LIGHT, a Member of the Tumor Necrosis Factor Ligand Superfamily, Prevents Tumor Necrosis Factor-α-mediated Human Primary Hepatocyte Apoptosis, but Not Fas-mediated Apoptosis*

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LIGHT is a member of a tumor necrosis factor (TNF) superfamily, and its receptors have been identified as lymphotixin-β receptor (LTβR) and the herpesvirus entry mediator (HVEM)/ATAR/TR2, both of which lack the cytoplasmic sequence termed the “death domain.” The present study has demonstrated that LIGHT inhibits TNFα-mediated apoptosis of human primary hepatocytes sensitized by actinomycin D (ActD), but not Fas- or TRAIL-mediated apoptosis. Furthermore, LIGHT does not prevent some cell lines such as HepG2 or HeLa from undergoing ActD/TNFα-induced apoptosis. This protective effect requires LIGHT pretreatment at least 3 h prior to ActD sensitization. LIGHT stimulates nuclear factor-κB (NF-κB)-dependent transcriptional activity in human hepatocytes like TNFα. The time course of NF-κB activation after LIGHT administration is similar to that of the pretreatment required for the anti-apoptotic effect of LIGHT. LIGHT inhibits caspase-3 processing on the apoptotic protease cascade in TNFα-mediated apoptosis but not Fas-mediated apoptosis. In addition, increased caspase-3 and caspase-8 activities in ActD/ TNFα-treated cells are effectively blocked by LIGHT pretreatment. However, LIGHT does not change the expression of TNFRp55, TNFRp75, and Fas. These results indicate that LIGHT may act as an anti-apoptotic agent against TNFα-mediated liver injury by blocking the activation of both caspase-3 and caspase-8.

Members of the TNF1 ligand superfamily play multiple roles in the development of the immune system and the osteogenesis, and mediate the effective function of both innate and adaptive immune responses (1). These proteins include TNFα, lymphotoxin-α or -β, Fas ligand (FasL), CD27L, CD30L, CD40L, 4–1BB (2), TRAIL (3), RANKL/OPGL (4–8), TWEAK (9), APRIL/TALL-2 (10, 11), AITRL (12), VEG1 (13), and BAFF/ TALL-1 (11, 14). Recently, LIGHT has been identified as a member of the TNF ligand superfamily and shown to be about 30% identical to FasL and lymphotixin-β (15). Further studies have revealed that LIGHT can bind to LTβR and HVEM/ ATAR/TR2 (15–19). TNFα, lymphotoxin-α,-β, and LIGHT exhibit distinct and overlapping patterns in the binding of four cognate receptors. TNFα and lymphotoxin-α of homotrimer (LTα) bind two receptors, TNFRp55 and TNFRp75 (1); and LTα forms heterotrimers with lymphotixin-β (LTβ), which can bind LTβR. The complexity of receptor/ligand engagement suggests a functional redundancy of these cytokines, however, these gene disruptions in mice indicate that the TNF and LT systems play important roles in the development of the immune system (20–25). Moreover, these systems are involved more directly in several immune responses. TNFα induces cell death in many types of cells, and stimulates expression of several chemokines. LTβR signaling stimulates secretion of chemokines such as interleukin-8, and causes cell death in some types of cells under certain culture conditions, such as colon adenocarcinoma HT-29 cells (26, 27). LTβR and HVEM are associated with TRAFs, which bind to the cytoplasmic domains of the TNF receptor family. LTβR binds TRAF2, TRAF3, and TRAF5, whereas HVEM binds TRAF2 and TRAF5. TRAF2 and TRAF5, but not TRAF3, stimulate the activation of several transcriptional pathways including NF-κB (18, 19, 28–30), although it is reported that splice variants of TRAF3 are capable of inducing NF-κB activation (31).

Because LIGHT engages LTβR and HVEM as the cellular receptors, it is expected to have biological functions similar to those of LTα and LTβ. As previous studies have shown, LIGHT induces cell death in HT-29 cells as does LTα (32–34), it causes growth arrest in RD cells following the developmental changes to smooth muscles cells, and it stimulates secretion of interleukin-8 and RANTES (regulated on activation normal T cell expressed and secreted) from the cells (35). It has also been reported that LIGHT is one of the CD28-independent co-stimulatory molecules in T cells (36). Recently, the studies on transgenic mice expressing recombinant LIGHT and on mice administrated soluble HVEM proteins for blocking LIGHT activity have revealed that LIGHT is required for the expansion of T cells and it plays an important role in T cell homeostasis (37, 38).

TNFα plays an important role in the proliferation of hepatocytes in vitro and in vivo (39–42), and also acts as a mediator of cell death in several liver injury models (43–46). Hepatocytes are normally resistant to TNFα-mediated cytotoxicity, so...
that they require sensitization with transcription inhibitors such as ActD for maximal TNFα cytotoxicity (47). This suggests that TNFRP55, an essential receptor for TNFα signaling, stimulates two distinct pathways: a signal mediated by NF-κB to maintain the survival of cells, and another with activation of a series involving the caspase family that results in cell death. Recent studies have indicated that the survival signal(s) seems to play a dominant role against the death signal(s). Because LIGHT stimulates activation of NF-κB in nonadherent cells (35), it is expected to be an anti-apoptotic mediator.

In this present study, we investigated the effects of LIGHT on human primary hepatocytes, and demonstrated that LIGHT blocks ActD/TNFα-induced hepatocyte apoptosis, but that it does not suppress ActD/agonistic anti-Fas antigen monoclonal antibody (oFas)- or ActD/TRAIL-mediated apoptosis. We also suggest that LIGHT stimulates NF-κB transcriptional activity in the human primary hepatocytes and inhibits the apoptotic caspase cascade induced by TNFα.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human primary hepatocytes were purchased from Cell Systems (Kirkland, WA). The cells were cultured in a basal medium composed of Ham’s F-12 and Leibovitz L-15 (1:1 medium) (Invitrogen, Carlsbad, CA), 0.2% (v/v) bovine serum albumin (Invitrogen), 5 mM glucose (Wako Pure Chemical Inc., Osaka, Japan), 10° M dexamethasone (Wako), and 10° M bovine insulin (Invitrogen) supplemented with 10% (v/v) fetal calf serum (JRH, Lenexa, KS). The cells had been expanded 10-fold, and were stored at –80°C until use. Human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The soluble form of human LIGHT protein was produced as previously described (35). Recombinant human TNFα, LTα, and TRAIL proteins, and anti-LTβR polyclonal antibody (AF829) were purchased from R&D System (Minneapolis, MN). Agonistic monoclonal antibody (mAb) against Fas antigen (oFas; CH-11) was purchased from MBL (Nagoya, Japan). Anti-TNFFR55 mAb (utr9) and anti-TNFFR75 mAb (utr1) were purchased from BMA (August, Switzerland). Anti-HVEM mAb was kindly provided by Dr. Trune (48). Rabbit antiserum raised against caspase-3 and FITC-conjugated anti-mouse IgG mAb were purchased from Pharmingen (San Diego, CA). Rabbit FITC-conjugated anti-goat IgG polyclonal antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA). Goat antiserum against actin (1-19), goat horseradish peroxidase-conjugated anti-rabbit Ig, and donkey horseradish peroxidase-conjugated anti-goat Ig were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ActD was purchased from Wako. Several protease inhibitors were purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Cytotoxicity Assay—Hepatocytes were plated onto 96-well type I collagen-coated plates in a basal medium containing 1% newborn calf serum (NCS). They were then incubated with 333 nM ActD for 30 min, followed by the addition of various amounts of TNFα, LTα, LIGHT, TRAIL, or oFas for 18 h. The lactate dehydrogenase (LDH) activity in the culture supernatants was determined using the Cytotoxicity Test-WAKO (Wako). Briefly, culture supernatants were treated with the substrate solution for 45 min, and their absorbance at 620 nm was measured with a plate reader according to the manufacturer’s instructions. To examine its anti-apoptotic effect, LIGHT was added to the hepatocyte cultures for the indicated times before administration of ActD. Data were expressed as the mean ± S.E. for three samples.

Determination of Apoptosis by Flow Cytometry—Hepatocytes were plated onto 6-well type I collagen-coated plates. They were then cultured for 18 h in a basal medium containing 10% NCS. After the medium was changed to the basal medium containing 1% NCS, the cells were treated with 333 nM ActD for 30 min, followed by the addition of 100 ng/ml TNFα, LTα, LIGHT, or oFas. Apoptosis was determined using the Early Apoptosis Detection Kit (Kamiya Biochemical, Seattle, WA). Briefly, 14 h after each cytokine addition, the cell cultures were washed, fixed with 70% ethanol, and stained with 0.1% propidium iodide for 30 min. They were then analyzed using a FACScan using the CELLQuest program (Becton Dickinson, San Jose, CA) at an excitation of 488 nm.

Reverse Transcription-PCR Analysis—Hepatocytes cultured in type I collagen-coated 75-cm² flasks were lysed with 0.6 ml of ISOGEN (NipponGene, Japan) by mixing vigorously. The lysates were then mixed with CHCl₃, and centrifuged. The aqueous phases were collected, and RNA precipitation was performed with isopropyl alcohol. Precipitated RNA was then resuspended, treated with DNase I (Gene Hunter, Nashville, TN), and extracted with the RNeasy Kit (Qiagen, Chatsworth, CA). Reverse transcription was performed using the TaqMan reverse transcription reagent kit (PE Biosystems, Foster City, CA). A pair of primers (5′-CCCTACACATACACCCCTTTGGAAGT-3′ as a forward primer, and 5′-AGCCCTAGCTGATACAGATTCCGTGAAT-3′ as a reverse primer) were designed to amplify the fragments of the CYP3A4 gene. The primers of the LTβR and HVEM genes were described previously (35). PCR was performed using the Advantage GC-cDNA polymerase mix (Clontech, Palo Alto, CA). As a control, PCR was performed using RNA without a reverse transcription process.

Flow Cytometry—The hepatocytes were harvested and stained with anti-TNFFR55, anti-TNFFR75, anti-HVEM, anti-LTβR, or oFas, in phosphate-buffered saline containing 1% FCS and 0.05% NaN₃ for 30 min on ice. The cells were then washed with the above buffer, the cells were exposed to FITC-conjugated anti-mouse Ig antibodies, and analyzed in FACSVantage (BD Biosciences, Mountain View, CA). Cells treated solely with secondary antibodies were used as an unstained negative control.

RESULTS

Characterization of Human Primary Hepatocytes—Primary hepatocytes are known to lose several of their biological functions during in vitro cultivation. Consequently, the mRNA expression of CYP3A4, a hepatocyte marker belonging to the cytochrome P-450 family, was determined by reverse transcriptase-PCR in the human primary hepatocytes in this experiment. As shown in Fig. 1A, the specific PCR product of the CYP3A4 gene was detected. Because LIGHT associates with two receptors, LTβR and HVEM, it was also investigated whether these receptors were expressed in the hepatocytes. The specific PCR products of both receptor genes were detected by reverse transcriptase-PCR (Fig. 1A), and the expression of both receptor proteins on the cell surface were determined by...
Fig. 1. Characterization of the human primary hepatocytes in this experiments. A, total RNA was prepared from human primary hepatocytes and analyzed by reverse transcriptase-PCR using CYP3A4-, LTβR-, and HVEM-specific primers based on sequences of each gene. To check whether specific PCR occurred, PCR without the reverse transcriptase (RT) process (-RT) was carried out as a control. B, LTβR; and C, HVEM proteins on the cell surface of human primary hepatocytes were determined by FACS analysis. Human primary hepatocytes were stained with specific antibodies against LTβR and HVEM, following FITC-conjugated secondary antibodies, and each receptor expression level was determined by FACS analysis (--). Cells treated solely with second antibodies were used as an unstained negative control (---). FACS analysis using LTβR- and HVEM-specific antibodies (Fig. 1, B and C). Therefore, the hepatocytes used in our experiments were confirmed to be biologically normal, and to be capable of responding to LIGHT through LTβR and/or HVEM.

LIGHT Has No Apoptotic Effect on Human Primary Hepatocytes—Several ligands of the TNF superfamily are able to induce apoptosis against some kinds of cells. To investigate whether LIGHT has cytotoxic effects on human primary hepatocytes, we examined LDH activity in culture supernatants 18 h after administration of LIGHT, TNFα, LTα, or αFas. As shown in Fig. 2, the level of LDH activity did not increase in response to any cytokines at concentrations ranging from 0.3 to 100 ng/ml. TNFα is reported to be required for pretreatment with transcriptional inhibitors like ActD causing apoptosis in cells such as murine hepatocytes (50–53). Therefore, the apoptotic effect of cytokines in the presence of ActD was examined. The LDH activity increased when TNFα, LTα, or αFas were added in the presence of ActD. However, the LDH level did not increase 18 h after LIGHT treatment even in the presence of ActD. Furthermore, the viability of the cells was not reduced over a period of 18 h after LIGHT treatment (data not shown). LIGHT engages two known cellular receptors, LTβR and HVEM, neither of which contain the so-called cytoplasmic death domain. However, death ligands, such as TNFα and Fas ligand (FasL), engage receptors containing a death domain in each cytoplasmic region, TNFRp55 or CD95, respectively. As one possible reason that LIGHT did not cause apoptosis in hepatocytes, it may be because of the lack of a death domain in both LTβR and HVEM.

To investigate the type of cell death induced by these cytokines, hepatocytes stained with annexin-V or propidium iodide in the above conditions were analyzed by FACS. As shown in Fig. 3, there was no difference in annexin-V staining patterns between hepatocytes treated with ActD alone and those treated with ActD plus LIGHT. In contrast, hepatocytes treated with ActD plus the other TNF family ligands, such as TNFα, LTα, or αFas, showed an increase of annexin-V staining levels more than those treated with ActD alone. Propidium iodide staining also yielded similar results to those seen with annexin-V staining (Fig. 3). These results demonstrate that LIGHT does not induce apoptosis in human primary hepatocytes even in the presence of ActD, whereas the other TNF family ligands induce apoptosis in hepatocytes pretreated with ActD. This indicates a functional difference between LIGHT and the other TNF family ligands.

LIGHT Pretreatment Prevents Human Primary Hepatocytes from Undergoing ActD/TNFα-induced Apoptosis, but Neither ActD/αFas- Nor ActD/TRAIL-induced Apoptosis—Recently, TNFα has been reported to induce expression of not only the death factor(s) but also a putative survival factor(s) via NF-κB activation. Both LTβR and HVEM are linked to an NF-κB activation pathway. To investigate whether LIGHT has an inhibitory effect on the hepatocyte apoptosis induced by other TNF family ligands, we examined the pretreatment effect of LIGHT on ActD/TNFα−, ActD/αFas−, or ActD/TRAIL-induced apoptosis. The LDH release from hepatocytes was dose
dependently increased by the addition of TNFα, TRAIL, or αFas in the presence of ActD. However, the LDH released 18 h after ActD/TNFα administration was effectively blocked by LIGHT pretreatment (100 ng/ml) 7 h prior to ActD treatment. This indicates that LIGHT allowed the hepatocytes to avoid the apoptosis induced by ActD/TNFα administration. However, this pretreatment did not prevent the ActD/αFas- or ActD/TRAIL-induced hepatocyte apoptosis (Fig. 4). Therefore, LIGHT has an anti-apoptotic effect on ActD/TNFα-treated hepatocytes, but not in ActD/αFas- or ActD/TRAIL-treated cells.

We next examined the time point at which LIGHT administration has an inhibitory effect on ActD/TNFα-induced apoptosis. As shown in Fig. 5, LIGHT pretreatment 1 h prior to ActD administration did not decrease the LDH release as compared with control. However, more than 3 h prior to ActD administration, the LDH levels were shown to decrease in hepatocytes treated with LIGHT. Therefore, pretreatment with LIGHT is required at least 3 h prior to ActD administration for an adequate anti-apoptotic effect.

LIGHT Has No Anti-apoptotic Effect on HepG2 Treated with ActD/TNFα—To investigate whether LIGHT has the same anti-apoptotic effect on tumor cells, the human hepatoma HepG2 was examined using the above assay system (Fig. 6). HepG2 treated with ActD/TNFα caused a dose-dependent increase in LDH release as in the hepatocytes. However, LIGHT pretreatment 6 h prior to ActD administration did not reduce the LDH release as compared with the control. Similar results were also obtained with HeLa cells (data not shown). Therefore, LIGHT pretreatment failed to prevent ActD/TNFα-induced apoptosis of several human tumor cells.

LIGHT Induces NF-κB Activation in Human Primary Hepatocytes—Our previous study demonstrated that LIGHT induces NF-κB activation of the human rhabdomyosarcoma cell line RD through either LTβR or HVEM signal transduction (35). We investigated the effect of LIGHT on NF-κB activation in human primary hepatocytes using a NF-κB-mediated SEAP reporter expression system (Clontech). SEAP activity in the hepatocyte culture supernatants was increased within 8 h after exposure to LIGHT as shown in Fig. 7. This increase continued for 24 h (data not shown). TNFα resulted in a more rapid induction of SEAP levels than did LIGHT, but αFas resulted in no induction. These results clearly indicate that LIGHT can induce NF-κB in human primary hepatocytes like TNFα.

LIGHT Inhibits Both Caspase-3 and Caspase-8 Activation in ActD/TNFα- but Not ActD/αFas-treated Hepatocytes—Several caspases play important roles in TNFα- or FasL-mediated apoptosis. To evaluate the anti-apoptotic effect of LIGHT on hepatocytes, Western blot analysis was performed to investigate the processing of caspase-3 using cell lysates from ActD/TNFα-treated hepatocytes pretreated with or without LIGHT. 4 h after ActD/TNFα incubation, the cleavage product of caspase-3, p20, was clearly detected, indicating that caspase-3 activation had occurred by that time (Fig. 8). Furthermore, 14 h after ActD/TNFα incubation, both the decrease in the pro-caspase-3 level and the appearance of another cleavage product, p17, along with p20, were observed in the ActD/TNFα-treated cells. The processed product p20 did not increase in the cell extract at 14 h when compared with that at 4 h. Because both processed forms were further processed in their own pro-

Fig. 3. TNFα, LTα, and αFas, but not LIGHT, induce apoptosis in ActD-sensitized hepatocytes. Human primary hepatocytes, treated as described in the legend to Fig. 2, were stained with FITC-conjugated annexin-V or propidium iodide, and were analyzed by FACS analysis.

Fig. 4. LIGHT protects hepatocytes from ActD/TNFα-mediated cell death, but not from that caused by ActD/αFas or ActD/TRAIL. Human primary hepatocytes were pretreated with or without 100 ng/ml LIGHT at 7 h before ActD administration combined with various concentrations of TNFα, αFas, or TRAIL, and were further treated as described in the legend to Fig. 2. Data are expressed as mean ± S.E., n = 3.
tolytic pathway, they might be highly unstable. In the presence of LIGHT, however, more procaspase-3 remained as compared with the unpretreated control, and p20 was absent 4 h after ActD/TNF/H9251 incubation (Fig. 8). 14 h after ActD/TNF/H9251 treatment, both cleavage products of caspase-3 were observed as was the case in the unpretreatment control even in the presence of LIGHT. On the other hand, in the ActD/Fas-treated cells, there was much less caspase-3 processing than in ActD/TNF/H9251-treated cells at 4 and 14 h. There was no difference in the amount of the cleavage products observed in both reactions with and without LIGHT (Fig. 8). When we examined caspase-8 processing by Western blot analysis, we were able to detect clearly neither the decrease of procaspase-8 nor its cleavage products because of the nonspecific binding of anti-caspase-8 antibodies (data not shown). Therefore, to ensure the inhibitory effect of LIGHT on caspase-8 activation, we determined the caspase-8 protease activity in ActD/TNFα-treated hepatocytes with or without LIGHT preadministration (Fig. 9). 4 h after TNFα apoptotic induction, a 5-fold increase in caspase-8 activity was observed in ActD/TNFα-treated cells as compared with that in untreated cells. In contrast, the increase in caspase-8 activity was strongly inhibited in ActD/TNFα-treated hepatocytes when pretreated with LIGHT. The specificity of the caspase-8 activity was confirmed by the disappearance of proteolytic activity caused by a caspase-8-specific
inhibitor, IETD-fmk. LIGHT preadministration also inhibited caspase-3 activation, which was consistent with the result of Western blot analysis. These findings indicate that LIGHT effectively inhibits ActD/TNFα/H9251-mediated caspase-3 and -8 activation, but not /H9251Fas-mediated caspase activation.

**DISCUSSION**

LIGHT belongs to the TNF ligand superfamily and is known to have several biological effects. These include: the apoptosis induction in certain tumor cell lines (32–34), the growth arrest on RD cells (35), and the co-stimulatory function of CD8-positive T cells (36), in a similar manner to those reported for TNFα and LTα. Recent studies of LIGHT transgenic mice have revealed that constitutive expression of LIGHT causes loss of tolerance to autologous tissues leading to autoimmune syndromes (37, 38). Whereas there are several studies regarding the biological activities of LIGHT on the immune system and against tumors, little is known about its effects on normal hepatocytes. TNFα has distinct biological effects on hepatocytes by causing either cellular proliferation during liver regeneration (39–42) or apoptosis in ActD-sensitized cells (47). The biological effect of TNFα on hepatocytes is dependent on the conditions of the hepatocytes. In this paper we have shown that LIGHT failed to induce apoptosis in ActD-sensitized hepatocytes, whereas TNFα, LTα, and αFas effectively induced the apoptosis, although both LTβR and HVEM proteins, specific receptors of LIGHT, were expressed on the hepatocytes (Fig. 1), the receptors examined were expressed on the primary hepatocytes, although each expression level was significantly different from that of the others. At 6 h of incubation with LIGHT, the expression of TNFRp55, TNFRp75, and Fas was unchanged, although there was an increase in HVEM expression. Because the amount of TNFRp55 on hepatocytes was not downregulated by LIGHT, we confirmed that the protective effect of LIGHT on ActD/TNFα-induced apoptosis was not because of the lack of available TNFRp55.

**FIG. 8.** LIGHT attenuates TNFα-mediated caspase-3 processing, but not that by αFas. Cell extracts from untreated, ActD/TNFα-, or ActD/αFas-treated hepatocytes pretreated with or without LIGHT were obtained at 4 and 14 h after ActD administration, and were determined by Western blotting with anti-caspase-3 antibodies. The presence of pro-caspase-3, p20, and p17 are indicated by arrows.

**FIG. 9.** LIGHT inhibits TNFα-mediated activation of caspase-3 and caspase-8. Caspase-3-like and caspase-8 enzyme activities in cell extracts were determined at 4 h after ActD administration, and each activity was shown as the -fold increase relative to the control cell activity. Results are from two independent experiments.

**FIG. 10.** LIGHT does not alter the expression levels of TNFRp55, TNFRp75, or Fas on human primary hepatocytes. Human primary hepatocytes incubated with or without 100 ng/ml LIGHT for 6 h were stained with antibodies against each antigen, following FITC-conjugated secondary antibodies. Each receptor expression level was determined by FACS analysis. Cells treated solely with secondary antibody were used as an unstained negative control. Untreated controls received no LIGHT treatment.
and LIGHT induced NF-κB activation in the hepatocytes (Fig. 7). We also revealed that LIGHT mRNA was predominantly expressed in both human adult and embryo liver, it functioned as a sort of priming factor for the proliferation of hepatocytes, and it stimulated the production of several chemokines in the hepatocytes, including LARC (data not shown). Thus, our results clearly demonstrate that LIGHT can stimulate some specific signal transductions through LTβR and/or HVEM in human primary hepatocytes, although it is not able to induce apoptosis of the hepatocytes even in the presence of ActD. TNFRp55, an essential receptor for TNFα signaling, stimulates TRAF-dependent NF-κB activation while it also induces apoptotic signals leading to activation of the caspase-proteolytic cascade via its downstream signal molecule TRADD (49). Both cytoplasmic domains of LTβR and HVEM proteins are known to associate some TRAF family proteins, and stimulate NF-κB activation (18, 19, 28–30). Therefore, we had speculated that the TRAF-mediated signaling of both LTβR and HVEM may be similar to that of TNFRp55, because the apoptosis induced by TNFα in human primary hepatocytes requires transcriptional inhibition by RNA synthesis inhibitors like ActD, and NF-κB activation is critical for the induction of resistance to TNFα cytotoxicity (49–52).

In the present study we attempted to determine whether LTβR and HVEM possess a so-called death domain, and we found that LIGHT prevented ActD-sensitized hepatocytes from TNFα-mediated apoptosis, but that it was not effective against αFas- and TRAIL-mediated apoptosis (Fig. 4). For an adequate anti-apoptotic effect, the pretreatment with LIGHT was required at least 3 h prior to ActD sensitization (Fig. 5), indicating that the time period for expressing or activating an anti-apoptotic factor(s) may be essential for the maximal effect. Because TNFα alone is not able to induce cell death in hepatocytes, it may stimulate both a death signal(s) and a survival pathway(s) simultaneously in the cells. There are some differences between the survival signal of LIGHT and that of TNFα with regard to NF-κB activation. In the present study, NF-κB activation mediated by LIGHT in hepatocytes was found to be less than that in TNFα-treated cells (Fig. 7). Recently Matushima et al. (53) have reported that NF-κB-inducing kinase and inhibitor of κB kinase-α (IKKα) are essential for the induction of NF-κB through LTβR, but not TNFRp55. It suggests that LTβR and TNFRp55 stimulate NF-κB activation through each distinct signal transduction pathway. Alternatively, the requirement of the pretreatment with LIGHT for the anti-apoptotic effect may be simply because of the transcriptional inhibition by ActD. In any case, it remains to be determined whether the anti-apoptotic signals through LTβR are the same as those through TNFRp55. This question may be answered by the observation of how the hepatocytes, which have the mutant TNFRp55 lacking the ability to associate TRAF proteins, respond to TNFα and LIGHT administration in the presence of ActD. It was found that both LTβR and HVEM are constitutively expressed in hepatocytes (Fig. 1). HVEM is known to associate with TRAF1, -2, and -5, but not with TRAF6 (18, 19), whereas LTβR associates with TRAF2, -3, and -5 (28–30). In nonhepatic cells, TRAF2 and -5 were demonstrated to play a key role in modulating NF-κB activation. In normal hepatocytes, however, we still have not identified the dominant receptor required for LIGHT-mediated anti-apoptotic effects and its downstream TRAF molecules. Further studies are needed to address these issues.

It is well known that TNFα or αFas induce apoptosis by mediating the proteolytic functions of caspases. We performed Western blot analysis to investigate caspase-3 processing during ActD/TNFα- or ActD/αFas-induced apoptosis, with or without LIGHT pretreatment (Fig. 8). 4 h after TNFα administration, one of the processed forms of caspase-3, p20, was detected in the extract from ActD/TNFα-treated cells, but not from the LIGHT-pretreated cells. 14 h after the induction, however, we observed two processed forms of caspase-3, p20 and p17, and the amount of pro-caspase-3 was dramatically decreased in the ActD/TNFα-treated cells, whereas there was a much smaller decrease in the amount of procaspase-3 in the LIGHT-pretreated cells. These findings indicate that caspase-3 activation is inhibited by LIGHT pretreatment during hepatocellular apoptosis, and that this protective activity becomes effective at a minimum of 4 h but weaker at 14 h after the induction of apoptosis. Such a short term effect of LIGHT may result from a decrease or inactivation of LIGHT-induced anti-apoptotic factor(s) by protein synthesis inhibition caused by ActD-mediated transcriptional inhibition. The increase in caspase-3-like activity induced by ActD/TNFα treatment was also significantly blocked by LIGHT (Fig. 9). Furthermore, whereas the caspase-8 processing was not detected by our experiments (data not shown), an increase in caspase-8 activity was significantly blocked by LIGHT, as shown in Fig. 9. Although LIGHT did not rescue hepatocytes from ActD/αFas- or TRAIL-induced apoptosis (Fig. 4), caspase-3 was found to be processed even in ActD/αFas-mediated apoptosis, whereas its processing occurred later and was weaker than that in ActD/TNFα-mediated apoptosis (Fig. 8). Recently, Kunstle et al. (54) have reported that caspase-3 inhibitors can prevent hepatocytes from apoptosis in a galactosamine (GalN)/LPS-mediated liver injury model, but not in a concanavalin A-mediated model. Because GalN was used as a transcriptional inhibitor for hepatocytes in the same manner of ActD, and concanavalin A is known to induce FasL in several types of cells including T cells, it may be that the cell death in GalN/LPS-treated mice is similar to that seen in the ActD/TNFα-mediated process and that apoptosis in concanavalin A-treated mice is caused by FasL. In a concanavalin A-mediated model, caspase-3 activation was not shown to be a critical step for hepatocellular cell death, even though caspase-3 is an important mediator of Fas in lymphocyte apoptosis (55). Our data are consistent with the above findings. Furthermore, Nagaki et al. (56) have reported that TNFα prevents hepatocyte apoptosis in GalN/TNFα-treated mice, but not in αFas-treated mice. They have also shown that TNFα-mediated caspase-8 activation was resistant to TNFα pretreatment. Their data are consistent with our results except in the case of caspase-8 activation (Fig. 9). It is not clear whether this disagreement is the result of differences between our in vitro and in vivo models or whether it reflects differences in the survival signal(s) associated with LIGHT and TNFα. We examined the effect of LIGHT on the expression of receptors for TNFα and FasL using FACS to determine another possible target for anti-apoptotic signals of LIGHT (Fig. 10). The expression of TNFRp55, TNFRp75, and Fas, however, did not change in the hepatocytes during LIGHT treatment. It may be that the anti-apoptotic effect of LIGHT is not because of down-regulation of available TNFRp55.

LIGHT can induce apoptosis in cell lines such as Hep3B2T, MDA-MB-231, WiDr, and HT-29 in the presence of IFN-γ (31–33, 57). These cells express both LTβR and HVEM as do the primary hepatocytes. LTβR affects apoptosis in HT-29 cells by interacting with TRAF3 (30), and HVEM binds TRAF2 and -5, which do not induce apoptosis but activate NF-κB and JNK/AP-1 pathways (18, 19). DNA synthesis in the primary hepatocytes by LIGHT plus IFN-γ administration was determined by a bromodeoxyuridine incorporation assay to investigate the effect of LIGHT on the IFN-γ-sensitized hepatocytes. Whereas DNA synthesis was shown to be inhibited by IFN-γ, LIGHT did
not induce apoptosis of the IFN-γ-sensitized hepatocytes (data not shown). The reason why the primary hepatocytes are resistant to IFN-γ/LIGHT-mediated apoptosis is unknown. It may be that LIGHT induces several distinct signal pathways depending on the condition of the cells. The present study is the first to show that cellular signals, using TNF family receptors lacking a death domain, can exert protective effects against the apoptotic signals from receptors possessing a death domain. Further studies are needed to identify a putative anti-apoptotic factor(s) activated and/or induced by LIGHT in hepatocytes. Studies using a GaIN/LPS-induced liver injury model are now under way to investigate the possibility of LIGHT for clinical trials.

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