thus cFLIP would likely prevail over caspase-8 in the competition for binding to the limited amount of FADD, thereby increasing the fraction of cFLIP bound to FADD in DISC. This intracellular change may be responsible for sensitizing cells to the Fas-mediated NF-κB activation pathway, which otherwise is supplanted by the death-inducing pathway in most cell-types. This situation may be analogous to the sensitization of cells toward Fas-mediated NF-κB activation by inhibiting the apoptotic pathway with caspase inhibitors, but it is obviously different from the CHX-sensitized NF-κB activation which is accompanied by extensive cell death through the down-regulation of cFLIP (22, 42). Sensitization toward Fas-mediated NF-κB activation by serum starvation may have some physiological relevance under inflammatory conditions when quiescent fibroblasts become activated to produce IL-6 and IL-8 by infiltrating FasL-bearing or FasL-shedding lymphocytes.
REFERENCES


FOOTNOTES

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‡ These two authors contributed equally to this work.

¶ To whom correspondence should be addressed: Department of Biology, College of Science, Yonsei University, 134 Shinchon-Dong, Sudaemoon-Gu, Seoul 120-749, Korea. Tel.: 82-2-2123-4084; Fax: 82-2-312-2242; E-mail: thlee@yonsei.ac.kr

Abbreviations used are: FADD, Fas-associated death domain protein; sFasL, soluble Fas ligand; TNF, tumor necrosis factor α; c-IAP2, cellular inhibitor of apoptosis protein 2; TRAF, TNF receptor associated factor; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; CHX, cycloheximide; MAPK, mitogen activated protein kinase; FCS, fetal calf serum; PKC, protein kinase C; PMA, Phorbol-12-myristate-13-acetate; Anis, anisomycin; EMSA, electrophoretic mobility shift assay; HU, hydroxyurea; DISC, death inducing signaling complex.
FIGURE LEGENDS

FIG. 1. p44 (ERK1) and p42 MAPK (ERK2) are activated by sFasL without significant activation of JNK and p38, and the phosphorylation of ERK1/2 requires MEK1/2. A, confluent GM6112 cultures maintained in low serum containing medium (0.25% FCS) for 24 h were stimulated with 1:10 dilution of sFasL-containing CHO-K1-sFasL cell supernatant for the indicated times. As controls, the cultures were treated with conditioned medium (CM) of the parental (untransfected) CHO-K1 cells maintained in serum-free medium or with TNF (10 ng/ml). Cell lysates were immunoblotted using anti-phosphotyrosine antibody [αPY(ab-1)] or anti-phospho-p38 antibody. The same lysates were also incubated with GST-c-Jun fusion protein and JNK activity was measured by the phosphorylation of c-Jun. B, GM6112 cells were pretreated with 50 µM PD098059 for 2 h and sequentially stimulated with sFasL, TNF or anti-Fas CH11 antibody for 15 min. ERK1/2 activation was examined by immunoblotting of the lysates with anti-phospho-ERK antibody.

FIG. 2. Effect of pretreatment with a high dose of PMA on the activation of ERK1/2 by sFasL or TNF. GM6112 cells were either left untreated or treated with 200 ng/ml PMA for 24 h prior to stimulation with a 1:10 dilution of conditioned medium (CM) from parental CHO-K1 or from sFasL-containing CHO-K1 cells, with TNF, or with PMA (10 ng/ml) for 15 min. Lysates were then generated, blotted, and probed with anti-phospho-ERK antibody.

FIG. 3. IL-6 and IL-8 genes are induced by treating serum-starved GM6112 cells with sFasL. A, confluent GM6112 cultures grown in complete medium (10% FCS) were stimulated with 10% sFasL preparation alone or with sFasL plus 1 µg/ml anisomycin (Anis) for the indicated times. For control experiments, the cultures were treated with TNF, anisomycin (1 µg/ml) or both for 3 h. RNA isolated from these cells was examined by Northern blot analysis for the presence of IL-8
mRNA as described elsewhere (left panel). Northern blot analysis was also performed with RNA samples prepared from the GM6112 cells treated with various concentrations of anisomycin alone or cotreated with 10% sFasL preparation, as indicated (right panel). B, The serum-starved GM6112 cells which were maintained in low serum (0.25% FCS) medium for 4 days were stimulated for 3 h with the indicated dilutions of sFasL in the absence or presence of a suboptimal concentration of anisomycin (50 ng/ml). RNA blot was prepared, and was probed with ^32^P-labeled IL-6 and IL-8 cDNA fragments. All blots were also probed with a ^32^P-labeled pHe7 internal reference cDNA specific for an invariant mRNA species.

**FIG. 4. Treatment with sFasL alone induces NF-κB activation in serum-starved GM6112 cells.** Confluent GM6112 cells grown in 10% FCS-containing medium were either serum-starved by maintaining the cultures in 0.25% FCS-containing medium for 4 days (indicated by plus sign) or kept in 10% FCS-containing medium for another 4 days (indicated by minus sign). The resulting cultures were exposed to 10% sFasL preparation for the indicated times. The cultures were also treated with 5 µg/ml cycloheximide (CHX) or with a combination of CHX with the same concentration of sFasL. Nuclear extracts were prepared, and electrophoretic mobility shift assays (EMSA) were performed with a radiolabeled double-strand NF-κB probe derived from the c-IAP2 promoter (left panel). Nuclear extracts were also prepared from confluent cultures which were maintained under serum-starved condition for the indicated times and then exposed to 10% sFasL preparation. EMSA using the same radiolabeled c-IAP2 NF-κB probe was performed in the absence or presence of 100-fold excess unlabeled c-IAP2 NF-κB oligonucleotides [cold (c-IAP2)] or 300-fold excess of IL-6 NF-κB oligonucleotides [cold (IL-6)] for competition (right panel).

**FIG. 5. The levels of FADD and procaspase-8 expression are reduced in serum-starved**
GM6112 cells. Confluent cultures were incubated in 0.25% FCS-containing medium for the indicated time. The cultures were also arrested at G1/S cell cycle stage by treating with 4 mM hydroxyurea for 1 day. Total cell lysates were prepared and processed for Western blot analysis using a series of antibodies reactive to the indicated proteins.

FIG. 6. Influence of antisense FADD and procaspase-8 expression on sFasL-induced NF-κB-dependent reporter gene activation. A, GM6112 cells which reached 70% confluence in a 10-cm culture dish were transfected with the antisense FADD or caspase-8 expression plasmids as indicated, along with 2 µg of NF-κB luciferase reporter plasmid and 3 µg of pCDM8-β-galactosidase by the CaPO4-DNA precipitation method using HEPES buffer (pH 6.95). Total amount of transfected DNA was maintained constant at 15 µg in each transfection. After 4 h, the medium was removed and the cells were shocked with 15% glycerol for 1.5 min. The cells were carefully washed with medium to remove traces of glycerol and allowed to recover in fresh medium for 4 h. In order to minimize differences due to variations in transfection efficiency the cells were evenly split into 4 wells in a 6-well-dish. After incubation of the cells for 24 h in a 10% serum-containing medium, two wells were left untreated and the other two were treated with sFasL for 8 h. Cell lysates were prepared and processed for luciferase assays. The results shown are the average of three independent experiments, with each group run in duplicate. B, The experimental design was the same as for A, except that, instead of NF-κB luciferase plasmid, pCMV-luciferase reporter plasmid was used. After treating transfectants with sFasL for 24 h, cells were processed for luciferase assay. C, HEK293 cells were seeded into 12-well-dishes and grown to 70% confluence. Transfection was carried out by the CaPO4-DNA precipitation method using BES buffer as described (26) with a total of 1.0 µg DNA containing the indicated antisense expression plasmid and the reporter plasmid (0.2 µg) in the absence or presence of Fas expression plasmid (0.2 µg).
h post-transfection, transfectants were left untreated and treated sFasL for 8 h. Cell lysates were prepared and processed for luciferase assay. The results shown are the average of five independent experiments.
**FIG. 1**

A. Time course of sFasL (min) and CM (15 min) treatment with TNF (15 min). Key markers:
- pp44 (ERK1)
- pp42 (ERK2)
- pp38
- GST-c-Jun

B. Effects of sFasL, TNF, and anti-Fas (CH11) with and without PD098059 pretreatment. Key markers:
- ERK1
- ERK2

PD098059 pretreated
FIG. 2
A. GM6112 grown in the complete medium

B. Serum-starved GM6112

FIG. 3
FIG. 5
FIG. 6
Non-apoptotic signaling pathways activated by soluble Fas ligand in serum-starved human fibroblasts: Mitogen-activated protein kinases and NF-κB-dependent gene expression

Jin-Hyung Ahn, Sun-Mi Park, Ho-Sung Cho, Myung-Shik Lee, Jong-Bok Yoon, Jan Vilcek and Tae H. Lee

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