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Overexpression of Bcl-2 Enhances LIGHT- and Interferon-γ-mediated Apoptosis in Hep3BT2 Cells*

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LIGHT is a member of the tumor necrosis factor superfamily and is the ligand for LT-β R, HVEM, and decoy receptor 3. LIGHT has a cytotoxic effect, which is further enhanced by the presence of interferon-γ (IFN-γ). Although LIGHT/IFN-γ can activate caspase activity, neither benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone nor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone can completely inhibit LIGHT/IFN-γ-mediated apoptosis. Moreover, overexpression of Bcl-2 further enhances LIGHT/IFN-γ-mediated apoptosis. It appears that LIGHT and IFN-γ act synergistically to activate caspase-3, with the resultant cleavage of Bcl-2, removal of the BH4 domain, leading to conversion of Bcl-2 from an antiapoptotic to a proapoptotic form in p53-deficient hepatocellular carcinoma Hep3BT2 cells. Thus, LIGHT seems to be able to override the protective effect of Bcl-2 and induce cell death. Although benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone can prevent the cleavage of Bcl-2 by LIGHT/IFN-γ, they only partially inhibit apoptosis in Hep3BT2 cells that are overexpressing Bcl-2. In contrast, both LIGHT/IFN-γ-mediated apoptosis and Bcl-2 cleavage are inhibited by free radical scavengers, indicating that free radicals may play an essential role in LIGHT/IFN-γ-mediated apoptosis at a step upstream of caspase-3 activation. These results suggest that LIGHT signaling may diverge into multiple, separate processes.

Members of the tumor necrosis factor (TNF)[1] superfamily are known to be potent mediators of immune responses (1). These proteins include TNF-α, TNF-β (LT-α), LT-β, FasL, CD27L, CD30L, CD40L, 4–1BB (2), TRAIL (3), TWEAK (4), RANKL/TRANCE/ODF/OPGL (5–9), APRIL/TALL-2 (10, 11), AITRL (12), VEGI/TL1 (13, 14), and BAFF/TALL-1/THANK/Blys (11, 15–17). With the exception of LT-α, all members of the TNF superfamily are type II membrane proteins that can also exist as soluble cytokines following release by membrane metalloproteases. A recently identified member of the TNF superfamily, named LIGHT[2] by Mauri *et al.* (18), has been shown to be 33 and 30% identical to FasL and LT-β, respectively. Further studies indicated that LIGHT can bind to lymphotoxin-β receptor (LT-βR) and herpesvirus entry mediator (HVEM)/TR2/ATAR (18–21). LT-βR is the receptor for membrane-bound LT-α/LT-β trimers (22) and is involved in the development of peripheral lymph nodes and spleen architecture (23, 24). Activation of LT-βR induces secretion of the chemokines interleukin-8 and RANTES from several tumor cell lines, indicating a role in neutrophil recruitment during an inflammatory response (25). HVEM has been shown to be the receptor for LT-α and herpes simplex virus envelope glycoprotein D (18), suggesting a potential role in the regulation of the immune response to viral infection. Recently, it has been further demonstrated that LIGHT can bind to soluble FasL decoy receptor 3(DcR3)/TR6 (26, 27), which is amplified in many tumor cells and is able to neutralize the cytotoxic effects of FasL and LIGHT.

In a previous study, we showed that LIGHT is cytotoxic to tumor cells that express both LT-βR and HVEM (28). However, LIGHT is not cytotoxic to hematopoietic cells that only express TR2/HVEM, such as peripheral blood lymphocytes, Jurkat cells, or $CD8^+$ tumor-infiltrating lymphocytes. It was also revealed that introduction of TR2/HVEM into PC-3 cells, which only express LT-βR, converts PC-3 cells from a LIGHT-resistant to a LIGHT-sensitive phenotype (28). This suggests that LIGHT triggers distinct biological responses based on the expression patterns of its receptors on the target cells. LIGHT can enhance the secretion of IFN-γ by activated T cells, and IFN-γ can dramatically enhance LIGHT-mediated apoptosis in human breast cancer cells (MDA-MB-231) as well as the p53-deficient human adenocarcinoma, HT-29. Furthermore, LIGHT induces apoptosis in the caspase-3-deficient tumor cell line MCF-7 (28), indicating that LIGHT is able to induce cell apoptosis in the absence of caspase-3 activation. However, the underlying mechanism of LIGHT-mediated apoptosis has not been elucidated. Recently, LIGHT was reported to be a CD28-independent co-stimulatory molecule in T cell growth and differentiation, Moreover, blockade of the LIGHT signaling pathway by LT-βR.Fc fusion protein can suppress the onset of graft

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[1] The abbreviations used are: TNF, tumor necrosis factor; IFN-γ, interferon-γ; TGF-β1, transforming growth factor-β1; LT-βR, lymphotoxin-β receptor; HVEM, herpesvirus entry mediator; ROS, reactive oxygen species; C60, carboxyfullerene; MnTBAP, manganese (III) tetrakis (5,10,15,20-benzoic acid) porphyrin; RANTES, regulated on activation normal T cell expressed; z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ac-YVAD-CMK, acetyl-Tyr-Val-Ala-Asp-chloromethylketone; MCA-YVAD.APK (DNP), 7-(methoxycoumarin-4-yl)acetyl-Tyr-Val-Ala-Asp-Ala-Pro-Lys(DNP)-OH; MCA-DEVD.APK (DNP), 7-(methoxycoumarin-4-yl)acetyl-Asp-Glu-Val-Asp-Ala-Pro-Lys(DNP)-OH.

[2] The name LIGHT was derived from "homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes."

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versus host disease in mouse models (29). Thus, LIGHT is a pleiotropic molecule that initiates diverse biological functions depending on the receptor expression profile of target cells and the cytokines secreted by T cells.

Bcl-2 is one of the key regulators of apoptosis, which is the cell suicide program critical for development, tissue homeostasis, prevention of cancer growth, and protection against pathogens. Bcl-2 promotes cell survival by inhibiting the adapters needed for activation of the proteases (caspases) that dismantle the cell (30). Bcl-2 resides predominantly on the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane through the insertion of its hydrophobic C terminus into the membrane. Bcl-2 exerts broad antiapoptotic effects by inhibiting the production of reactive oxygen species (ROS) and enhancing the steady state of mitochondrial transmembrane functions (reviewed in Refs. 31 and 32). In addition, Bcl-2 can protect cells from various death-inducing agents, such as UV light (33), ceramide (34), nitric oxide (35), and TNF-mediated apoptosis (36). Furthermore, Bcl-2 has been implicated in the prevention of cell death via a caspase-independent mechanism (37). Therefore, it is important to determine whether LIGHT-mediated apoptosis can be inhibited by Bcl-2.

Here we report that LIGHT and IFN-γ act synergistically to activate caspases to digest Bcl-2 within its loop region, thus removing the BH4 domain and converting Bcl-2 from an antiapoptotic to a proapoptotic form. Although caspase inhibitors can prevent Bcl-2 cleavage and block its enhanced sensitivity to LIGHT/IFN-γ-mediated apoptosis, wild type and Bcl-2-overexpressing Hep3BT2 cells are still susceptible to LIGHT/IFN-γ-induced cell death in the presence of caspase inhibitors. In addition, hepatocellular carcinoma Hep3BT2 cells overexpressing caspase-resistant Bcl-2 are also susceptible to LIGHT/IFN-γ-mediated apoptosis, suggesting that the apoptotic signals triggered by LIGHT/IFN-γ might bypass Bcl-2 to induce cell death. In contrast, a potent free radical scavenger, the C3 form of carboxyfullerene (C60), inhibits both Bcl-2 cleavage and LIGHT/IFN-γ-mediated cell death in a dose-dependent manner. This indicates that free radicals are involved in the early stage of LIGHT/IFN-γ-mediated apoptosis, and LIGHT/IFN-γ might be able to bypass mitochondria to mediate caspase-independent cell death.

EXPERIMENTAL PROCEDURES

Cell Culture—The human hepatoma cell line Hep3BT2 (kindly provided by Dr. C.-K. Chou) was maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies) at 37 °C in 5% (v/v) CO_2. The human breast cancer cell line MCF-7 (ATCC number HTB22) was maintained in minimum essential Eagle's medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 0.01 mg/ml bovine insulin (Life Technologies).

Vector Construction and Site-directed Mutagenesis—The cDNA encoding full-length human Bcl-2 was cloned into pcDNA3 (Invitrogen). Substitution of Asp^{31} and Asp^{34} in human Bcl-2 by glutamic acids was performed by overlap extension using polymerase chain reaction (38). The primers used for polymerase chain reaction amplification were as follows: Bcl-2-31E mutant, 5′-TACGAGTGGGAAGCGGGAG-3′ (sense), 5′-CTCCCGCTTCCCACTCGTA-3′ (antisense); Bcl-2-34E mutant, 5′-GATGCGGGAGAAGTGGGCG-3′ (sense), 5′-CGCCCACTTCTCCCGCATC-3′ (antisense). The fidelity of Bcl-2 mutants was confirmed by dideoxynucleotide termination sequencing, and these were then subcloned into pcDNA3.

Generation of Bcl-2 Stable Transfectants—Bcl-2 cDNA and mutant constructs were transfected into Hep3BT2 using LipofectAMINE™ (Life Technologies). Stable transfectants were selected with 800 mg/ml G418 (Sigma), followed by Western blot analysis to confirm the expression of Bcl-2.

Antibodies and Other Reagents—The expression of Bcl-2 and caspase-3 was detected by using anti-Bcl-2 monoclonal antibody (clone 124; Roche Molecular Biochemicals) and anti-CPP32 (Transduction Laboratories) antibodies. The broad range caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK), caspase-1 inhibitor, Ac-YVAD-CMK, and caspase-3 inhibitor, z-DEVD-FMK, were purchased from Calbiochem. The fluorescent substrates, MCA-YVAD.APK (DNP) and MCA-DEVD.APK (DNP) were obtained from Calbiochem. Two regioisomers of $C_{60}(C(COOH)_2)_3$, C_3 and D_3 (kindly provided by Dr. T. Y. Luh) were synthesized and purified following the method of Lamparth and Hirsch (39, 40). Recombinant human TGF-β1 and human IFN-γ were purchased from R&D Systems and Roche Molecular Biochemicals, respectively. The recombinant human soluble form of LIGHT was prepared as described (28).

Immunoblot Analysis—Cell lysates were prepared by the addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Equal amounts of protein were subjected to electrophoresis, transferred onto nitrocellulose membrane (Hybond-C extra, Amersham Pharmacia Biotech), and reacted with appropriate antibodies in PBS containing 5% nonfat dry milk, 0.02% Tween 20. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents subsequently (Amersham Pharmacia Biotech).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Test—The survival rate of cells was determined by MTT test. Briefly, cells were seeded in 96-well flat bottom plates at a density of 5×10^3 cells/well. After treatment, 10 μl of 5 mg/ml MTT per well was added and incubated at 37 °C for 4 h. Cells were then lysed by the addition of 50 μl of 10% SDS in 0.4 N HCl per well and incubated at 37 °C for another 16 h. The optical density of each sample was determined by measuring the absorbance at 570 *versus* 650 nm using an enzyme-linked immunosorbent assay reader (TECAN, RainBow).

Measurement of Caspase Activity—Cytosolic extracts were prepared by freezing and thawing of cells in extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) as described (41). Cell lysates (50 μg) were diluted with 500 μl of ICE standard buffer (100 mM HEPES-KOH buffer, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mg/ml ovalbumin) and incubated at 30 °C for 60 min with 20 μM fluorescent substrates. Fluorescence intensity was measured using a fluorescence spectrophotometer (Hitachi F-4500) at an excitation wavelength of 325 nm and emission wavelength of 392 nm.

RESULTS

Bcl-2 Enhances LIGHT/IFN-γ-mediated Apoptosis—Bcl-2 is well known as an antiapoptotic regulator, and it has been shown that Hep3BT2 cells overexpressing Bcl-2 are resistant to transforming growth factor β-1 (TGF-β1)-mediated apoptosis (42). We tested whether Bcl-2 has similar effects on LIGHT/IFN-γ-mediated apoptosis. We found that LIGHT and IFN-γ have a mild cytotoxic effect on either wild type or Bcl-2-overexpressing Hep3BT2 cells. The survival rate of both cells lines subjected to LIGHT (300 ng/ml) or IFN-γ (300 units/ml) treatment for 72 h is 76 and 88%, respectively (Fig. 1, *A* and *B*). However, in the presence of low dose IFN-γ (50 units/ml), LIGHT-mediated apoptosis is dramatically enhanced. The increased concentration of IFN-γ (300 units/ml) cannot further enhance the LIGHT-mediated cytotoxic effect in both cells. In addition, it is surprising to find that Bcl-2 has no protective effect against LIGHT and/or IFN-γ-mediated cell death (Fig. 1, *B* and *C*), although overexpression of Bcl-2 can protect Hep3BT2 cells from TGF-β1-mediated apoptosis (Fig. 1*D*). Compared with wild type Hep3BT2 cells (75% survival), Bcl-2 overexpression renders Hep3BT2 cells more sensitive to apoptosis induced by LIGHT (50 ng/ml) in conjunction with IFN-γ (100 units/ml) in all of the clones tested (40% survival) (Fig. 1*C*). Therefore, Bcl-2 clearly enhances the sensitivity of Hep3BT2 cells to LIGHT/IFN-γ-mediated apoptosis.

Caspase-3 Is Activated by LIGHT/IFN-γ to Cleave Bcl-2—To clarify the mechanism of Bcl-2-enhanced apoptosis, the expression of Bcl-2 was monitored. The phosphorylation status of Bcl-2 was the same before and after LIGHT/IFN-γ treatment (data not shown). After the addition of IFN-γ alone for 16–24 h, Bcl-2 is still intact, and the level of procaspase-3 is maintained

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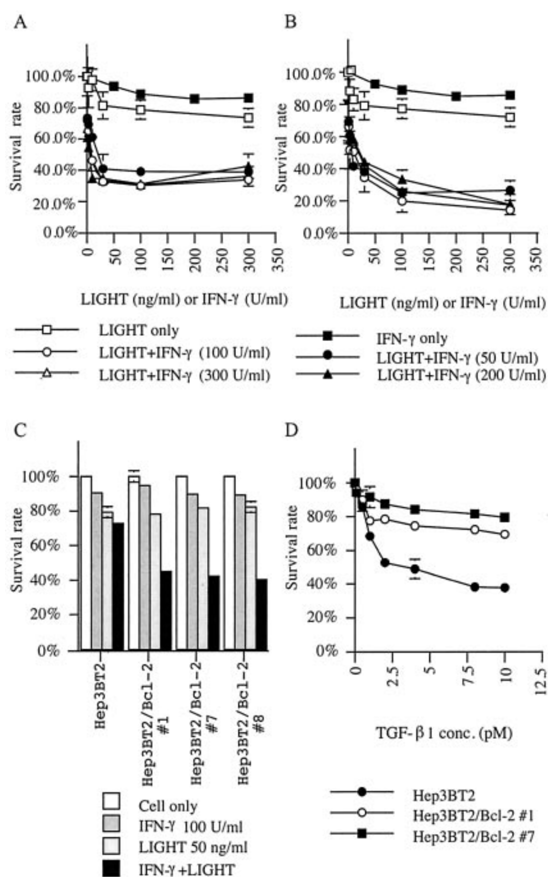

FIG. 1. **Bcl-2 enhances LIGHT-mediated cell death but protects against TGF-β1-mediated apoptosis in Hep3BT2 cells.** *A* and *B*, synergistic effect of LIGHT/IFN-γ-induced cell death. Hep3BT2 cells (*A*) and Hep3BT2 cells overexpressing Bcl-2 (clone 8) (*B*) were treated with LIGHT, IFN-γ, or LIGHT/IFN-γ at different concentrations. The synergistic effects can be observed in the combined treatments. *C*, Hep3BT2 cells or Hep3BT2 cells overexpressing Bcl-2 (clones 1, 7, and 8) were incubated with IFN-γ (100 units/ml) and/or LIGHT (50 ng/ml) for 72 h. *D*, Hep3BT2 or Hep3BT2/Bcl-2 cells (clones 1 and 7) were cultured in serum-free medium for 48 h and then incubated with TGF-β1 at various concentrations as indicated for 48 h. Cell viability was determined by MTT assays, while the percentage of cell survival was determined by measurement of A_{280} for cells treated with cytokines compared with cells cultured in medium alone.

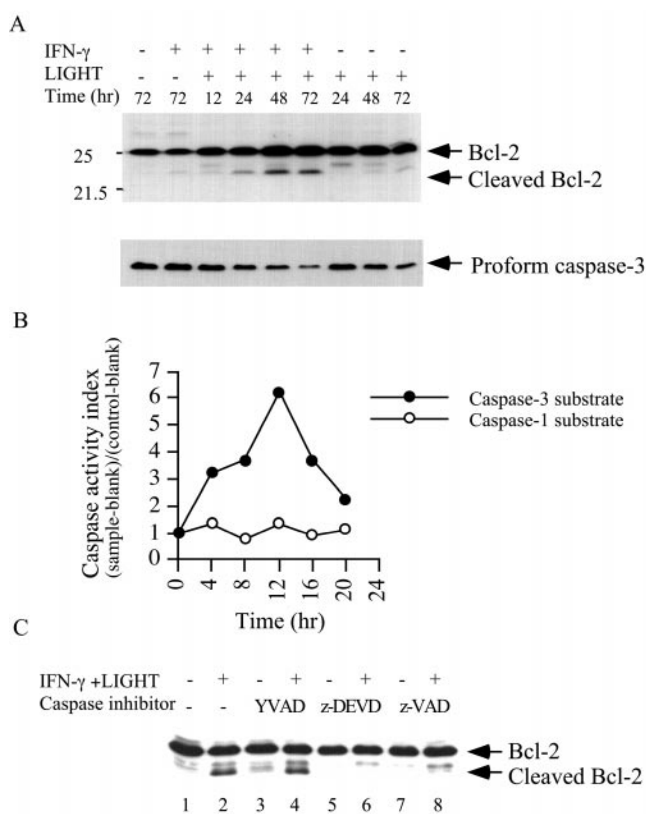

FIG. 2. **Caspase-3-like protease-mediated cleavage of Bcl-2 in LIGHT-induced cell death.** *A*, cleavage of Bcl-2 in LIGHT-mediated cell death. Hep3BT2/Bcl-2 cells were incubated with IFN-γ (100 units/ml) and LIGHT (50 ng/ml) for various time intervals as indicated, and then the cell lysates were fractionated on SDS-PAGE for Western blot analysis using anti-Bcl-2 monoclonal antibody (*upper panel*) or anti-CPP32 polyclonal antibody (*lower panel*) as probe. *B*, activation of caspase-3-like proteases in LIGHT/IFN-γ-mediated cell death. Hep3BT2 cells overexpressing Bcl-2 were incubated with IFN-γ (100 units/ml) and LIGHT (50 ng/ml), and the activities of caspase-1- and caspase-3-like proteases were determined by incubating the cell lysates with MCA-YVAD.APK (DNP) or MCA-DEVD.APK (DNP), respectively, as described under "Experimental Procedures." *C*, inhibition of Bcl-2 cleavage by caspase inhibitors. Hep3BT2/Bcl-2 cells were pretreated with 50 μM YVAD-FMK (*lanes 3* and *4*), z-DEVD-FMK (*lanes 5* and *6*), or z-VAD-FMK (*lanes 7* and *8*) at 37 °C for 1 h and then cultured in medium supplemented with IFN-γ (100 units/ml) and LIGHT (50 ng/ml) (*lanes 2*, *4*, *6*, and *8*) for 48 h. Data shown are representative of three independent experiments.

at the same level (data not shown), while a cleaved 23-kDa Bcl-2 was observed after the addition of LIGHT (50 ng/ml) for 72 h (Fig. 2*A*). In the presence of IFN-γ (100 units/ml), Bcl-2 (26 kDa) was cleaved to a 23-kDa species with a concomitant decrease in levels of procaspase-3 after 24-h treatment (Fig. 2*A*). In contrast, Bcl-2 remains intact in the case of TGF-β1-mediated apoptosis (data not shown). To confirm the activation of caspase-3, fluorescent caspase substrates were incubated with LIGHT/IFN-γ-treated Hep3BT2 cell lysates. We found that fluorescence increased significantly when lysates of cells treated for 4 h with LIGHT/IFN-γ were incubated with the caspase-3 substrate, MCA-DEVD.APK (DNP), but not when incubated with the caspase-1 substrate, MCA-YVAD.APK (DNP) (Fig. 2*B*). This demonstrated that a caspase-3-like enzyme, but not a caspase-1-like enzyme, is activated by LIGHT/IFN-γ. To confirm the correlation between Bcl-2 cleavage and caspase-3 activation, membrane-permeable caspase inhibitors were added to cells. We found that both the caspase-3-like enzyme inhibitor, z-DEVD-FMK, and general caspase inhibitor, z-VAD-FMK, inhibited the cleavage of Bcl-2 (Fig. 2*C*, *lanes 6* and *8*), while the caspase-1-like enzyme inhibitor, YVAD-FMK, had no effect on Bcl-2 cleavage (Fig. 2*C*, *lane 4*). In addition, various specific protease inhibitors, such as leupeptin, phenylmethylsulfonyl fluoride, pepstatin, and aprotinin, were also tested, but none of these had any effect on Bcl-2 cleavage (data not shown). This suggested that caspase-3-like proteases are responsible for Bcl-2 cleavage. Furthermore, we transfected Bcl-2 into a caspase-3-deficient (43), LIGHT/IFN-γ-sensitive human breast cancer cell line, MCF-7, and then incubated the transfected cells with LIGHT/IFN-γ. In contrast to what we observed in Hep3BT2, Bcl-2 was not cleaved in MCF-7 cells after LIGHT/IFN-γ treatment for up to 4 days (Fig. 3*C*, *lane 2*). Thus, we concluded that caspase-3 is responsible for the Bcl-2 cleavage induced by LIGHT/IFN-γ in Hep3BT2 cells.

Mapping of Bcl-2 Cleavage Site—Two putative caspase recognition sites, ^{28}YEWD^{31} and ^{31}DAGD^{34}, have been found in the loop region of Bcl-2. These sequences are typical of caspase-1 and caspase-3 recognition sites, respectively. To determine the cutting site of Bcl-2, we mutated Asp^{31} and Asp^{34} to Glu^{31} (Bcl-2-31E) and Glu^{34} (Bcl-2-34E), respectively (Fig. 3*A*). Both the Bcl-2-31E and Bcl-2-34E mutants were resistant to caspase-3 cleavage (Fig. 3*B*, *lanes 6*, *8*, *10*, and *12*), indicating that both Asp^{31} and Asp^{34} (located at the P4 and P1

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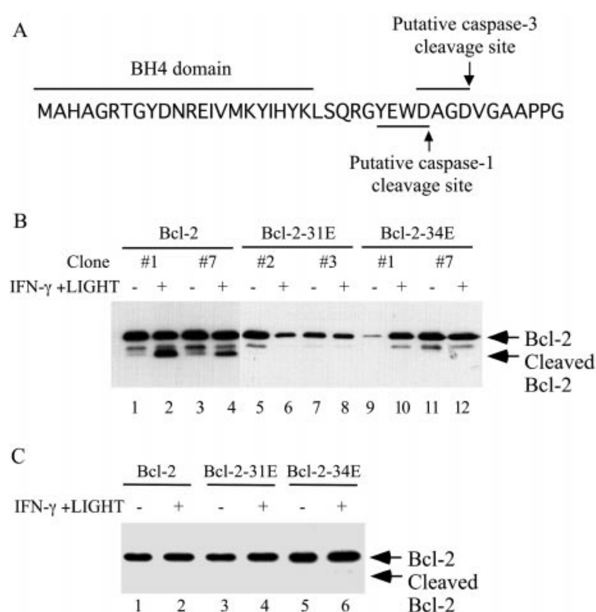

FIG. 3. **Mapping of caspase cleavage site on Bcl-2.** *A*, positions of putative caspase cleavage sites in the Bcl-2 loop region. *B*, Hep3BT2 cells overexpressing wild type Bcl-2 or Bcl-2 mutants (31E and 34E) were incubated with IFN-γ (100 units/ml) and LIGHT (50 ng/ml) for 48 h. Lysates from Hep3BT2 cells overexpressing wild type Bcl-2 (*lanes 1–4*), the Bcl-2-31E mutant (*lanes 5–8*), or the Bcl-2-34E mutant (*lanes 9–12*) were fractionated on SDS-PAGE for Western blot analysis using anti-Bcl-2 monoclonal antibody as probe. *C*, MCF-7 cells were transiently transfected wild type Bcl-2 (*lanes 1* and *2*), Bcl-2 mutants Bcl-2-31E (*lanes 3* and *4*), or Bcl-2-34E (*lanes 5* and *6*). After 24 h, the culture media were supplemented with IFN-γ (100 units/ml) and LIGHT (50 ng/ml) (*lanes 2*, *4*, and *6*), and cells were incubated for a further 72 h. Cell lysates were subjected to Western blot analysis using anti-Bcl-2 monoclonal antibody as probe.

positions of the caspase-3 cleavage site, respectively) are essential for caspase recognition. Since a caspase-1-like enzyme is not activated by LIGHT/IFN-γ (Fig. 2*B*), and inhibition of caspase-1 is unable to prevent Bcl-2 cleavage (Fig. 2*C*), these data further confirmed that caspase-3 is responsible for LIGHT/IFN-γ-mediated Bcl-2 cleavage. Furthermore, Bcl-2, Bcl-2-31E, and Bcl-2-34E mutants were all resistant to LIGHT/IFN-γ-induced cleavage in the caspase-3-deficient cell line, MCF-7 (Fig. 3*C*, *lanes 2*, *4*, and *6*).

The Effects of Bcl-2 Mutations and Caspase Inhibitors on LIGHT/IFN-γ-mediated Apoptosis—We wanted to establish whether caspase inhibitors and the Bcl-2-31E and Bcl-2-34E mutants could restore the antiapoptotic function of Bcl-2 and protect Hep3BT2 cells from LIGHT/IFN-γ-mediated apoptosis. To understand whether Bcl-2 mutants are antiapoptotic, Hep3BT2 cells overexpressing Bcl-2-31E and Bcl-2-34E mutants were treated with TGF-β1 as control to determine their ability to prevent TGF-β1-mediated apoptosis. As shown in Fig. 4*A*, both Bcl-2-31E and Bcl-2-34E mutants, as well as wild type Bcl-2, provide same protective effect as that of wild type Bcl-2 to Hep3BT2 cells against TGF-β1-mediated apoptosis. This indicated that both Bcl-2-31E and Bcl-2-34E mutants still maintain their antiapoptotic ability. We then asked whether the Bcl-2-31E and Bcl-2-34E mutants could protect Hep3BT2 cells from LIGHT/IFN-γ-mediated apoptosis. The survival rate of Hep3BT2 cells overexpressing Bcl-2-31E and Bcl-2-34E (77% survival) was higher than that of cells overexpressing wild type Bcl-2 (42% survival). Surprisingly, both Bcl-2-31E and Bcl-2-34E mutants were still susceptible to LIGHT/IFN-γ-induced apoptosis, although the survival rates of Bcl-2-31E and Bcl-2-34E mutants are restored to the same level as that of wild type Hep3BT2 cells (68%) (Fig. 4*B*).

We then asked whether caspase inhibitors could protect Hep3BT2/Bcl-2 cells from LIGHT/IFN-γ-mediated apoptosis. As shown in Fig. 4*D*, the caspase-1 inhibitor, YVAD-FMK, had no effect on the survival of Hep3BT2/Bcl-2 cells, while the caspase-3 inhibitors z-DEVD-FMK and z-VAD-FMK increased their survival rate from 45 to 75%, which is similar to the survival rate (~75%) of Hep3BT2 overexpressing Bcl-2-31E and Bcl-2-34E mutants (Fig. 4*B*). However, neither z-DEVD-FMK nor z-VAD-FMK could further increase the survival rate of Hep3BT2 cells (Fig. 4*C*). The failure of caspase inhibitors to protect Hep3BT2 cells cannot be attributed to their inability to penetrate cell membrane, since we have shown that caspase inhibitors can inhibit Bcl-2 cleavage (Fig. 2*C*) and protect Hep3BT2 cells from TGF-β1-mediated apoptosis (~70% survival) under the same assay conditions (Fig. 4*E*). These results support our hypothesis that LIGHT and IFN-γ act synergistically to activate a caspase-3-like protease to cleave Bcl-2, thus converting its activity from antiapoptotic to proapoptotic. The inhibition of caspase-3-like activity by z-VAD-FMK or mutation of the caspase-3 cleavage site in Bcl-2 (mutants Bcl-2-31E and Bcl-2-34E) can restore the survival rate of Bcl-2-overexpressing Hep3BT2 cells to the same level as that of wild type Hep3BT2.

Free Radicals Are Involved in the Upstream of Caspase-3 Activation—ROS have been shown to participate in TNF-α-mediated apoptosis (44–47) and other apoptotic events (48–50), so we tested whether free radical inhibitors could protect cells from LIGHT/IFN-γ-induced apoptosis. We found that the potent, water-soluble C3 form of carboxyfullerene (C60), which has been shown to be a very effective neuroprotective antioxidant both *in vitro* and *in vivo* (51), inhibited apoptosis of both Hep3BT2 and Hep3BT2/Bcl-2 cells. In contrast, the relatively less cell-permeable D3 form of C60 only had a partial protective effect against LIGHT/IFN-γ-induced apoptosis (Fig. 5, *A* and *B*). A similar protective effect is also observed in another LIGHT/IFN-γ-sensitive cell line, HT-29 (data not shown). Neither the superoxide dismutase mimetic, MnTBAP, nor the inducible nitric-oxide synthetase inhibitor, L-NAME, had a significant protective effect against LIGHT/IFN-γ-induced apoptosis in both wild type and Bcl-2-overexpressing Hep3BT2 cells, although MnTBAP has partial protective effect against LIGHT/IFN-γ-induced apoptosis in HT-29 adenocarcinoma cells (data not shown). To further clarify the stage at which free radicals contribute to LIGHT/IFN-γ-mediated apoptosis, we examined whether carboxyfullerenes could inhibit Bcl-2 cleavage by caspase-3. As shown in Fig. 5*C*, carboxyfullerenes could inhibit the cleavage of Bcl-2 (Fig. 5*C*, *lanes 3* and *4*) and the activation of caspase-3-like activity (Fig. 5*D*), indicating that the production of free radicals occurs upstream of caspase-3 activation. Thus, we concluded that ROS play critical roles in LIGHT/IFN-γ-induced apoptosis.

DISCUSSION

Previous studies have shown that LIGHT can transduce CD28-independent costimulatory signals that enhance IFN-γ secretion by preactivated T cells and further increase cytotoxic T lymphocytes activity (28, 29). Although LIGHT alone is not a potent cytotoxic factor for tumors *in vitro*, tumor cells transfected with LIGHT are rejected *in vivo*. We speculated that the cytotoxic effect of LIGHT observed *in vivo* might result from its ability to enhance IFN-γ secretion by preactivated T cells, thus allowing LIGHT to act synergistically with IFN-γ in tumor cell killing. This speculation is supported by the observation that LIGHT alone has little cytotoxic effect to induce Hep3BT2 cell apoptosis, while IFN-γ can synergistically proceed apoptotic processes with LIGHT on Hep3BT2 cells and Bcl-2-overexpressing cells (Fig. 1, *A* and *B*). This phenomenon is consistent with previous observation in other tumor cell lines, such as

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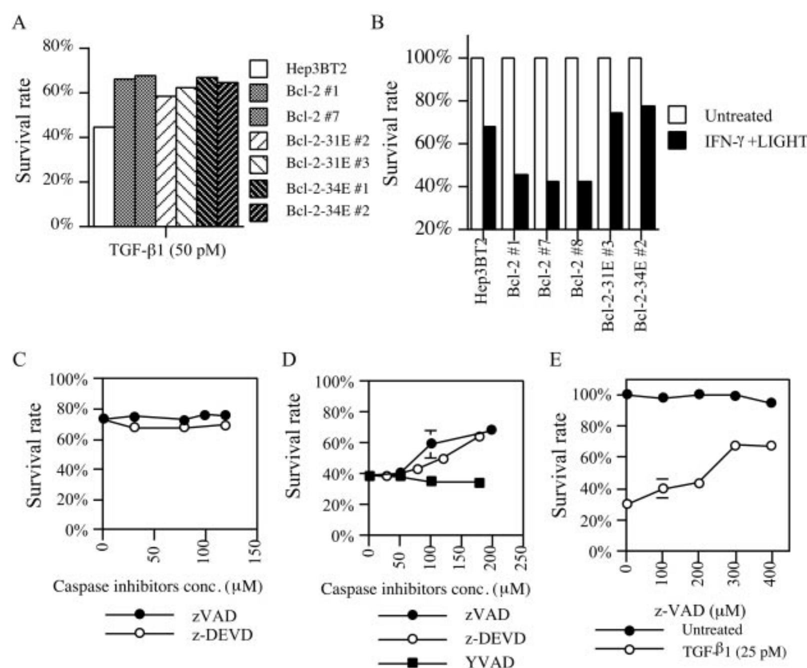

FIG. 4. **Effects of Bcl-2 mutants and caspase inhibitors in LIGHT/IFN-γ-mediated apoptosis.** *A*, both Bcl-2-31E and Bcl-2-34E mutants protect Hep3BT2 cells from TGF-β1-mediated apoptosis. Hep3BT2, Hep3BT2/Bcl-2, Hep3BT2/Bcl-2-31E, or Hep3BT2/Bcl-2-34E cells were serum-starved for 48 h and then cultured in Dulbecco's modified Eagle's medium supplemented with 50 pM TGF-β1 for a further 48 h. Cell viability was determined by MTT assay, and the percentage of survival for cells in each treatment was compared with that for the cells cultured in the absence of TGF-β1. *B*, partial protective effect of Bcl-2-31E and Bcl-2-34E mutants against LIGHT-mediated cell death. Hep3BT2, Hep3BT2/Bcl-2, Hep3BT2/Bcl-2-31E, or Hep3BT2/Bcl-2-34E cells were cultured in medium supplemented with 100 units/ml IFN-γ and 50 ng/ml LIGHT for 72 h. Cell viability was determined by MTT assay. *C* and *D*, failure of caspase inhibitors to protect Hep3BT2 cells from LIGHT/IFN-γ-mediated apoptosis. Hep3BT2 (*C*) and Hep3BT2/Bcl-2 cells (*D*) were pretreated with DEVD-FMK, z-VAD-FMK, or YVAD-FMK for 1 h and then cultured in medium supplemented with 100 units/ml IFN-γ and 50 ng/ml LIGHT for 72 h. Cell viability was determined by MTT assay. *E*, caspase inhibitor z-VAD-FMK can protect Hep3BT2 cells from TGF-β1-induced apoptosis. Hep3BT2 cells were cultured in serum-free medium for 48 h and then incubated with z-VAD-FMK before the addition of TGF-β1 (25 pM). Data shown are representative of three independent experiments.

MDA-MB-231 and HT29 (28).

To elucidate the mechanisms of LIGHT/IFN-γ-mediated apoptosis, we tested the protective effect of Bcl-2 and caspase inhibitors on several tumor cells. We demonstrated that overexpression of Bcl-2 enhances the cytotoxic effect of LIGHT/IFN-γ in hepatocellular carcinoma Hep3BT2 cells. This enhanced cytotoxicity occurs via the activation of caspase-3, which cleaves Bcl-2 to remove its BH4 domain. This observation is in accord with previous observations that Bcl-2 is the substrate of caspase-3, and recombinant caspase-3 can cleave Bcl-2 at the loop region to remove BH4 domain *in vitro* (55). In addition, it has been reported that the BH4 domains of Bcl-2-like proteins are critical for the inhibition of apoptosis and for interaction with CED-4 (52) or Bax (53), and the BH4 domain-deficient Bcl-2 has been shown to translocate to mitochondria and promote release of cytochrome *c* to induce cell apoptosis (54). Thus, all of the evidence supports the argument that the enhanced cytotoxicity in Bcl-2-overexpressing Hep3BT2 cells is via the cleavage of Bcl-2 by caspase-3, thus converting Bcl-2 from antiapoptotic to proapoptotic.

The conversion of Bcl-2 to a Bax-like death effector also can be triggered by Fas-mediated apoptosis (55), alphavirus infection (56), interleukin-2 deprivation (57), and chemotherapeutic agents (57, 68). Our observations provide evidence of another route by which Bcl-2 can be converted to a proapoptotic form, via the actions of LIGHT/IFN-γ. Although mutation of Asp^{31} and Asp^{34} to glutamic acids (Bcl-2-31E, Bcl-2-34E) prevents cleavage of Bcl-2 and abrogates alphavirus-induced apoptosis (56), these caspase-resistant Bcl-2 mutants only abolish the enhanced cytotoxic effect of wild type Bcl-2 and still cannot protect cells against LIGHT/IFN-γ-mediated apoptosis (Fig. 4*B*).

Two principal pathways for caspase activation have been demonstrated. One pathway requires the participation of mitochondria and the assembly of apoptosome complex after cytochrome *c* release (the intrinsic pathway), while other signals can bypass mitochondria and activate caspases directly by recruiting adaptor proteins to death receptors (the extrinsic pathway) (70–72). Bcl-2 can block the activation of caspase cascade initiated by cytochrome *c* release from mitochondria (73, 74) but not other death receptor-mediated caspase activation independent of cytochrome *c* release (75, 76). In this study, we found that caspase-resistant Bcl-2 mutants cannot inhibit LIGHT/IFN-γ−mediated apoptosis (Fig. 4*B*). In addition, the endogenous Bcl-2 is undetectable in wild type Hep3BT2 cells (data not shown); thus, the cytotoxic effect mediated by LIGHT/IFN-γ does not result from the cleavage of endogenous Bcl-2 by caspase-3. Therefore, we speculated that the apoptotic signals triggered by LIGHT/IFN-γ might bypass mitochondria to induce cell death uninhibitable by Bcl-2.

Since the caspase-3-like protease was activated within 12 h after LIGHT/IFN-γ treatment (Fig. 2*B*), while cleavage of Bcl-2 was observed in 16–24 h (Fig. 2*A*), the caspase-3 activation is Bcl-2-uninhibitable and might be initiated by other caspase cascades. Although caspase-3 is responsible for Bcl-2-enhanced cytotoxic effect, caspase-3 is dispensable in LIGHT/IFN-γ-mediated apoptosis, since the caspase-3-deficient MCF-7 cells are sensitive to LIGHT/IFN-γ-mediated apoptosis, and caspase inhibitors cannot protect cells from apoptosis mediated by LIGHT/IFN-γ. In addition, caspase inhibitors z-VAD-FMK and z-DEVD-FMK cannot prevent LIGHT/IFN-γ−mediated apoptosis, indicating that LIGHT/IFN-γ could mediate caspase-independent cell death (Fig. 6). Thus, although caspase is activated by LIGHT/IFN-γ, it might be not the major factor to

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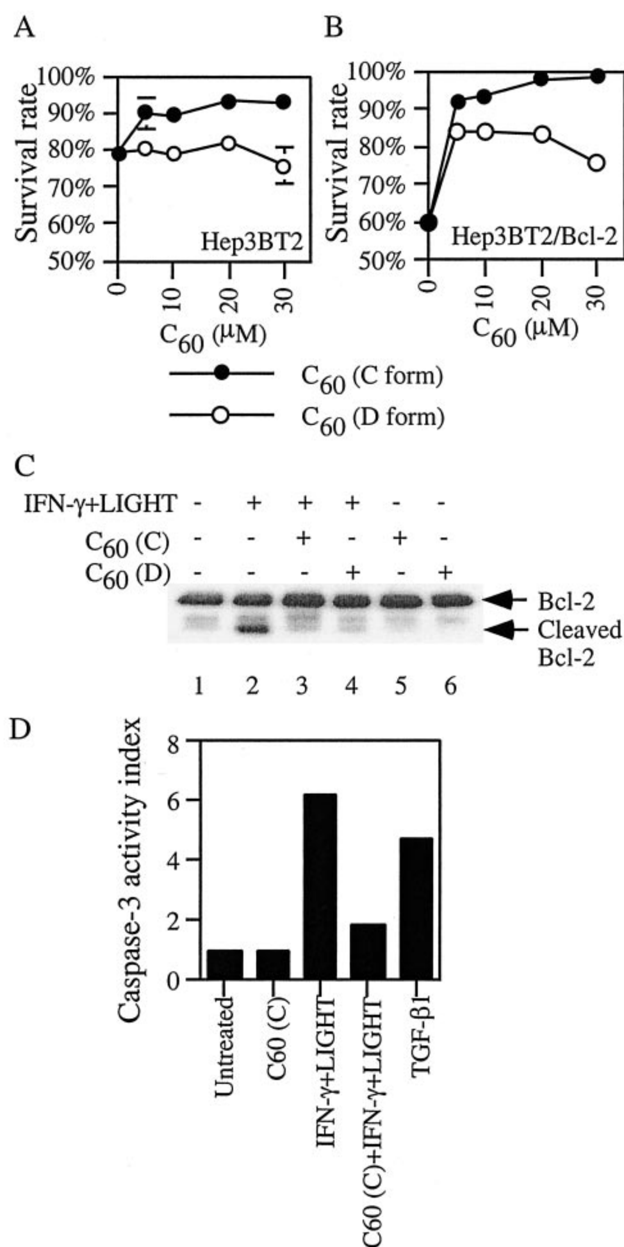

FIG. 5. **Free radical scavenger carboxyfullerenes rescue cells from LIGHT/IFN-γ-induced death.** Hep3BT2 cells (*A*) and Hep3BT2 cells overexpressing Bcl-2 (*B*) were incubated with carboxyfullerenes for 1 h, followed by the addition of LIGHT (50 ng/ml) and IFN-γ (100 units/ml) for 72 h. The cell survival rate was determined by MTT assay. *C*, carboxyfullerenes can inhibit the cleavage of Bcl-2. Hep3BT2/Bcl-2 cells pretreated with a 25 μM concentration of the C3 or D3 isoform of carboxyfullerene were cultured in the absence or presence of LIGHT (50 ng/ml) and IFN-γ (100 units/ml) for 72 h. Cell lysates were fractionated on SDS-PAGE for Western blot analysis using anti-Bcl-2 monoclonal antibody as probe. *D*, carboxyfullerenes inhibit caspase-3-like protease activation triggered by LIGHT/IFN-γ. Hep3BT2 cells were pretreated with 25 μM carboxyfullerene (C3 form), followed by incubation with LIGHT (50 ng/ml) and IFN-γ (100 units/ml) for 16 h or with 25 pM TGF-β1 for 6 h after 48-h starvation. Caspase-3-like protease activity was determined by incubating the cell lysates with the fluorescent caspase substrate MCA-DEVD.APK (DNP). Data shown are representative of three independent experiments.

induce cell death. During the preparation of this manuscript, a novel member of the TNF receptor family, TAJ, which lacks the death domain in the cytoplasm, has also been reported to mediate caspase-independent cell death (77). Thus, it will be interesting to ask whether other members of TNF receptor superfamily, which lack the death domain in the cytoplasm,

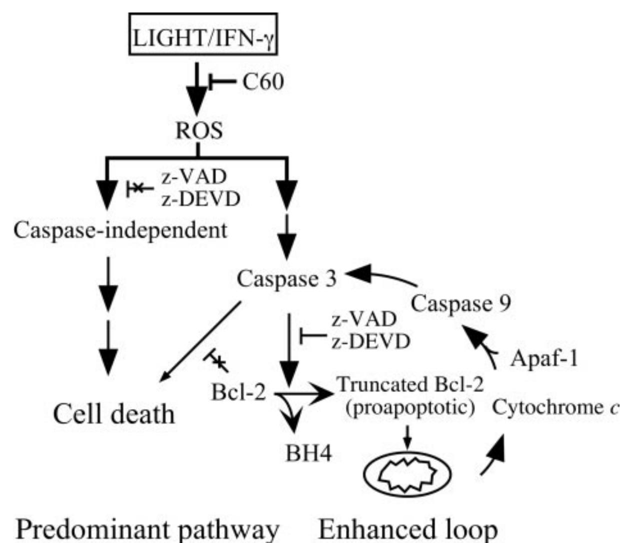

FIG. 6. **Putative model of LIGHT/IFN-γ-mediated cell death on Hep3BT2 cells.** Activation of caspase-3 is one of the factors contributing to LIGHT/IFN-γ-mediated cell death. After caspase-3 activation, Bcl-2 is cleaved at the loop region and is converted from antiapoptotic to proapoptotic. The BH4 domain-deficient, truncated Bcl-2 fragment increases cytochrome *c* release from mitochondria, thus further activating caspase-3 to cleave Bcl-2 and other cellular substrates. In caspase-3-deficient MCF-7 cells or in the presence of caspase inhibitors, LIGHT/IFN-γ still can activate a caspase-independent pathway to induce cell death, although Bcl-2 cleavage is abolished. Free radicals are generated upstream of the unidentified caspase-independent apoptotic signaling pathway and caspase-3 activation, which can be inhibited by the water-soluble C3 form of carboxyfullerene.

also induce cell death independent of caspase activation.

The aspartate-specific cysteine proteases, known as caspases, are wildly recognized as key players in initiation or effector steps of apoptosis. However, caspases are not the only molecules that mediate apoptosis, and several reports have demonstrated the existence of other apoptotic pathways (58–60). For example, it has been also shown that the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp can block caspase activity and inhibit Fas-mediated apoptosis but not BAX-induced death (61). Furthermore, the caspase-3-deficient cell line, MCF-7, is sensitive to TNF and staurosporine-induced apoptosis (62), and NO-induced apoptosis cannot be inhibited by caspase inhibitors (37). Thus, LIGHT/IFN-γ-induced apoptosis may involve an unidentified caspase-independent pathway. However, we cannot completely rule out the possibility that LIGHT/IFN-γ-mediated apoptosis occurs via the activation of other unidentified caspases, which are not inhibited by z-VAD-FMK and z-DEVD-FMK.

Recently, several groups have reported that cell apoptosis, which cannot be rescued by caspase inhibitors, can be inhibited by the overexpression of manganese superoxide dismutase (47) or by the oxygen free radical scavenger, *N*-acetyl-L-cysteine (63). ROS-mediated apoptosis has also been demonstrated in many model systems (44, 45, 64, 65). In this study, we observed that LIGHT/IFN-γ-induced apoptosis could be inhibited completely by free radical scavenger carboxyfullerenes. Carboxyfullerenes (C60) have been described as free radical sponges, and the C3 and D3 isomers of C60 have been shown to be potent scavengers of hydroxyl radicals ($OH^{\cdot}$) and superoxide anions ($O_2^{\cdot-}$) in solution, although the D3 isomer is less potent than the C3 isomer (51). Derivatives of C60 have been shown to act as potent antioxidants in several models of oxidative stress (51, 65, 66), and C60 has also been found to inhibit radical-initiated lipid peroxidation (67). In this study, we have demonstrated that LIGHT/IFN-γ-induced apoptosis is inhibited by carboxy-

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fullerenes (Fig. 5, *A* and *B*), but not by L-NAME, an inducible nitric-oxide synthetase inhibitor (data not shown). This indicated that reactive oxygen species, but not nitric oxide, are responsible for LIGHT/IFN-γ-induced apoptosis. Moreover, superoxide dismutase mimetic, MnTBAP, has a partial protective effect on HT-29 cells, but not Hep3BT2; thus, superoxide also contributes to LIGHT/IFN-γ-induced apoptosis in HT-29 cells (data not shown). Furthermore, production of ROS seems to occur upstream of caspase-3 activation, since inhibition of free radical production also prevents the activation of caspase-3 (Fig. 5*D*). This is in accordance with a previous observation that overexpression of Mn^{2+}-superoxide dismutase can suppress the activation of caspase-3 and inhibit apoptosis induced by TNF-α (47). However, previous studies showed that Bcl-2 might interfere with the generation or action of ROS and protect cells from apoptosis (48). In contrast, we found that ROS scavenger, but not Bcl-2, rescued cells from apoptosis, suggesting that the apoptotic signals induced by LIGHT/IFN-γ bypass the protective effect of Bcl-2.

Result from this study demonstrated that apoptosis induced by LIGHT/IFN-γ occurs via a novel pathway. First, caspases may not be instrumental in this process because caspase inhibitors cannot inhibit cell apoptosis. Second, ROS induced by LIGHT/IFN-γ is generated at a relatively early step of apoptosis and bypasses the protective effect of Bcl-2. Third, ROS are not by-products but appear as potent mediators to induce cell death mediated by LIGHT/IFN-γ, as that observed in TNF-α apoptotic signaling cascades (78–80). Even the superoxide radicals are produced mostly at the mitochondrial electron transport chain when oxygen is reduced by a single electron; the superoxides can also be produced by other organelles, such as the endoplasmic reticulum and nuclear and plasma membranes. ROS produced at sites other than mitochondria have been also reported to be involved in some apoptotic systems (69). Therefore, it will be interesting to clarify the source(s) of ROS induced by LIGHT/IFN-γ in the future.

The finding that LIGHT can bind to both LT-βR and TR2/HVEM/ATAR (18–21) further complicates the mechanism of LIGHT/IFN-γ-mediated apoptosis, since knowledge of the downstream signaling pathways associated with both LT-βR and TR2/HVEM/ATAR is still very limited. The relationships between ROS production and the apoptosis cascades that occur downstream of receptor signaling remain to be elucidated.

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