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Mechanism of LIGHT/Interferon-γ-Induced Cell Death in HT-29 Cells

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Abstract LIGHT is a member of tumor necrosis factor (TNF) superfamily, and previous studies have indicated that in the presence of interferon-γ (IFN-γ), LIGHT through LTβR signaling can induce cell death with features unlike classic apoptosis. In present study, we investigated the mechanism of LIGHT/IFN-γ-induced cell death in HT-29 cells, where the cell death was profoundly induced when sub-toxic concentrations of LIGHT and IFN-γ were co-treated. LIGHT/IFN-γ-induced cell death was accompanied by DNA fragmentation and slight LDH release. This effect was not affected by caspase, JNK nor cathepsin B inhibitors, but was partially prevented by p38 mitogen-activated protein kinase (MAPK) and poly (ADP-ribose) polymerase (PARP) inhibitors, and abolished by aurintricarboxylic acid (ATA), which is an inhibitor of endonuclease and STATs signaling of IFN-γ. Immunobloting reveals that LIGHT/IFN-γ could induce p38 MAPK activity, Bak and Fas expression, but down-regulate Mcl-1. Besides, LIGHT/IFN-γ could not activate caspase-3 and -9, but decreased mitochrondrial membrane potential. Although LIGHT could not affect IFN-γ-induced STAT1 phosphorylation and transactivation activity, which was required for the sensitization of cell death, survival NF-κB signaling of LIGHT was inhibited by IFN-γ. These data suggest that co-presence of LIGHT and IFN-γ can induce an integrated interaction in signaling pathways, which lead to mitochondrial dysfunction and mix-type cell death, not involving caspase activation. J. Cell. Biochem. 93: 1188–1202, 2004.

Key words: LIGHT; IFN-γ; p38 MAPK; caspase; mitochondrial; NF-κB

Members of the tumor necrosis factor (TNF) ligand superfamily play multiple roles in cell activation, proliferation, differentiation, and apoptosis. Among the list of molecules belonging to the TNF superfamily, many of them can trigger both canonical apoptotic and necrotic death events [Fiers et al., 1999; Wallach et al., 1999; Wilson and Browning, 2002]. Examples of these proteins are TNF-α, lymphotoxin (LT)-α or -β, Fas ligand (FasL), TRAIL [Wiley et al., 1995], TWEAK [Chicheportiche et al., 1997], and LIGHT. FasL interacts with the death domain containing Fas receptor leads to prototypical apoptosis, which is mediated by caspase activation and a cascade of events eventually culminating in the various hallmarks of apoptosis [Wallach et al., 1999]. Like the apoptotic effect of FasL, TNF-α can also induce caspase activation and cell apoptosis through type I TNFR, whose intracellular death domain couples to the caspase machinery [Thorburn, 2004]. Even though ample evidences delineate the classic apoptotic mechanisms triggered by both cytokines, recent studies, however, further indicated that both cytokines could trigger cell death of necrosis-type when the cellular caspase signaling is absent [Fiers et al., 1999; Khwaja and Tatton, 1999; Holler et al., 2000; Matsumura et al., 2000; Wilson and Browning, 2002].

LIGHT is a member of TNF family that is predominantly expressed in lymphoid tissues [Mauri et al., 1998; Tamada et al., 2000a]. By signaling through two identified receptors, herpesvirus entry mediator (HVEM) and LT-β receptor (LTβR), LIGHT profoundly participates in multiple immunological functions.

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Decoy receptor 3 (DcR3), on the other hand, is a soluble receptor of LIGHT and can neutralize its biological actions [Yu et al., 1999]. In vitro studies show that LIGHT is a costimulatory molecule for T cells and could lead to enhanced proliferation, Th1-type cytokine production, and NF-κB translocation [Wallach et al., 1999; Tamada et al., 2000a]. In vivo study further confirms this effect. Blockade of endogenous LIGHT effectively prevents the development of graft-versus-host disease (GVHD), whereas local overexpression of LIGHT enhances tumor rejection [Tamada et al., 2000b]. Signaling via HVEM may be important for these LIGHT-mediated activities.

LIGHT could also induce cell death in certain tumor cell lines [Harrop et al., 1998; Zhai et al., 1998; Chen et al., 2000; Rooney et al., 2000; Zhang et al., 2003]. Studies have demonstrated that LIGHT induces this event through interaction with the non-death domain containing receptor LTβR [Wu et al., 1999; Rooney et al., 2000]. Upon binding with LIGHT, LTβR recruits several TNFR-associated factors (TRAFs), which are coupling adaptors to trigger multiple signaling cascades [Nakano et al., 1996; Force et al., 1997]. Further study has indicated that TRAF3 coupling is primarily involved in mediating LTβR-induced cell death [Force et al., 1997; Wu et al., 1997, 1999; Rooney et al., 2000], whereas TRAF2 and TRAF5 association play an important role in the activation of NF-κB [Nakano et al., 1996; Chang et al., 2002]. Compared to the dramatic and rapid effects of Fas and TNF-α, LIGHT-induced tumor cell death is relatively moderate and slow, and often requires the sensitization by interferon-γ (IFN-γ). In tumor cells, the mutual priming effect on sensitivity to cell death induced by LIGHT and IFN-γ was demonstrated [Sugarman et al., 1985; Zhai et al., 1998; Chen et al., 2003]. Previous studies including ours further showed that caspase inhibitor has only a partial effect to prevent LIGHT/IFN-γ-induced cell death in different tumor cells [Wilson and Browning, 2002; Chen et al., 2003; Zhang et al., 2003]. Supporting these observations, LIGHT-induced apoptosis of MCF-7 breast cancer cell, which is caspase-3 deficient [Zhai et al., 1998]. In contrast, apoptosis signal-regulating kinase (ASK) contributed to the caspase-independent pathway of LTβR-induced cell death [Chen et al., 2003]. In addition, LIGHT-sensitized IFN-γ-mediated cell death probably through down-regulation of anti-apoptosis Bcl-2 family members was suggested [Chen et al., 2003; Zhang et al., 2003]. Nevertheless, the contribution of pro-apoptotic and anti-apoptotic Bcl-2 family members displayed cell type specificity, because these members are differentially changed in LIGHT/IFN-γ treated hepatoma and breast cancer cells [Chen et al., 2003; Zhang et al., 2003]. Moreover, the different cell death features mediated by LTβR, i.e., apoptosis, necrosis, or mix type, were demonstrated and compared in WEHI 164 fibroblastoid line and HT-29 adenocarcinoma [Wallach et al., 1999]. Following LTβR activation, apoptotic death was shown in WEHI 164, but mixed type of death was shown in HT-29.

Even though several reports have begun to explore the death mechanism by which LIGHT/IFN-γ induces cell death, which is somewhat unusual to Fas and TNF-α, the cell-specific mechanism and death feature have not been fully understood. In this study, we attempt to further elucidate the death mechanism of LIGHT/IFN-γ in HT-29 adenocarcinoma cells, which has been previously shown to undergo a mix-type cell death following LTβR activation through an agonist LTβR antibody stimulation [Wilson and Browning, 2002]. In addition to verify the role of caspases, we extend to examine the roles of several cellular molecules already known to regulate cell death and survival. These include Bcl-2 family members, p38 mitogen-activated protein kinase (MAPK), JNK, STAT1, NF-κB, Fas, and cathepsin B. The results indicate that LIGHT/IFN-γ-induced cell death is mitochondria-dependent, while is caspase-3-independent. Activation of p38 MAPK, up-regulation of Bak, Fas, and inhibition of NF-κB signaling contribute to cell death.

MATERIALS AND METHODS

Cell Culture and Reagents

Human colon adenocarcinoma HT-29 cells obtained from American Type Culture Collection (Manassas, VA) were grown at 37°C in 5% CO_2 using DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Recombinant human IFN-γ was purchased from Roche Molecular Biochemicals (Mannheim, Germany). TNFα and IFN-β were purchased from PeproTech (London, UK). SB203580, CA074Me, ZVAD-fmk, aurintricarboxylic acid (ATA), and genistein were purchased from

Calbiochem (San Diego, CA). SP600125 was purchased from TOCRIS (Ellisville, MO). [γ-^{32}P]ATP (6,000 Ci/mmol) and the enhanced chemiluminescence detection agent were purchased from NEN (Boston, MA). Rabbit polyclonal antibody specific for p38 MAPK, Bak, Bax, Bad, Mcl-1, STAT1, Fas-FITC, protein A/G beads, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Abs against active (phosphorylated) STAT1 (Y701) was purchased from New England Biolabs (Beverly, MA). CytoTox96 LDH cytotoxicity detection kit was purchased from Promega (Madison, WI). pGL2-ELAM-Luc (κB-Luc) under the control of one NF-κB binding site was constructed. The luciferase reporter construct of human IRF-1 gene promoter (-1.3 kb) containing gamma-activated sequence (GAS) element was kindly provided by Dr. Yoshihiro Ohmori (Cleveland Clinic Foundation, Cleveland, OH). All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA). Generation of anti-LTβR monoclonal antibody was prepared as described [Chen et al., 2003]. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Generation of LIGHT, LTβR-Fc, and DcR3-Fc

The cDNA of extracellular region of LIGHT was cloned into pIZ/V5-His-FLAG (Invitrogen, Carlsbad, CA) by polymerase chain reaction. The primers used for polymerase chain reaction were designed to introduce an *EcoR*I site as described in the followings: 5′-CGAATTCA-GATCTGACGGACCTGCAGGCTCC-3′ (sense) and 5′-GGAATTCCTTCACACCATGAAAGCC-C-3′ (antisense). The PCR products were ligated at the *EcoR*I site of pIZ/V5-His-FLAG to create pIZ/V5-His-FLAG-LIGHT. The construct was autosequenced (MB Mission Biotech Corp., Taipei, Taiwan) for verification. The pIZ/V5-His-FLAG-LIGHT construct was transfected into Sf21 cells by Lipofectin^{TM} (Invitrogen). Stable transfectants were selected with 500 μg/ml Zeocin (Invitrogen). Protein was purified by agarose beads conjugated with anti-FLAG antibody (M2) and followed by dialysis in PBS as described [Chen et al., 2003]. LTβR·Fc and DcR3·Fc fusion proteins were produced as previously described [Zhai et al., 1998; Hsu et al., 2002]. The LTβR·Fc and DcR3·Fc fusion proteins were extracted from the supernatants of recombinant virus-infected Sf21 cells using protein A-Sepharose beads, and eluted with 0.1 M glycine buffer (pH 3.0), followed by dialysis against PBS. Human IgG1 (hIgG1) was used as a control in all experiments to test the effects of Fc fusion proteins.

Cytotoxicity Assay

For crystal violet assay, cells (5×10^4) were seeded in 96-well microtiter plates and treated with IFN-γ or LIGHT for 72 h. Then cells were washed with PBS, fixed with methanol, and washed with distilled water. The crystal violet assay, which is an assay of cytotoxicity, was used to determine the surviving fraction of cells after treatment. The cells were stained with 1% crystal violet, rinsed with cold water, and air-dried. Acetic acid (33%) was then added to each well. Living cells in each well that survived despite drug treatment took up crystal violet and stained deep purple. Cell absorption was determined at 540 nm with a microplate reader. To calculate the surviving fraction of cells we divided the mean optical density of treated wells by the mean optical density of untreated control wells after correcting for the optical density of blank wells. In some experiments, the integrity of the plasma membrane of HT-29 cells after drug treatment was determined by monitoring the leakage of LDH. The CytoTox96 nonradioactive cytotoxicity assay kit was used, and LDH released into the medium was determined according to the manufacturer's instructions. Briefly, equal volume of 2× substrate solution (1 mM pyruvate, 0.2 mM NADH in 0.1 M Tris-HCl, pH 7.4) was added to the medium, and the mixtures were incubated at 37°C for 10 min. Samples were read at the absorbance at 340 nm in a microtiter plate reader. Cells treated with lysis buffer for 10 min was used as a positive control for necrosis. Background reading from vehicle-treated sample was subtracted from the readings. Data were then calculated by the percentage of lysis buffer-treated group.

DNA Laddering

Cells were collected, washed with PBS twice, and then lysed in lysis buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 0.5% sodium lauryl sarkosinate, and 0.5 mg/ml proteinase K) for 3 h at 56°C and treated with 0.5 mg/ml RNase A for an additional 1 h at 56°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) and the DNA concentration was determined at

260 nm by spectrophotometry. Loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/v) low melting point agarose, 0.25% (w/v) bromophenol blue) and samples were loaded onto presolidified, 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. Agarose gels were run at 50 V for 90 min in TBE buffer. Gels were observed and photographed under UV light.

Immunoblotting Analysis

After agent treatment, the medium was aspirated. Cells were rinsed twice with ice-cold PBS, and whole-cell lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was then added to each well. After cell harvest, cell lysates were centrifuged. Equal amounts of the soluble protein were denatured in SDS, electrophoresed on a 10% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) containing 5% nonfat milk for 1 h at room temperature. After immunoblotting with the first specific antibodies, membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. After three washes with TBST, the protein bands were detected with enhanced chemiluminescence detection reagent according to the vendor's instruction before exposure to photographic film.

Immunoprecipitation and Kinase Assay

To determine p38 MAPK activity, anti-p38 antibody (1 μg) and protein A/G agarose beads were added to the prepared cell extracts as mentioned above. Immunoprecipitation proceeded at 4°C overnight. The precipitated beads were washed three times with 1 ml of ice-cold cell lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 2 mM dithiothreitol). The immune-complex kinase assay of one half of the immunoprecipitates was performed at 30°C for 30 min in 20 μl of kinase reaction buffer containing 1 μg of myelin basic protein (MBP), 25 μM ATP, and 3 μCi of [γ-^{32}P]ATP. The reaction was terminated with 5 μl Laemmli sample buffer, and the products were resolved by 12% SDS–PAGE. The phosphorylated MBP was visualized by autoradiography. The other half of the immunoprecipitates was subjected to SDS–PAGE and immunoblotting to verify that equal amounts of kinases were undergoing kinase reaction.

Assessment of Mitochondrial Membrane Potential

Briefly 5×10^5 HT-29 cells in DMEM containing 10% FBS were exposed to drugs for 24 h. Cells were detached with trypsin–EDTA and washed with PBS. Mitochondrial membrane potential ($\Delta\psi m$) was measured by flow cytometry using the rhodomine-6G (Sigma), a fluorescent dye being shown to be selectively accumulated in the mitochondria of living cells by a mechanism which depends on $\Delta\psi m$. For doing this, cells were re-suspended in 0.5 ml PBS containing 10 μg/ml of rhodomine-6G for 15 min at 37°C and then immediately submitted for flow analysis (Becton Dickinson, Franklin Lakes, NJ).

Flow Cytometry

For the assay of Fas, cells treated with LIGHT/IFN-γ, were harvested and washed twice with FACS washing buffer (1% FCS and 0.1% NaN_3 in PBS), followed by incubation with monoclonal anti-Fas-FITC antibody at 4°C for 20 min. After washing with FACS washing buffer three times, the fluorescence of cells was analyzed with a FACScan flow cytometer. In the experiment of propidium iodide (PI) staining, trypsinized cells were washed with ice-cold PBS, fixed in 70% ethanol at 0°C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of PI staining buffer (0.5% Triton X-100/PBS, 1 mg/ml RNase A) at 37°C for 10 min, and stained with 0.5 ml of 80 μg/ml PI for 30 min. Before cytometric analysis, cells were filtered on a nylon mesh filter. Fluorescence emitted from the PI–DNA complex was quantitated after laser excitation of the fluorescent dye by a FACScan flow cytometry (Becton Dickinson).

Transfection Assays

HT-29 cells (5×10^5) were seeded into 12-well plates overnight before transfection with the indicated plasmids. Twenty-four hours later, the cells were transfected with 1 μg pGL20E-LAM-Luc (κB-Luc), 1 μg IRF-1 promoter construct, and 1 μg β-galactosidase expression

vector (pCR3lacZ; Pharmacia, Uppsala, Sweden) by using LipofectAMINE PLUS Reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, cells were added to drugs for 12 h. Cells were then washed once with cold PBS. To prepare cell lysates, 100 μl lysis buffer (Promega) was added to each well to scrape cells from dishes, followed by centrifugation at 13,000 rpm for 30 s to collect supernatant. Equal amount of protein (about 10–20 μg) in 5 μl cell lysate was incubated with equal volume of luciferase substrate (Steady Glo reagent, Promega) in a 96-well microplate, and the luminescence was measured in a microplate luminometer (Rosys-Anthos, Tecan, Austria). Luciferase activity was normalized to transfection efficiency monitored by the co-transfected pCR3lacZ. Activating agents-induced response was presented as the relative activity as compared to control cells transfected with empty vector of κB or IRF-1 luciferase construct, and pCR3lacZ.

Statistical Evaluation

Values were expressed as the mean ± SEM from at least three independent experiments performed in duplicate. Analysis of variance was used to assess the statistical significance of the differences, and a P value of less than 0.05 was considered statistically significant.

RESULTS

LIGHT/IFN-γ Induce Cell Death in HT-29 Cells

Previous reports have shown that LIGHT induced cell death in certain tumor cell lines in the presence of IFN-γ. Thus, we attempt to test the LIGHT/IFN-γ-induced cell death in HT-29 cells. We found LIGHT could dramatically and dose-dependently enhance IFN-γ-mediated cell death at 72 h by crystal violet assay in HT-29 cells (Fig. 1A). LIGHT/IFN-γ also induced DNA fragmentation at 48 h (Fig. 1B). However, we also found that LIGHT/IFN-γ could induce a weak LDH release, a maker of necrosis (Fig. 1C). Thus, LIGHT/IFN-γ-induced cell death displays characteristics of both apoptosis and necrosis. The LIGHT/IFN-γ-induced cell death in HT-29 cells was specially abrogated by recombinant soluble LIGHT receptors, LTβR-Fc and DcR3-Fc, of which correlated the specific binding of its receptors (Fig. 1D).

SB203580 and ATA Prevent LIGHT/IFN-γ-Induced Cell Death and Cell Cycle Arrest

The death-inducing ligands such as TNF-α, FasL, and TRAIL can induce apoptosis via activation of many signaling pathways [Nagata, 1997; Ashkenazi and Dixit, 1998; Walczak and Krammer, 2000]. Thus, we investigated whether caspases were involved in the LIGHT/IFN-γ-mediated cell death in HT-29 cells. For this purpose, we examined the effect of a pancaspase inhibitor, ZVAD-*fmk*, on the LIGHT/IFN-γ-induced cell death in HT-29 cells. We found that pre-incubation of cells with the broad range caspase inhibitor ZVAD-*fmk* resulted in no protective effect (Fig. 2). In addition to caspases, poly (ADP-ribose) polymerase (PARP) is emerging as an important activator of caspase-independent cell death which associates with DNA fragmentation, we thus examined the PARP inhibitor, 3-aminobenzamide (3AB), which is known to produce selective inhibition of poly(ADP-ribosyl)ation [Kuo et al., 1998; Hong et al., 2004]. Results showed that 3AB could partially prevent LIGHT/IFN-γ-induced cell death from 61 to 23% (Fig. 2), suggesting the occurrence of nuclear event in LIGHT/IFN-γ-induced action. Furthermore, it has been reported that a lysosomal cystein proteinase, cathepsin B, was involved in TNF-induced cell death [Nakayama et al., 2002]. Thus, we also examined the effect of a specific inhibitor of cathepsin B, CA074Me. The results indicated that cathepsin inhibitor failed to reverse the cell death induced by LIGHT/IFN-γ.

To verify that IFN-γ exerts a permissive effect on cell death, we examined two agents, which were reported to interrupt the essential initiating signals for IFN-γ-induced death, JAK/STAT1. Genistein is a non-selective tyrosine kinase and can inhibit JAK [Maziere et al., 2001]. ATA, initially regarded as an endonuclease inhibitor [Benchokroun et al., 1995], was shown by our previous study to elicit a non-selective inhibition of IFN-γ signaling [Chen et al., 2002]. Correlated with these notions, we found LIGHT/IFN-γ-induced cell death indeed was attenuated by genistein and ATA (Fig. 2).

It is becoming increasingly clear that several protein kinase signal transduction pathways are involved in cell death. JNK and p38 MAPK are in this case playing a role in cell death [Eby et al., 2000; Cuadrado et al., 2003; Sah

et al., 2003]. Another reason to explore both kinases is based on previous observation that ASK-dependent signaling participates in LTβR-mediated cell death [Chen et al., 2003] and that p38 MAPK and JNK are downstream signaling targets of ASK [Tobiume et al., 2001]. To investigate whether both kinases participate in LIGHT/IFN-γ-induced cell death, we used p38 MAPK and JNK inhibitors, SB203580 and SP600125, respectively, to test the protective effect. We observed that SB203580 could inhibit LIGHT/IFN-γ-induced cell death, but SP600125 was without effect (Fig. 2).

Not only verified by crystal violet for cell death, we also performed PI staining to confirm cell apoptosis. Results indicated that LIGHT/IFN-γ could induce cell cycle arrest at sub-G_1 state, which was accompanied by the decreased cell cycle at G_0/G_1 state (Fig. 3A,B). In consistent with that SB203580 and ATA could effectively prevent LIGHT/IFN-γ-induced cell death as assayed by crystal violet they also could reverse LIGHT/IFN-γ-induced cell cycle arrest.

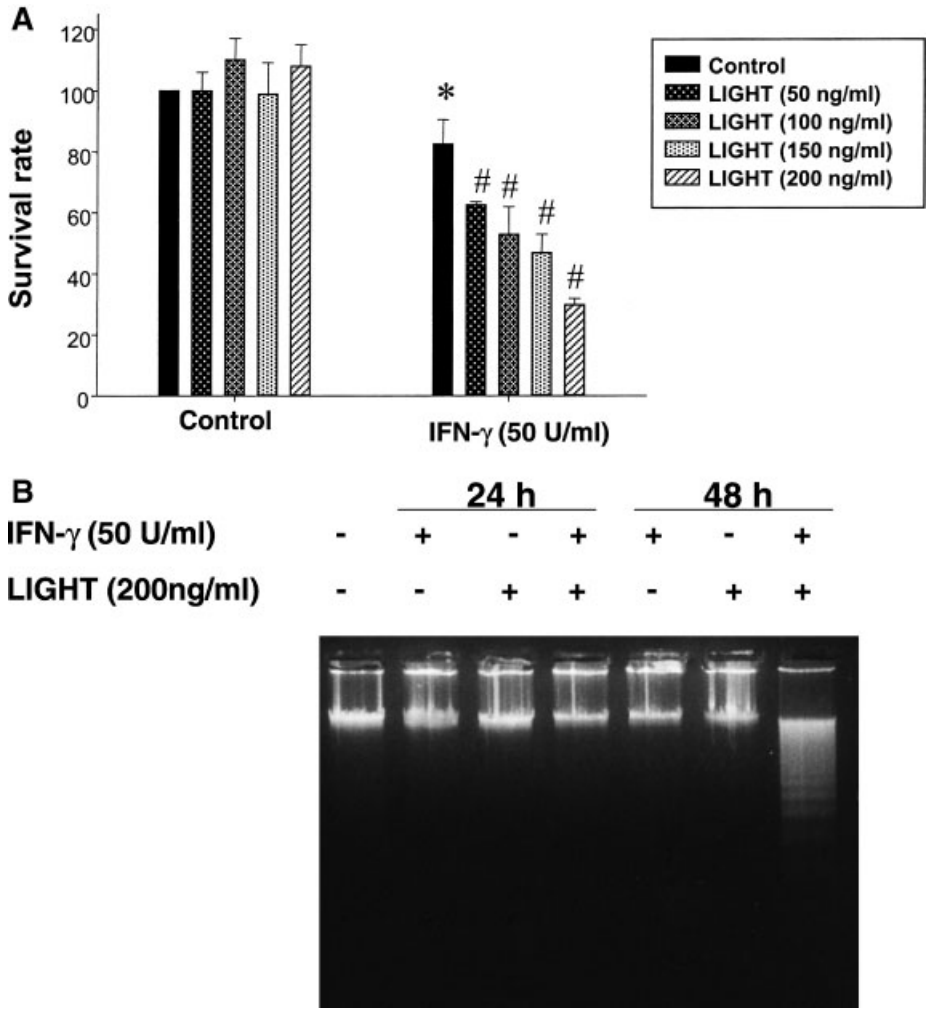

Fig. 1. LIGHT/interferon-γ (IFN-γ)-induced cell death in HT-29 cells. **A**: HT-29 cells were added with the indicated concentrations of LIGHT in the presence or absence of IFN-γ (50 U/ml). After 72 h, viability was estimated by crystal violet assay. **B**: HT-29 cells were added with LIGHT (200 ng/ml) in the presence or absence IFN-γ (50 U/ml). After 24 and 48 h, DNA was extracted and analyzed by agarose gel electrophoresis. **C**: LDH activity was assayed after the addition of LIGHT (200 ng/ml) and IFN-γ for 72 h. **D**: Effect of soluble receptor fusion proteins on LIGHT/IFN-γ-induced cell death. Preincubated with IgG1 (200 ng/ml), LTβR-Fc (200 ng/ml), and decoy receptor 3 (DcR3)-Fc (200 ng/ml) for 30 min, then treated with LIGHT (200 ng/ml) in the presence or absence of IFN-γ (50 U/ml). After 72 h, viability was estimated by crystal violet assay. Each value represents the mean ± SEM of at least three independent experiments, which were performed in duplicate. *Statistically significant inhibition ($P < 0.05$) as compared to the control response. #Statistically significant reverse effect as compared to the LIGHT/IFN-γ-induced response.

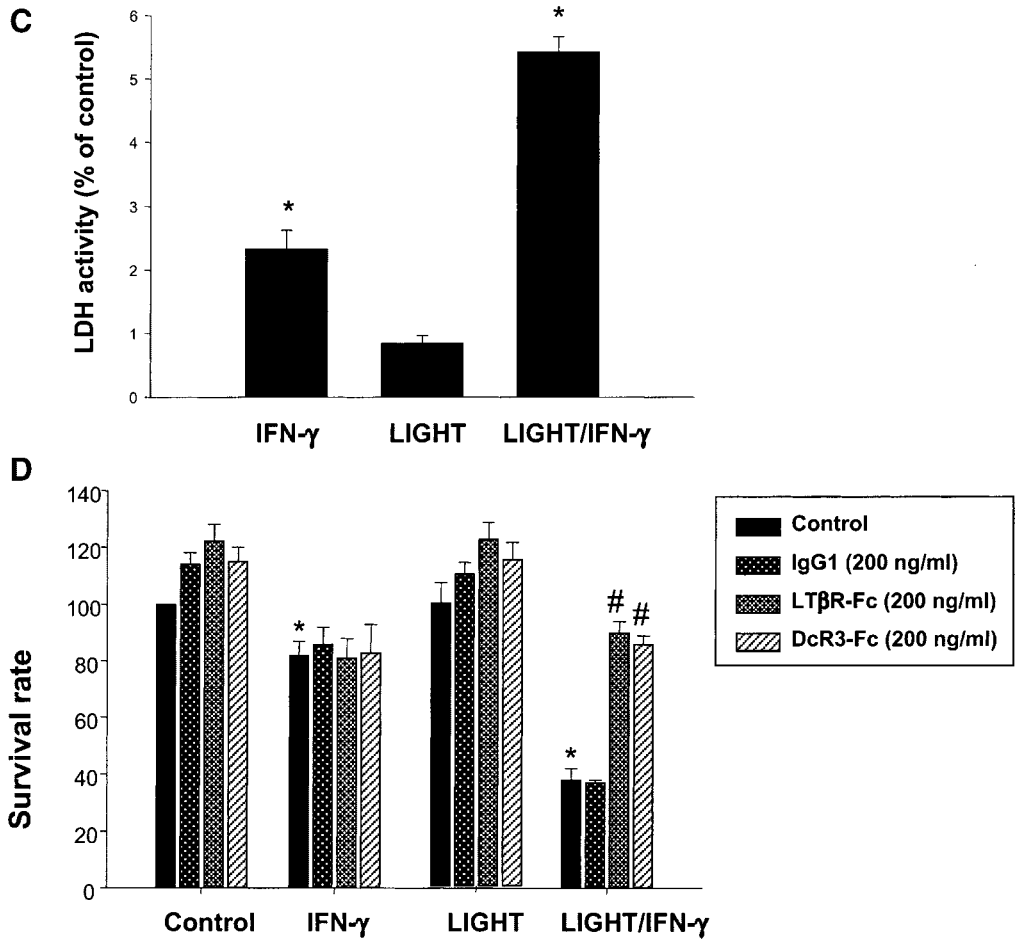

Fig. 1. (*Continued*)

LIGHT/IFN-γ Decrease Mitochondria Membrane Potential and Affect Bcl-2 Family Member Expressions

To evaluate the mechanisms by which LIGHT/IFN-γ-induced cell death in HT-29 cells, we determined the signaling pathway of the apoptosis. We first evaluated the caspase-3 and -9 activation by Western blotting and activities by using cleavage of specific substrates. As correlated with the results that ZVAD could not inhibit LIGHT/IFN-γ-induced cell death, we found that LIGHT/IFN-γ did not induce caspase-3 or -9 cleavage and activation (data not shown).

Although LIGHT/IFN-γ did not activate caspases, we next investigated other possible mechanisms involved in LIGHT/IFN-γ-mediated cell death. We examined the change of mitochondrial potential by flow cytometry. As shown in Figure 4A, a slight decrease of membrane potential was observed at 24 and 30 h after addition of LIGHT/IFN-γ. Members of pro-apoptotic Bcl-2 family, such as Bax, Bad, and Bak, are involved in the loss of mitochondrial membrane potential and contribute to the cell apoptosis [Wang, 2002]. As shown in Figure 4B, IFN-γ and LIGHT could not alter the expression of Bax and Bad, but increase the expression of Bak obviously at 24 h, which correlated with the early stage of LIGHT/IFN-γ-induced cell death and changes of mitochondrial membrane potential. In contrast, the level of protective member of Bcl-2 family, Mcl-1, was decreased by IFN-γ and LIGHT. Cytokine co-incubation led to an enhanced reduction of Mcl-1 at 24 h. These findings suggest LIGHT/IFN-γ might induce HT-29 cell death via a mitochondria-dependent and caspase-independent pathway.

LIGHT/IFN-γ Induce p38 MAPK Activity

Since LIGHT/IFN-γ-induced cell death could be inhibited by p38 MAPK inhibitor, SB203580, we investigated if LIGHT/IFN-γ could induce

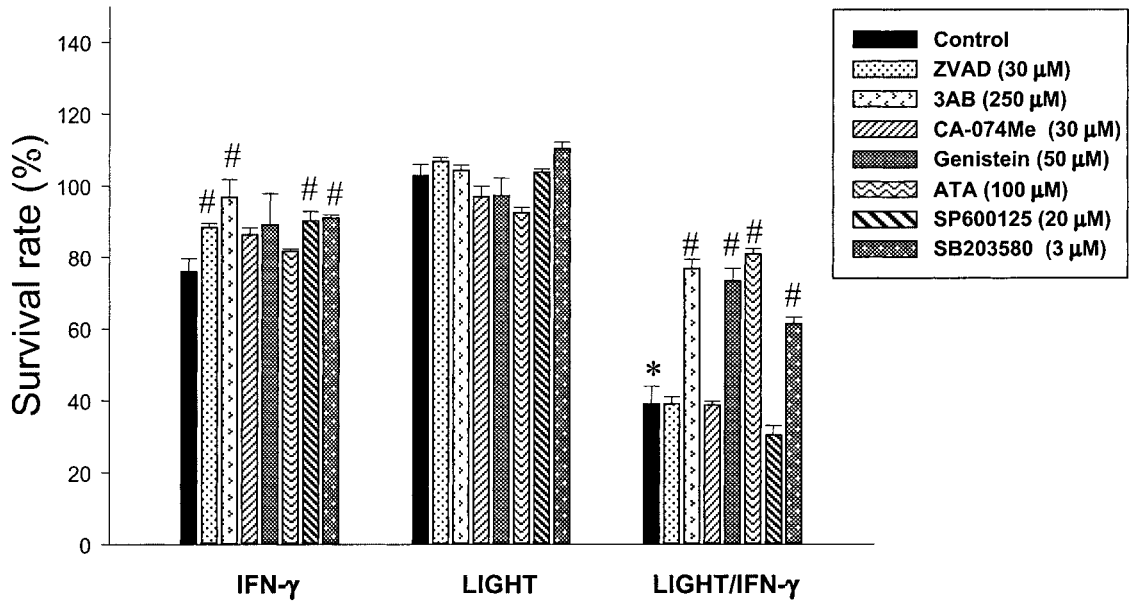

Fig. 2. Effects of various inhibitors on LIGHT/IFN-γ-induced cell death. HT-29 cells were pretreated with ZVAD-*fmk* (30 μM), 3-aminobenzamide (3AB) (250 μM), CA074Me (30 μM), genistein (30 μM), aurintricarboxylic acid (ATA) (100 μM), SP600125 (20 μM), and SB203580 (3 μM), and they were then added with LIGHT (200 ng/ml) in the presence or absence of IFN-γ (50 U/ml). After 72 h, viability was estimated by crystal violet assay. Each value represents the mean ± SEM of at least three independent experiments, which were performed in duplicate. *Statistically significant inhibition ($P < 0.05$) as compared to the control response. #Statistically significant reverse effect as compared to the LIGHT/IFN-γ-induced response.

p38 MAPK activation. An immunokinase assay indicated that IFN-γ and LIGHT alone could slightly induce p38 MAPK activity, and the effect of LIGHT was more apparent than that of IFN-γ. Combined IFN-γ and LIGHT treatment has the additive effect on p38 MAPK activity (Fig. 5).

LIGHT Could not Affect IFN-γ-Induced STAT1 Signaling, but Potentiate IFN-γ-up-Regulated Fas

To understand whether the cross-interaction between signaling pathways transduced by both cytokines participates in cell death sensitization, we examined IFN-γ-induced STAT1 signaling and LIGHT-induced NF-κB activation. As shown in Figure 6A, phosphorylated STAT1 at tyrosine 701 was detected as early as 30 min after IFN-γ treatment, and maintained for at least 1.5 h. IFN-γ also slightly induced STAT1 phosphorylation at serine 727, but its extent only achieved 130% of the control level. We found LIGHT could not alter the STAT1 phosphorylation, either at Y701 or S727, caused by IFN-γ.

We further traced the signal mediator IRF-1, which is downstream of STAT1 activation and involved in IFN-γ-induced cell apoptosis [Tanaka et al., 1994; Tamura et al., 1995; Kano et al., 1999]. We found that IRF-1 promoter activity controlled by STAT1 was induced by IFN-γ at 12 h, but LIGHT could not affect this response (Fig. 6B). Thus, sensitizing cell death by LIGHT is not associated with the up-regulation of cell apoptosis-related genes through a STAT1-dependent mechanism. In contrast, ATA, as expected, could inhibit IFN-γ-induced IRF-1 activity, while p38 MAPK inhibitor, SB203580, could not (Fig. 6B).

Previous studies have demonstrated that Fas is expressed on HT-29 cells, and IFN-γ up-regulates the expression of Fas. This action is contributed to apoptosis induced by higher concentrations of IFN-γ alone (Xu et al., 1998). Consistent with these observations, we confirmed that IFN-γ could up-regulate Fas expression at 24 and 48 h. Moreover, we found that LIGHT alone even though could not induce Fas expression, it obviously potentiated IFN-γ-up-regulated Fas expression at 48 h (Fig. 6C). This result implies that increased Fas expression is involved in LIGHT-IFN-γ-induced cell death.

IFN-γ Attenuates LIGHT-Induced NF-κB Activity

Because NF-κB is an important factor involved in the regulation of cell survival [Perkins, 2000], we investigated whether LIGHT/IFN-γ-

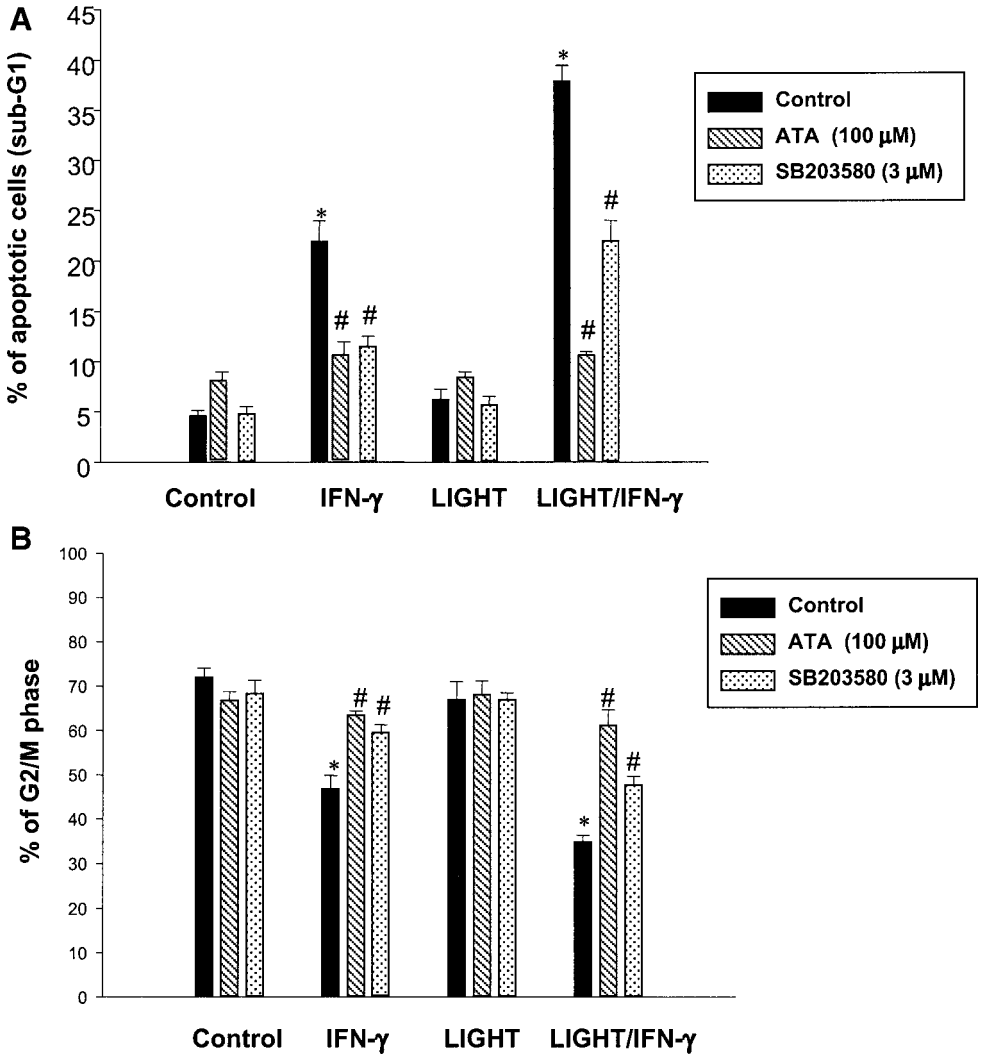

Fig. 3. Effects of ATA and SB203580 on LIGHT/IFN-γ-induced hypodiploid. HT-29 cells were pretreated with ATA (100 μM) and SB203580 (3 μM) for 30 min, and they were then added with LIGHT (200 ng/ml) in the presence or absence of IFN-γ (50 U/ml). Following 72 h incubation, cells were stained by propidium iodide (PI) and cell cycle distribution, including sub-G_1 (**A**) and G_2/M phases (**B**), was determined by flow cytometry. Each value represents the mean ± SEM of at least three independent experiments, which were performed in duplicate. *Statistically significant inhibition ($P < 0.05$) as compared to the control response. #Statistically significant reverse effect as compared to the LIGHT/IFN-γ-induced response.

induced cell death is associated with the inhibition of NF-κB. We found that LIGHT could induce 79% increase of luciferase activity as an index of NF-κB binding in HT-29 cells. Similar increasing pattern was also observed by stimulating LTβR with an agonistic antibody. Interestingly, we found IFN-γ co-treatment abolished LIGHT- and anti-LTβR-induced κB-luciferase activity at 12 h, but IFN-β did not exert such effect (Fig. 7A). When IFN-γ was added 1 h after LIGHT stimulation, NF-κB inhibition was insignificant (Fig. 7B). These data suggest that IFN-γ might modify LIGHT-induced early upstream signals leading to κB activation, and consequent downstream events related to NF-κB mediated gene transcription. The NF-κB inhibitory effect played by IFN-γ was not specific to LIGHT, since TNF-α-induced marked NF-κB activation was likewise abrogated by the presence of IFN-γ (Fig. 7A).

DISCUSSION

Receptors in the TNF family can initiate both canonical apoptotic and/or necrotic cell death, dependent on cell type examined [Fiers et al., 1999; Khwaja and Tatton, 1999; Holler et al., 2000; Matsumura et al., 2000; Wilson and

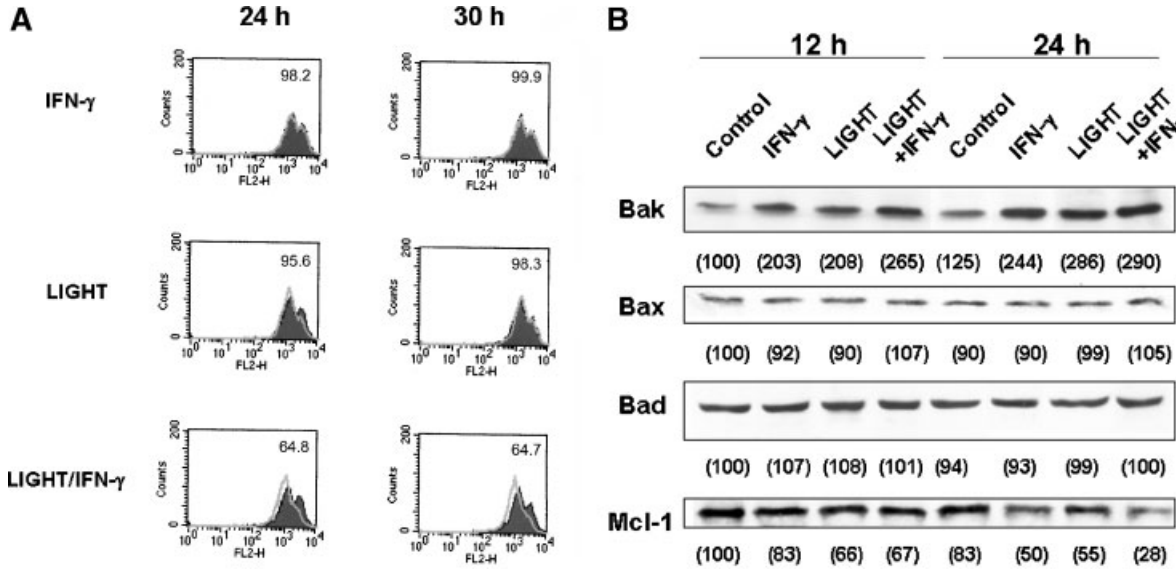

Fig. 4. HT-29 cells were treated with LIGHT (200 ng/ml) in the presence or absence of IFN-γ (50 U/ml) for the time indicated. **A**: Cells were then stained with rhodamin-6-G for the measurement of membrane potential by flow cytometry. The numbers in the histograms indicate the percent of mean fluorescence intensity relative to the control. **B**: Whole cell lysates were extracted for immunoblotting with Bax, Bak, Bad, and Mcl-1. Results were representative of three independent experiments.

Browning, 2002]. In this report, we investigated the mechanisms of LIGHT/IFN-γ-induced cell death in HT-29 cell line. In this model, IFN-γ plays a priming role for the cytotoxic action of LIGHT, and vice versa is the priming role of LIGHT in IFN-γ action. LIGHT/IFN-γ-induced cell death displays characteristics of both apoptosis and necrosis, since we found that LIGHT/IFN-γ could induce DNA fragmentation, sub-G_1 state and LDH release. This phenomenon is similar to the previous suggestion observed in HT-29 cells [Wilson and Browning, 2002].

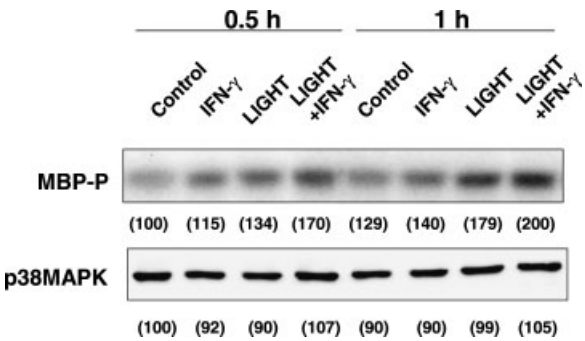

Fig. 5. LIGHT/IFN-γ-induced p38 mitogen-activated protein kinase (MAPK) activation. HT-29 cells were added with LIGHT (200 ng/ml) in the presence or absence IFN-γ (50 U/ml) at the time indicated. Whole cell lysates were immunoprecipitated with p38 MAPK antibody, and protein A/G beads overnight. One half of the immunoprecipitated p38 MAPK complex was subjected to SDS–PAGE, rendered for the immunoblotting, and probed with antibody specific for p38 MAPK. The other half of the immunoprecipitated p38 MAPK complex was rendered for kinase assay. After 30 min reaction at room temperature, the enzyme activity was terminated by adding SDS sample buffer and analyzed by autoradiography. The results are representative of three different experiments with similar results.

In our study, the broad range caspase inhibitor ZVAD could not block LIGHT/IFN-γ-induced cell death, and caspase-3 and -9 cleavages were not observed. Although complicated death events initiated by receptors in the TNF family have been reported to be caspase-dependent, there were others reported to be caspase-independent. In this context, for example, it was shown for TNF/zVAD-induced necrosis in L929, NIH3T3, and U937 cells [Fiers et al., 1999; Khwaja and Tatton, 1999], LIGHT-triggered cell death in Hep3B [Chen et al., 2000, 2003], and TAJ-mediated cell death in 293T cells [Eby et al., 2000]. However, membrane potential loss and Bak expression were observed when the cell death was induced by LIGHT/IFN-γ. Classical cell death pathway shows that membrane potential loss could cause the release of cytochrome c, which in turn binds to Apaf-1, and induces a conformational change in Apaf-1, allowing it to bind the nucleotide dATP or ATP. The nucleotide binding to the Apaf-1-cytochrome c complex triggers its oligomerization to form the apoptosome, which recruits procaspase-9. The binding of procaspase-9 to the apoptosome forms the

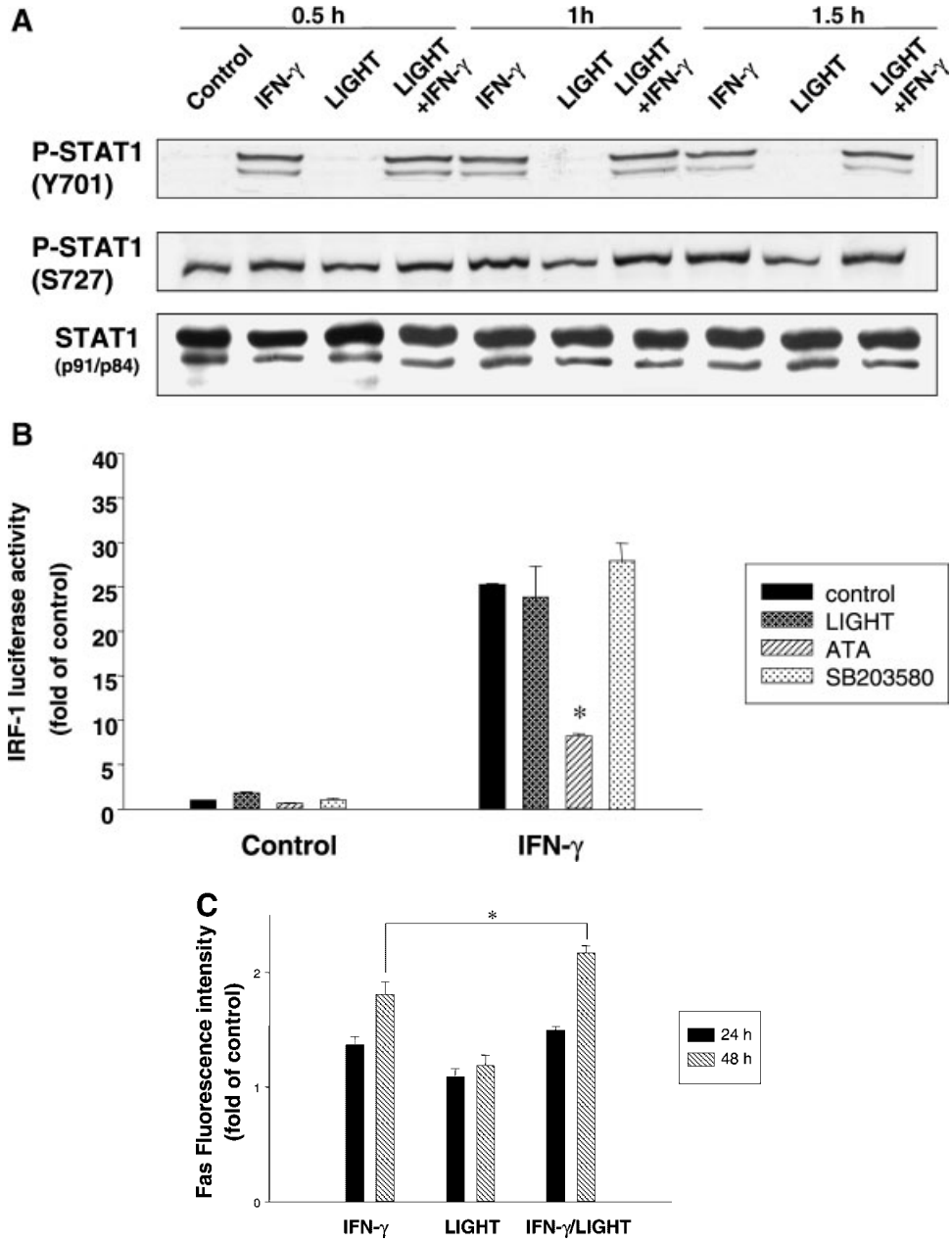

Fig. 6. LIGHT did not affect IFN-γ-induced STAT1 phosphorylation and IRF-1 transcriptional activity but potentiated the IFN-γ-up-regulated-Fas. HT-29 cells were added with LIGHT (200 ng/ml) in the presence or absence of IFN-γ (50 U/ml) for the time indicated. Total cell lysates were harvested for immunoblotting and detection with Abs specific to STAT1 (**A**) or for luciferase assay (**B**). Flow cytometry analysis of cell surface Fas expression using anti-Fas-FITC Ab immunostaining was shown in (**C**). Each value represents the mean ± SEM of at least three independent experiments, which were performed in duplicate. *Statistically significant inhibition ($P < 0.05$) as compared to the control response.

caspase-9 holoenzyme that cleaves and activates the downstream caspases, such as caspase-3 [Wang, 2002]. According to our results, it is suggested that LIGH/IFN-γ-induced membrane potential loss does not cause caspase-3 and -9 activation, and there might exist other caspase-independent pathways. This observation is in concert with a previous study that LIGHT induced apoptosis of MCF-7 breast cancers cells, which is caspase-3 deficient [Zhai et al., 1998].

There are some potential mediators of caspase-independent cell death that could account for LIGHT/IFN-γ-induced mitochondria-dependent, but caspase-independent cell death. It has been proposed that Bax and Bax-like proteins,

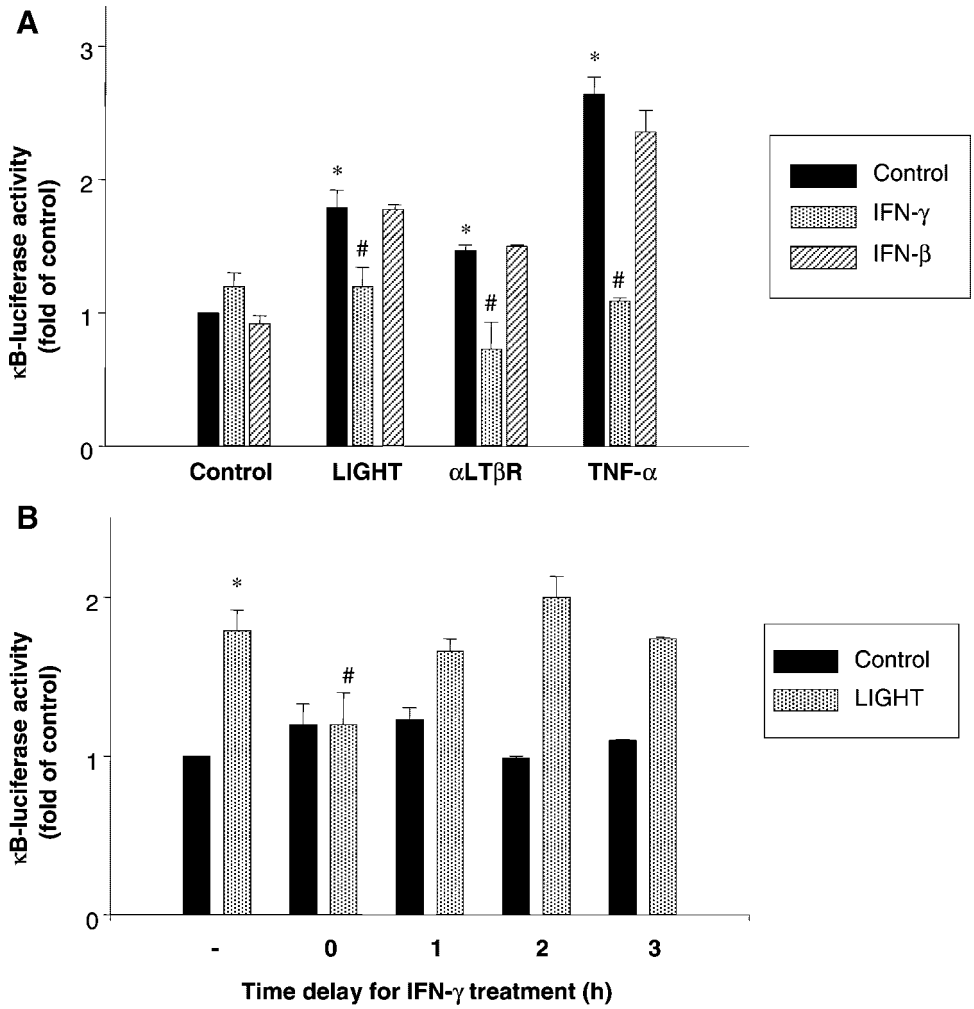

Fig. 7. IFN-γ inhibited LIGHT-induced κB-luciferase activity. HT-29 cells were cotransfected with 0.5 μg of κB luciferase plasmid and 0.5 μg β-gal-lacZ as ''Materials and Methods.'' After 24 h, cells were treated with LIGHT (200 ng/ml) in the presence or absence of IFN-γ (50 U/ml) (**A**) or cells were treated with LIGHT (200 ng/ml) followed by the delay addition of IFN-γ (50 U/ml) (**B**). After 12 h,cells were then lysed and fold of luciferase reporter gene activity was determined. Results were normalized for transfection efficiency with β-gal (for details see ''Materials and Methods''). Each value represents the mean ± SEM of at least three independent experiments, which were performed in duplicate. $*P < 0.05$ as compared to the control response of empty vector group. Each value represents the mean ± SEM of at least three independent experiments, which were performed in duplicate. *Statistically significant inhibition ($P < 0.05$) as compared to the control response.

such as Bak, might mediate caspase-independent cell death via their channel forming ability that could promote mitochondrial permeability transition or puncture the outer mitochondrial membrane [Adams and Cory, 1998]. Bax and Bax-like proteins have been known to kill mammalian cells even in the presence of caspase inhibitors, provoking chromatin condensation and membrane alterations, but without caspase activation [Xiang et al., 1996; McCarthy et al., 1997; Torriglia et al., 2000]. This effect leads to release more caspase-independent apoptotic molecules, such as apoptosis-inducing factor (AIF). AIF released from mitochondria can translocate to nucleus and induce chromatin condensation [Lorenzo et al., 1999]. In this study, the increased expression of Bak upon LIGHT/IFN-γ stimulation might thus play an intermediate key role linking mitochondria dysfunction and cell death. Besides, previous study has shown that overactivation of PARP initiates a nuclear signal that propagates to mitochondria, promotes the release of AIF, and thus, amplifies the large-scale fragmentation of DNA [Hong et al., 2004]. Since PARP inhibitor, 3AB could effectively inhibit LIGHT/IFN-γ induced-cell-death, there might exist a link between PARP and AIF in this event.

Endonuclease G is also a mitochondrial protein that has been shown to translocate to the nucleus and to mediate DNA fragmentation during apoptosis [Li et al., 2001]. In this study, endonuclease inhibitor ATA could inhibit LIGHT/IFN-γ-induced cell death. It is suggested that nuclear event observed in LIGHT/IFN-γ-induced cell death is mediated by mitochondria dysfunction. Moreover, ATA was also demonstrated to act in an endonuclease-independent manner to interact with several cellular targets. Inhibition of IFN-γ-induced STAT1 activation belongs to these actions [Chen et al., 2002] and might explain its protective effect on LIGHT/IFN-γ-induced cell death.

Previous studies have indicated the role of STAT-1-activated IRF-1 in apoptosis induced by IFN-γ and implicate in the enhanced susceptibility to TNF-α-mediated apoptosis [Kano et al., 1999; Suk et al., 2001]. In this study, we found that LIGHT could not potentiate IFN-γ-induced STAT1-phosphorylation and IRF-1-transcriptional activity, thus the role of IRF-1 in conferring susceptibility to LIGHT-induced cell death must be excluded. We also examined if up-regulation of Fas plays a role in LIGHT/IFN-γ-induced cell death. Previous report has shown that Fas is expressed on HT-29 cells and IFN-γ could up-regulate its expression [Xu et al., 1998]. Confirming this point, IFN-γ alone increased Fas expression, and LIGHT simultaneous addition further increased this response at 48 h. Since FasL is also constitutively expressed on HT-29 cells [Xu et al., 1998], we suggest Fas-mediated apoptosis possibility participate in the late phase of LIGHT/IFN-γ-induced cell death, even though we predict its involvement is minimal. This prediction is based on the non-effectiveness of caspase inhibitor on cell death. Furthermore, we did not observe any changes of FasL expression by LIGHT/IFN-γ (data not shown).

JNK, ERK, and p38 MAPK are often activated by many TNF family receptors both with and without death domains, and involved in cell death independently of caspase activation [Baker and Reddy, 1998]. In our study, p38 MAPK inhibitor could partially inhibit LIGHT/IFN-γ-induced cell death, while neither MEK inhibitor PD98059, nor JNK inhibitor SP600125 could. We also observed that LIGHT could potentiate IFN-γ-activated p38 MAPK activity. It is possible that upon LIGHT binding to its receptors, the activation of TRAFs associated kinases, such as ASK, may contribute to p38 MAPK activation [Chen et al., 2003]. As previously indicated, p38 MAPK activity results in cytochrome c release and Fas expression [Stephanou et al., 2001; Garcia-Fernandez et al., 2002], thus contributing to LIGNT/IFN-γ-induced cell death.

NF-κB signaling pathway is an important element to prolong cell survival, as many cell protective proteins are NF-κB-up-regulated target genes [Perkins, 2000]. Previous report has shown that inhibition of NF-κB activation sensitized some tumor cells to TNF-α-, TRAIL-, or FasL-induced cell death [Jeremias et al., 1998]. We also observed that LIGHT could activate NF-κB in HT-29 cells, and IFN-γ could repress LIGHT-induced NF-κB-dependent reporter gene expression. The kinetic study on inhibiting LIGHT-induced NF-κB activation shows that IFN-γ is not effective when added up to 1–3 h after LIGHT stimulation. It strongly suggested that suppression of NF-κB activation by IFN-γ most likely contributes to the enhanced cell death. In consistent with our finding, several studies have shown the cross-interaction between STAT and NF-κB signals. For example, STAT1 could inhibit of TNF-α-induced NF-κB activation by associated with TRADD [Wang et al., 2000; Wesemann and Benveniste, 2003].

In this study, we demonstrate that co-presence of LIGHT and IFN-γ is prerequisite to cause cell death in HT-29 cells, and this effect is caspase-independent but mitochrondria-dependent. The integrated summation of individual cell death signaling, such as p38 MAPK activation, Bak, Fas expression, and Mcl-1 down-regulation converge to render cells undergoing mitochondrial dysfunction and cell death. Moreover, protective NF-κB signaling induced by LIGHT can be masked by the presence of IFN-γ, leading to the enhanced susceptibility of cell death.

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