# Studies on Physiological Functions of the Tumor Necrosis Factor (TNF) Superfamily of Pro-inflammatory Cytokine

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# Abstract

Tumor necrosis factor (TNF) superfamily has a variety of members which have own physiological roles in many types of mammalian cells. Considerable research on TNFα has revealed that it has many physiological functions in many types of cells in addition to induction of cell death, and it induces two distinct signaling pathways, NFκB activation and cell death via caspase activation. However, the relationship of TNF superfamily in mechanisms for the determination of the cell death, and their pathophysiological roles for non-inflammatory diseases had remained unknown.

To elucidate the effect of TNF superfamily on cell death, I examined apoptotic activity of TNFα, homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and anti-Fas antibodies (αFas), on behalf of Fas ligands, in actinomycin D (ActD)-sensitized hepatocytes. 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. Moreover, LIGHT effectively suppressed the apoptosis mediated by ActD/TNFα but neither ActD/αFas nor ActD/TRAIL, via NFκB activation; thus,

suggesting that some TNF superfamily ligands can negatively regulate TNFα-mediated apoptosis.

To elucidate the involvement of TNFα in non-inflammatory diseases, ICAM-1 expression, which is expected to serve as a surrogate marker for TNFα expression, was examined in Wistar fatty rats (model of diabetic nephropathy). The expression increased in the glomeruli of kidney during the progression of the nephropathy; thus, suggesting that the pro-inflammatory cytokine TNFα has the potential to play pathophysiological roles in non-inflammatory diseases such as diabetic nephropathy. These results obtained from my studies indicate a part of a wide variety of physiological implications for TNF superfamily.

# Abbreviations

| | |
|---|---|
| ActD | actinomycin D |
| αFas | anti-Fas antibody |
| BSA | bovine serum albumin |
| FACS | fluorescence-activated cell sorting |
| FasL | Fas ligand |
| FITC | fuorescein isothiocyanate |
| GalN | D-galactosamine |
| HVEM | herpesvirus entry mediator |
| ICAM-1 | intercellular adhesion molecule-1 |
| IFNγ | interferon γ |
| IL-1 | interleukin-1 |
| LARC | liver activation regulated chemokine |
| LDH | lactate dehydrogenase |
| LFA-1 | lymphocyte function-associated antigen-1 |
| LIGHT | homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes |
| LT | lymphotoxin |

| | |
|---|---|
| LTβR | lymphotoxin β receptor |
| mAb | monoclonal antibody |
| NCS | newborn calf serum |
| NFκB | nuclear factor-κB |
| PAM | periodic acid-methenamine-silver |
| PAS | periodic acid-schiff |
| PBS | phosphate buffered saline |
| RANTES | regulated on activation normal T cell expressed and secreted |
| TNF | tumor necrosis factor |
| TNFR | TNF receptor |
| TRAF | TNF receptor-associated factor |
| TRAIL | tumor necrosis factor-related apoptosis-inducing ligand |

# General Introduction

Tumor necrosis factor α (TNFα), which is known as a pro-inflammatory cytokine, has many types of physiological functions, in addition to its role in the induction of inflammatory reactions and apoptosis in some types of tumor cells (Eigler et al., 1997). TNF and its receptor each belong to a superfamily composed of 19 and 25 members, respectively, based on sequence homology to TNFα and the receptor (Wallach et al., 2002; Aggarwal et al., 2012).

One of the main features of the TNF receptor family is the existence of a death domain (DD) that plays an important role in the induction of apoptosis. TNF receptor TNFRp55 possesses a DD, whereas some of the other receptors lack the DD (Hehlgans et al., 2002; Fig. 1A). Studies have identified two distinct signaling pathways that are stimulated through typical TNF receptors bound to TNFα; one is a caspase pathway triggered by caspase-8 activation via interaction with DD, TRADD, FADD and the caspase, another is an NFκB activation pathway mediated by TNF receptor-associated factors (TRAFs), which are coupled to many types of TNF receptors. Caspase activation is an especially important event for TNFα-mediated apoptosis. Moreover, substantial research had been conducted to thoroughly understand TNFα signaling before my study. However, the relationship of TNF superfamily in the pathway through TNFα signals apoptosis

remained unknown. For instance, several types of cells are resistant to TNFα-induced apoptosis even though TNFRp55 is expressed in cells where caspase activation is expected to be induced. One of reasons for the resistance could be based on NFκB activation simultaneously induced by TNFα. In addition, the complexity of TNFα signaling led me to develop an interest in physiological significance of TNFα and the onset of diseases. I performed two studies to elucidate not only the implication of TNF superfamily in TNFα receptor signaling through the apoptosis and its physiological implications, but also other physiological functions in addition to apoptosis and inflammatory reactions.

Simultaneous activation of NFκB and apoptosis by the TNFα pathway was thought to be the mechanism by which TNFα failed to induce apoptosis in several normal cells. As already described, some TNF receptor superfamily members lack the DD, which is essential for TNFα-mediated apoptosis; however, they retain the ability to activate NFκB. Thus, treating cells with ligands to these receptors leads only to the activation of NFκB and not the induction of apoptosis. Among categorized receptors, lymphotoxin β receptor (LTβR) and the herpesvirus entry mediator (HVEM) are reported to interact with a member of the TNF family. This ligand, known as LIGHT, is homologous to the lymphotoxins shows inducible

expression and competes with herpes simplex virus glycoprotein D for HVEM a receptor expressed by T lymphocytes. LIGHT is expected to cause the activation of NFκB but not caspase processing in treated cells. These previous findings raised the hypothesis that LIGHT may suppress TNFα-dependent apoptosis via NFκB activation (Fig. 1B).

In part 1, I investigated this hypothesis by establishing an actinomycin D (ActD)- and TNFα-mediated apoptosis assay system using human normal hepatocytes and then evaluated the effect of LIGHT on apoptosis. Pro-apoptotic ligands like TNFα and antibodies for Fas antigens, which bind receptors bearing DD, effectively induced apoptosis in ActD-treated cells, whereas LIGHT failed to cause apoptosis in these cells. Surprisingly, pretreatment with LIGHT rescued ActD- and TNFα-treated cells from apoptosis, although the treatment could not prevent apoptosis induced by anti-Fas antibodies and TRAIL.

The complexity of TNFα signaling, described in part 1, may enable TNFα to play a role in a wide variety of physiological functions and non-inflammatory diseases. To address the effect of TNFα on non-inflammatory diseases, sophisticated systems for the detection of TNFα gene expression in tissues as well as plasma samples were required. At the time when I tried to conduct my study, it

was difficult to easily detect cytokines such as TNFα in specific tissues. Only northern blotting, which detects transcripts of target genes was available for use in the targeting of specific tissues. It was easier to detect these molecules in liquid samples such as plasma and urine. Therefore, I needed a breakthrough idea to tackle this issue.

It was reported that TNFα induces the expression of adhesion molecules on endothelial cells (Mulligan et al., 1993), which causes migration of white blood cells into the cytokine-injected tissues (Munro et al., 1989). This suggests that adhesion molecules play important roles in the infiltration of inflammatory cells into regions of inflammation and their accumulation in these regions. I finally came up with an idea that ICAM-1 expression on cells of reporter tissues could serve as a surrogate marker for TNFα expression in targeted tissues. In parallel with insight, it was reported that TNFα induced the expression of ICAM-1 in the mesangial cells of kidney (Brennan et al., 1990; Sterzel et al., 1993).

In part 2, to elucidate the implications of TNFα and ICAM-1 in the nephropathy resulting from non-insulin dependent diabetes mellitus, which was thought to be categorized as a non-inflammatory disease, ICAM-1 expression was examined in the kidneys of a new genetically obese-hyperglycemic rat (Wistar

Fatty), a model that reflects the pathological characteristics of non-insulin-dependent diabetic nephropathy. Immunohistochemical analysis using anti-ICAM-1 antibodies revealed that ICAM-1 expression increased in the glomeruli of Wistar fatty rats during the progression of nephropathy, although no infiltration of immune cells was observed. To evaluate whether ICAM-1 caused the nephropathy in Wistar fatty rats, the effect of blocking ICAM-1 by chronic administration of anti-ICAM-1 antibodies on the urinary excretion of albumin by Wistar fatty rats was examined. However, the treatment failed to reduce the urinary excretion of albumin. These findings suggest that ICAM-1 expression could be a surrogate marker for an increase in TNFα expression in the kidney during diabetic nephropathy, although ICAM-1 is unlikely to act as a causal factor for the pathogenesis of Wistar fatty.

## A Figure

**Figure 1. Conceptual diagram.**

A, Relationship between TNFα ligands and their receptors applied in my studies

Schematic illustration showed the known interactions between members of TNF ligand family and members of TNF receptor family, and adaptor proteins and signaling pathways associated with those receptors, identified before my studies.

B, My hypothesis about TNFα-related signaling on anti-apoptosis

NFκB activation by TNF receptor family was assumed to suppress TNFα-mediated caspase activation. To investigate the effects of NFκB activation on caspase activation, LIGHT mediated NFκB activation was expected to inhibit the apoptosis under the condition that TNF signaling stimulates only caspase pathway in the presence of ActD.

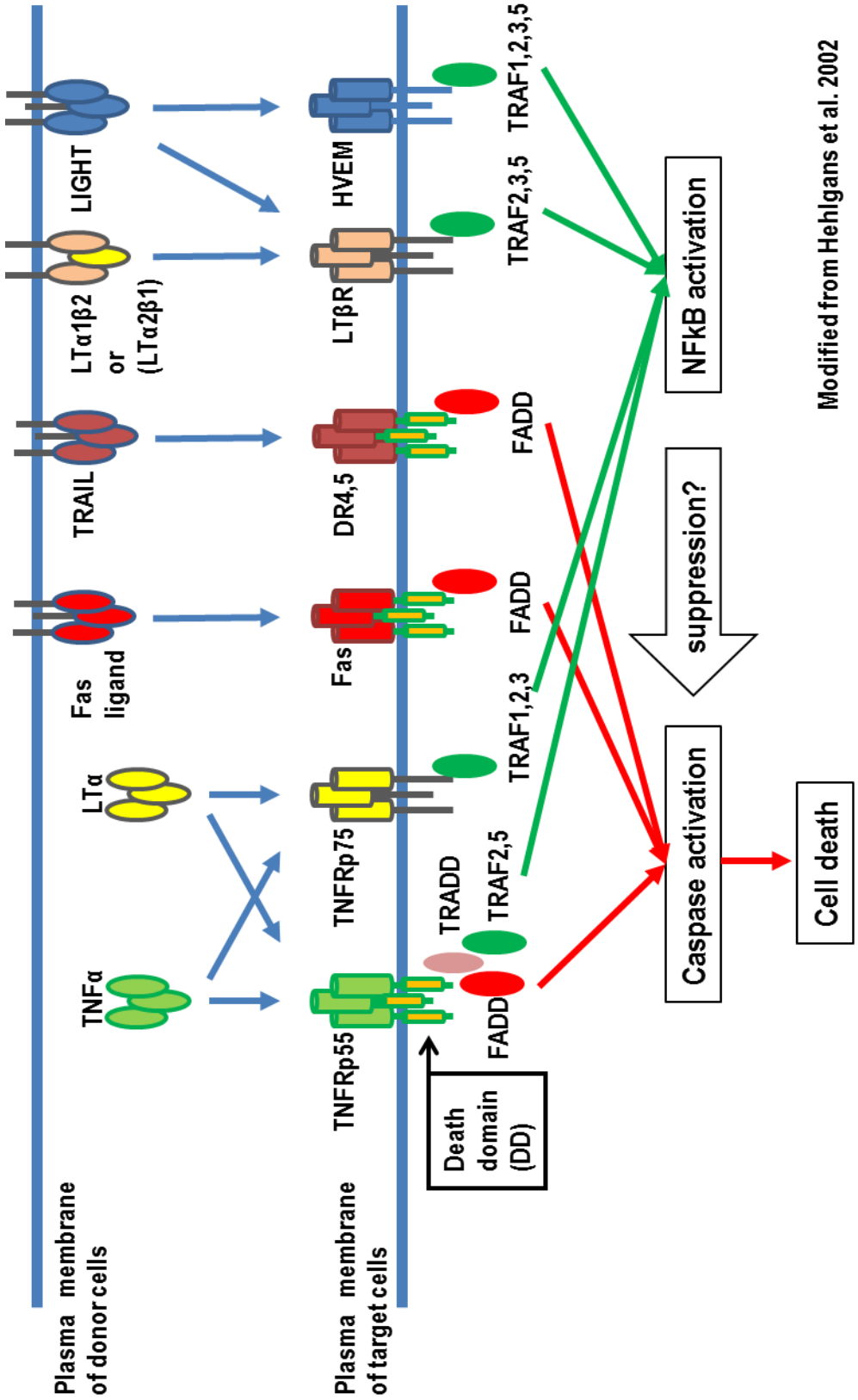
Plasma membrane of donor cells
Plasma membrane of target cells
TNFα
LTα
Fas ligand
TRAIL
LTα1β2 or (LTα2β1)
LIGHT
TNFRp55
TNFRp75
Fas
DR4,5
LTβR
HVEM
TRADD
FADD
TRAF2,5
TRAF1,2,3
FADD
FADD
TRAF2,3,5
TRAF1,2,3,5
Death domain (DD)
Caspase activation
Cell death
suppression?
NFkB activation
Modified from Hehlgans et al. 2002

Figure 1A

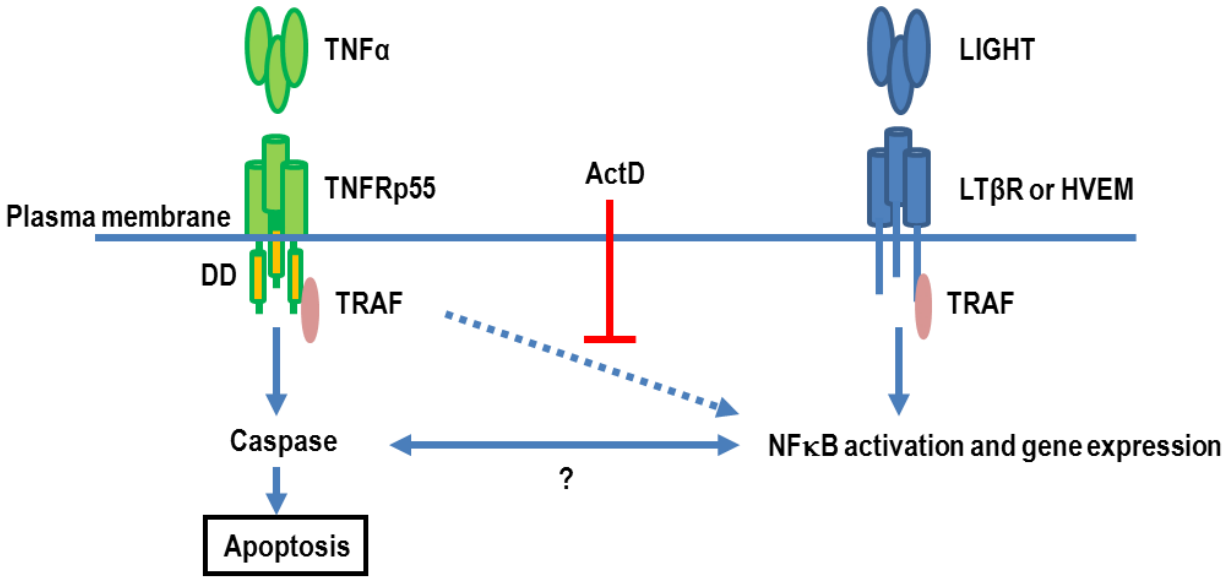
TNFα
LIGHT
TNFRp55
ActD
LTβR or HVEM
Plasma membrane
DD
TRAF
TRAF
Caspase
NFκB activation and gene expression
?
Apoptosis

Figure 1B

# Part 1

# 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

## Abstract

LIGHT is a member of tumor necrosis factor (TNF) superfamily. Its receptors have been identified as lymphotoxin 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. This protective effect requires LIGHT pretreatment at least 3 h prior to ActD sensitization. LIGHT stimulates nuclear factor-κB (NFκB)-dependent transcriptional activity in human hepatocytes such as TNFα. The time course of NFκB activation after LIGHT administration is similar to that of the pretreatment required for the anti-apoptotic effect of LIGHT. LIGHT inhibits caspase-3 processing during the apoptotic protease cascade in TNFα-mediated apoptosis but not Fas-mediated apoptosis. These results indicate that LIGHT may act as an anti-apoptotic agent against TNFα-mediated liver injury by blocking the activation of caspase-3.

## Introduction

Members of the TNF ligand superfamily play multiple roles in the development of the immune system and in osteogenesis. They also mediate the effective function of the innate and adaptive immune responses (Smith et al., 1994). These proteins include TNFα, lymphotoxin α (LTα), lymphotoxin β (LTβ), Fas ligand (FasL), CD27L, CD30L, CD40L, 4-1BB (Gruss et al., 1995), TRAIL (Wiley et al., 1995), RANKL/OPGL (Anderson et al., 1997; Lacey et al., 1998; Wong et al., 1997; Yasuda et al., 1998), TWEAK (Chicheportiche et al., 1997), APRIL/TALL-2 (Hahne et al., 1998; Shu et al., 1999), AITRL (Kwon et al., 1999), VEG1 (Zhai et al., 1999), and BAFF/TALL-1 (Shu et al., 1999; Schneider et al., 1999). Recently, LIGHT, which is homologous to the lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM. HVEM is a receptor expressed by T lymphocytes that has been identified as a member of the TNF ligand superfamily and shown to be about 30% identical to FasL and LT β (Mauri et al., 1998). Further studies have revealed that LIGHT can bind to lymphotoxin β receptor (LTβR) and to herpesvirus entry mediator

(HVEM)/ATAR/TR2 (Mauri et al., 1998; Montgomery et al., 1996; Kwon et al., 1997; Marsters et al., 1997; Hsu et al., 1997). TNFα, LTα, LTβ, and LIGHT exhibit distinct and overlapping patterns in the binding of four cognate receptors. TNFα and the homotrimer of LTα bind two receptors, TNFRp55 and TNFRp75 (Smith et al., 1994); and LTβ forms heterotrimers with LTα, which can bind LTβR. The complexity of receptor/ligand engagement suggests a functional redundancy of these cytokines. However, disruptions of these genes in mice indicate that the TNF and LT systems play important roles in the development of the immune system (Matsumoto et al., 1996; Neumann et al., 1997; Fu et al., 1997; Koni et al., 1997; Alimzhanov et al., 1997; Futterer et al., 1998). 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 (Degli-Esposti et al., 1997; Browning et al., 1996). Members of the TNF receptor family like LTβR and HVEM are associated with TNF receptor associated factors (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 regulators, including NFκB (Marsters et al., 1997; Hsu et al., 1997; Nakano et al., 1996; Mackay et al., 1996; VanArsdale et al., 1997), although it is reported that splice variants of TRAF3 are capable of inducing NFκB activation (van Eyndhoven et al., 1999).

Because LIGHT engages LTβR and HVEM as the cellular receptors, it is expected to have physiological functions similar to those of LTα and LTβ. As previous studies have shown, LIGHT and LTα induce cell death in HT-29 cells (Harrop et al., 1998; Zhai et al., 1998; Rooney et al., 2000). LIGHT also causes growth arrest in RD cells following the developmental changes to smooth muscles cells. Furthermore, LIGHT stimulates cellular secretion of interleukin-8 and RANTES (regulated on activation normal T cell expressed and secreted) (Hikichi et al., 2001). It has also been reported that LIGHT is one of the CD28-independent costimulatory molecules in T cells (Tamada et al., 2000). Recent studies of transgenic mice expressing recombinant LIGHT and of mice administered soluble HVEM proteins to block LIGHT activity have revealed that LIGHT is required for the expansion of T cells, and it plays an important role in T cell homeostasis (Wang et al., 2001; Shaikh et al., 2001).

TNFα plays an important role in the proliferation of hepatocytes *in vitro* and *in vivo* (Watanabe et al., 1997; Kirillova et al., 1999; Yamada et al., 1997; Webber et al., 1998) and acts as a mediator of cell death in several liver injury models (Iimuro et al., 1997; Pfeffer et al., 1993; Leist et al., 1995; Gantner et al., 1995). Hepatocytes are normally resistant to TNFα-mediated cytotoxicity. They require sensitization with transcription inhibitors such as ActD for maximal TNFα cytotoxicity (Leist et al., 1994). This suggests that TNFRp55, an essential receptor for TNFα signaling, stimulates two distinct pathways: one signal mediated by NFκB that maintains the survival of cells, and another with activation of a series of effectors 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 NFκB in nonhepatic cells (Hikichi et al., 2001), it is expected to be an anti-apoptotic mediator.

In the present study, I investigated the effects of LIGHT on human primary hepatocytes and demonstrated that LIGHT blocks ActD/TNFα-induced hepatocyte apoptosis, but it does not suppress ActD/agonistic anti-Fas antigen monoclonal antibody (αFas)- or ActD/TRAIL-mediated apoptosis. In addition, I

propose that LIGHT stimulates NFκB transcriptional activity in the human primary hepatocytes and inhibits the apoptotic caspase cascade induced by TNFα.

## Materials and Methods

### Cells and reagents

Human primary hepatocytes were purchased from Cell Systems (Kirkland, USA). The cells were cultured in a basal medium composed of Ham's F-12 and Leivobitz L-15 (1:1) medium (Invitrogen, Carlsbad, USA), 0.2% (v/v) bovine serum albumin (Invitrogen), 5 mM glucose (Wako Pure Chemical Inc., Osaka, Japan), 10 nM dexamethasone (Wako), and 10 nM bovine insulin (Invitrogen) supplemented with 10% (v/v) fetal calf serum (JRH, Lenexa, USA). The cells had been expanded 10-fold, and were stored at －160°C before use. The soluble form of human LIGHT protein was produced as previously described (Hikichi et al., 2001). Recombinant human TNFα, LTα, and TRAIL proteins and anti-LTβR polyclonal antibody (AF629) were purchased from R&D System (Minneapolis, USA). Agonistic monoclonal antibody (mAb) against Fas antigen (αFas; CH-11) was purchased from MBL (Nagoya, Japan). Anti-TNFRp55 mAb (utr9) and anti-TNFRp75 mAb (utr1) were purchased from BMA (Augst, Switzerland). Anti-HVEM mAb was kindly provided by Dr. Truneh (Harrop et al., 1998). Rabbit

antiserum raised against caspase-3 and FITC conjugated anti-mouse IgG mAb were purchased from Pharmingen (San Diego, USA). Rabbit FITC-conjugated anti-goat IgG polyclonal antibody was purchased from Vector Laboratories, Inc. (Burlingame, USA). Goat antiserum against actin (I-19), goat horseradish peroxidase-conjugated anti-rabbit Ig, and donkey horseradish peroxidase-conjugated anti-goat Ig were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). 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 αFas 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 αFas. Apoptosis was determined using the Early Apoptosis Detection Kit (Kamiya Biochemical, Seattle, USA). Briefly, 14 h after each cytokine addition, the cells were trypsinized, washed with the basal medium, and pelleted by centrifugation. After being resuspended, the cells were stained in a suspension buffer containing annexin-V or propidium iodide for 20 min. They were then analyzed on FACScan using the CELLQuest program (Becton Dickinson, San Jose, USA) at an excitation of 488 nm.

## Reverse transcriptase-PCR analysis

Hepatocytes cultured in type I collagen-coated 75-$cm^2$ flasks were lysed with 0.6 ml of ISOGEN (NipponGene, Japan) by mixing vigorously. The lysates were then mixed with $CHCl_3$ 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, USA), and extracted using the RNeasy Kit (Qiagen, Chatsworth, USA). Reverse transcription was performed using the TaqMan reverse transcription reagent kit (PE Biosystems, Foster City, USA). A pair of primers (5-CCTTACACATACACACCCTTTGGAAGT-3 as a forward primer, and 5-AGCTCAATGCATGTACAGAATCCCCGGTTA-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 (Hikichi et al., 2001). PCR was performed using the Advantage GC-cDNA polymerase mix (Clontech, Palo Alto, USA). As a control, PCR was performed using RNA without a reverse transcription process.

**Western blot analysis for caspase-3 processing**

Hepatocytes were plated onto type I collagen-coated 225-$cm^2$ flasks. They were treated with or without LIGHT (100 ng/ml) for 6 h in the basal medium containing 1% NCS, followed by the induction of apoptosis as described above. The cells were harvested with a cell scraper 4 or 14 h after the induction, and stored at −80°C until protein extraction. The pellets were resuspended with a lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 100 μM 4-amidinophenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 50 μg/ml antipain), and then mixed well at 4°C. After centrifugation, the protein concentration of each supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad). Samples were subjected to 10-20% SDS-PAGE, and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Bedford, USA) in a transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, and 15% methanol). These membranes were blocked in BlockAce (Dainippon Seiyaku, Japan) overnight at 4°C. Rabbit anti-caspase-3 antibody was used as primary antibody at a 1:1000 dilution in 10% BlockAce for 30 min. They were then washed in phosphate-buffered saline containing 0.05% Tween 20 (Bio-Rad). Goat anti-rabbit Ig antibody was used as secondary antibody

at a 1:5000 dilution for 30 min. Chemiluminescence (Amersham Biosciences) was then used to visualize immunoreactive protein complexes. The exposure times for the detection of procaspase-3 and its processed forms were 1 and 5 min, respectively.

**NFκB transcription assay by a reporter gene system**

Hepatocytes were plated onto 24-well type I collagen-coated plates. They were then cultured in the basal medium containing 10% FCS overnight. NFκB transcriptional activity was determined using the Mercury Pathway Profiling SEAP system (Clontech). Briefly, hepatocytes were transfected with 0.5 μg of pNFκB-SEAP vector, pTAL-SEAP vector as a negative control, or pSEAP2-control vector as a positive control, using FuGENE6 reagent (Roche Molecular Biochemicals). After they were cultured for 1 day, the media were changed to the basal medium containing 1% NCS, with or without 100 ng/ml LIGHT, TNFα, or αFas, respectively. SEAP activity was determined using the Great EscAPe chemiluminescence detection kit (Clontech) according to the manufacturer's instructions. The data were expressed as the mean ± S.E. for four samples.

## Flow cytometry

The hepatocytes were harvested and stained with anti-HVEM or anti-LTβR, in phosphate-buffered saline containing 1% FCS and 0.05% $NaN_3$ for 30 min on ice. The cells were then washed with the above buffer, and the cells were exposed to FITC-conjugated anti-mouse Ig antibodies, and analyzed in a FACSvantage (BD Biosciences, Mountain View, USA). 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 physiological functions during *in vitro* cultivation. Consequently, the expression of CYP3A4, a hepatocyte marker belonging to the cytochrome P-450 family, was determined by reverse transcriptase-PCR in human primary hepatocytes in this experiment. As shown in Fig. 2A, the specific PCR product of the CYP3A4 gene was detected. Because LIGHT associates with two receptors, LTβR and HVEM, the expression of these receptors was also investigated in the hepatocytes. The specific PCR products of both receptor genes were detected by reverse transcriptase-PCR (Fig. 2A) and the expression of both receptor proteins on the cell surface was determined by FACS analysis using LTβR- and HVEM-specific antibodies (Fig. 2B and 2C). Therefore, the hepatocytes used in our experiments were confirmed to be physiologically normal and 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 in some types of cells. To investigate whether LIGHT has cytotoxic effects on human primary hepatocytes, I examined LDH activity in culture supernatants 18 h after administration of LIGHT, TNFα, LTα, and αFas. As shown in Fig. 3, the level of LDH activity did not increase in response to any of these cytokines at concentrations ranging from 0.3 to 100 ng/ml. Pretreatment with transcriptional inhibitors like ActD has been reported to be required for TNFα to cause apoptosis in cells such as murine hepatocytes (Beg et al., 1996; Wang et al., 1996; Van Antwerp et al., 1996; Matsushima et al., 2001). Therefore, the apoptotic effect of cytokines was examined in the presence of ActD. LDH activity increased when TNFα and 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. LTα treatment did not show an obvious increase in LDH levels. Furthermore, the viability of 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 and CD95, respectively. The lack of a death domain in both LTβR and HVEM may be a possible reason that LIGHT did not cause apoptosis in hepatocytes.

To investigate the type of cell death induced by these cytokines, hepatocytes were stained with annexin-V that recognizes phosphatidylserine on outer plasma membrane caused by apoptosis induction or propidium iodide that stains nuclear DNA in plasma membrane-damaged cells by apoptosis in the above conditions, and then they were analyzed by FACS. As shown in Fig. 4, the intensity of annexin-V and propidium iodide staining in ActD-treated cells was the same as that of autofluorescence suggesting that ActD could not cause apoptosis in human primary hepatocytes. The annexin-V staining patterns in hepatocytes treated with ActD alone were the same as those in hepatocytes treated with ActD plus LIGHT. In contrast, hepatocytes treated with ActD plus the other TNF family ligands such as TNFα, LTα, and αFas showed higher levels of annexin-V staining than those seen in hepatocytes treated with ActD alone. Propidium iodide staining also yielded similar results to those seen with annexin-V staining (Fig. 4). 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 does not prevent ActD/Fas- or ActD/TRAIL-induced apoptosis**

TNFα has recently been reported to induce expression of not only the death factor(s) but also a putative survival factor(s) via NFκB activation. Both LTβR and HVEM are linked to an NFκB activation pathway. To investigate whether LIGHT has an inhibitory effect on the hepatocyte apoptosis induced by other TNF family ligands, I examined effects of LIGHT pretreatment on ActD/TNFα-, ActD/αFas-, and ActD/TRAIL-induced apoptosis. LDH release from hepatocytes was increased in a dose-dependent manner by the addition of TNFα, TRAIL, and 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 hepatocyte apoptosis induced by ActD/αFas or ActD/TRAIL (Fig. 5). Therefore, LIGHT has an anti-apoptotic effect on ActD/TNFα-treated hepatocytes but not on ActD/αFas- or ActD/TRAIL-treated cells.

I subsequently examined the time point at which LIGHT administration has an inhibitory effect on ActD/TNFα-induced apoptosis. As shown in Fig. 6, LIGHT pretreatment 1 h prior to ActD administration did not decrease the release of LDH compared with that of the control. However, initiating LIGHT treatment > 3 h prior to ActD administration decreased the LDH levels in hepatocytes. Therefore, pretreatment with LIGHT was required at least 3 h prior to ActD administration for an adequate anti-apoptotic effect.

**LIGHT induces NFκB activation in human primary hepatocytes**

Our previous study demonstrated that LIGHT induces NFκB activation of the human rhabdomyosarcoma cell line RD through either LTβR or HVEM signal transduction (Hikichi et al., 2001). I investigated effects of LIGHT on NFκB activation in human primary hepatocytes using an NFκB-mediated SEAP reporter expression system (Clontech). SEAP activity in the hepatocyte culture

supernatants increased within 8 h of exposure to LIGHT, as shown in Fig. 7. This increase continued for 24 h (data not shown). TNFα treatment resulted in a more rapid induction of SEAP levels than treatment with LIGHT, but αFas treatment did not result in induction. These results clearly indicate that LIGHT induced NFκB in human primary hepatocytes like TNFα.

**LIGHT inhibits caspase-3 activation in ActD/TNFα-treated hepatocytes but not in ActD/αFas-treated hepatocytes**

Several caspases play important roles in TNFα- and FasL-mediated apoptosis. To evaluate anti-apoptotic effects of LIGHT on hepatocytes, caspase-3 processing was investigated using western blot analysis in cell lysates of ActD/TNFα-treated hepatocytes with or without LIGHT pretreatment. Four hours after the initiation of ActD/TNFα treatment, the cleavage product of caspase-3, p20, was clearly detected; thus, indicating that caspase-3 activation had occurred by that time (Fig. 8). Furthermore, 14 h after the initiation of ActD/TNFα treatment, a decrease in the procaspase-3 level and the appearance of another cleavage product, p17, along with p20, were observed in the ActD/TNFα-treated cells. The level of processed product, p20, observed in the cell

extract 14 h after the initiation of ActD/TNFα treatment was not greater than the level observed at 4 h. Because both processed forms were further processed in their own proteolytic pathway, they might be highly unstable. In the presence of LIGHT, however, more procaspase-3 remained compared with that of the non-pretreated control, and p20 was absent 4 h after the initiation of ActD/TNFα treatment (Fig. 8). Both cleavage products of caspase-3 were observed (as was the case in the non-pretreated control) even in the presence of LIGHT for 14 h after the initiation of ActD/TNFα treatment, whereas the amount of pro-caspase-3 observed in the sample that received pretreatment was much higher than the sample that did not receive pretreatment. On the other hand, there was considerably lower caspase-3 processing in the ActD/αFas-treated cells than in the ActD/TNFα-treated cells at 4 and 14 h. There was no difference in the amount of the cleavage products observed in the reactions with and without LIGHT pretreatment (Fig. 8). I could not clearly detect a decrease in either procaspase-8 or its cleavage products because of the nonspecific binding of the anticaspase-8 antibodies (data not shown). These findings indicate that LIGHT effectively inhibits ActD/TNFα-mediated caspase-3 activation but not Fas-mediated caspase activation.

## Discussion

LIGHT belongs to the TNF ligand superfamily and is known to have several physiological effects. These include the induction of apoptosis in certain tumor cell lines (Harrop et al., 1998; Zhai et al., 1998; Rooney et al., 2000), growth arrest in RD cells (Hikichi et al., 2001), and a costimulatory function of CD8-positive T cells (Tamada et al., 2000) in a manner similar 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; thus, leading to autoimmune syndromes (Wang et al., 2001; Shaikh et al., 2001). Although there are several studies regarding the physiological activities of LIGHT on the immune system and against tumors, little is known about its effects on normal hepatocytes.

TNFα has distinct physiological effects on hepatocytes. It causes either cellular proliferation during liver regeneration (Watanabe et al., 1997; Kirillova et al., 1999; Yamada et al., 1997; Webber et al., 1998) or apoptosis in ActD-sensitized cells (Leist et al., 1994). The physiological effect of TNFα on hepatocytes is

dependent on the condition of the hepatocytes. In this part, I have shown that LIGHT did not induce apoptosis in ActD-sensitized hepatocytes, whereas TNFα, LTα, and αFas effectively induced apoptosis. These results were obtained even though both LTβR and HVEM proteins, the specific receptors of LIGHT, were expressed on the hepatocytes (Fig. 2). In addition, LIGHT induced NFκB activation in the hepatocytes (Fig. 7). I also revealed that LIGHT mRNA was predominantly expressed in both human adult and embryonic 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 liver activation regulated chemokine (LARC) (data not shown). Thus, the 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 the apoptosis of hepatocytes even in the presence of ActD. TNFRp55, an essential receptor for TNFα signaling, stimulates TRAF dependent NFκB activation. It also induces apoptotic signals leading to activation of the caspase proteolytic cascade via its downstream signal molecule TRADD (Liu et al., 1996). Both cytoplasmic domains of LTβR and HVEM proteins are known to associate with some TRAF family proteins and stimulate NFκB activation

(Marsters et al., 1997; Hau et al., 1997; Nakano et al., 1996; Mackay et al., 1996; VanArsdale 1997). I 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. In addition, NFκB activation is critical for the induction of resistance to TNFα cytotoxicity (Liu et al., 1996; Beg et al., 1996; Wang et al., 1996; Van Antwerp et al., 1996).

In part 1 of the present study, I attempted to determine whether LTβR and HVEM possess a so-called death domain. I found that LIGHT prevented ActD-sensitized hepatocytes from TNFα-mediated apoptosis, but it was not effective against Fas- and TRAIL-mediated apoptosis (Fig. 5). For an adequate anti-apoptotic effect, pretreatment with LIGHT was required for at least 3 h prior to ActD sensitization (Fig. 6); thus, 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 simultaneously stimulate both a death signal(s) and a survival pathway(s) in the cells. There are some differences between the survival signal of LIGHT and of TNFα with regard to NFκB activation. In the present study, NFκB activation

mediated by LIGHT in hepatocytes was found to be less than that mediated by TNFα (Fig. 7). Matsushima et al. (2001) recently reported that NFκB-inducing kinase and inhibitor of κB kinase-α (IKKα), but not TNFRp55, are essential for the induction of NFκB through LTβR. This suggests that LTβR and TNFRp55 stimulate NFκB activation through distinct signal transduction pathways. Alternatively, the requirement for LIGHT pretreatment to observe the anti-apoptotic effect may be because of 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 through the observation of how hepatocytes that have the mutant TNFRp55 lacking the ability to associate with TRAF proteins respond to TNFα and LIGHT administration in the presence of ActD. It was shown that LTβR and HVEM are constitutively expressed in hepatocytes (Fig. 2). HVEM is known to associate with TRAF1, 2, and 5, but not with TRAF6 (Marsters et al., 1997; Hau et al., 1997), whereas LTβR associates with TRAF2, 3, and 5 (Nakano et al., 1996; Mackay et al., 1996; VanArsdale et al., 1997). TRAF2 and 5 have been shown to play a key role in modulating NFκB activation in non-hepatic cells. In normal hepatocytes, however, I still have not identified the dominant receptor required for

LIGHT-mediated anti-apoptotic effects or its downstream TRAF molecules. Further studies are needed to address these issues.

It is well known that TNFα or αFas induce apoptosis by activating the proteolytic functions of caspases. I investigated caspase-3 processing during ActD/TNFα- and ActD/αFas-induced apoptosis, with and without LIGHT pretreatment using western blot analysis (Fig. 8). One of the processed forms of caspase-3, p20, was detected in an extract four hours after TNFα administration from ActD/TNFα-treated cells but not in an extract from LIGHT-pretreated cells. Fourteen hours after the induction, however, I observed two processed forms of caspase-3, p20 and p17, and the amount of pro-caspase-3 dramatically decreased in the ActD/TNFα-treated cells. 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 this protective activity is effective at a minimum of 4 h but is 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. Although LIGHT did not rescue hepatocytes from

ActD/αFas- and ActD/TRAIL-induced apoptosis (Fig. 5), caspase-3 was found to be processed even in ActD/αFas-mediated apoptosis. The processing of caspase-3 occurred later and was weaker than that in ActD/TNFα-mediated apoptosis (Fig. 8). It has recently been reported that caspase-3 inhibitors can protect hepatocytes from apoptosis in a galactosamine (GalN)/LPS-mediated liver injury model but not in a concanavalin A-mediated model (Kunstle et al. 1999). Because GalN was used as a transcriptional inhibitor for hepatocytes in the same manner as 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. Caspase-3 activation was not shown to be a critical step for hepatocellular cell death in a concanavalin A-mediated model even though caspase-3 is an important mediator of Fas in lymphocyte apoptosis (Enari et al., 1996). Furthermore, it has been reported that TNFα prevents hepatocyte apoptosis in GalN/TNFα-treated mice but not in αFas-treated mice (Nagaki et al. 2000). These data are consistent with my results.

LIGHT can induce apoptosis in cell lines such as Hep3BT2, MDA-MB-231, WiDr, and HT-29 in the presence of IFNγ (van Eyndhoven et al., 1999; Harrop et

al., 1998; Zhai et al., 1998; Chen et al., 2000). These cells, including the primary hepatocytes, express LTβR and HVEM. LTβR affects apoptosis in HT-29 cells by interacting with TRAF3 (VanArsdale et al., 1997), HVEM binds TRAF2 and 5, which do not induce apoptosis but activate the NFκB and JNK/AP-1 pathways (Marsters et al., 1997; Hau et al., 1997). To investigate the effect of LIGHT on IFNγ-sensitized hepatocytes, DNA synthesis in primary hepatocytes caused by treatment with LIGHT plus IFNγ was assessed using a bromodeoxyuridine incorporation assay. Although DNA synthesis was shown to be inhibited by IFNγ, LIGHT did not induce apoptosis in IFNγ–sensitized hepatocytes (data not shown). The reason why these primary hepatocytes are resistant to IFNγ/LIGHT-mediated apoptosis is unknown. It may be that LIGHT induces several distinct signaling pathways depending on the condition of the cells. The present study is the first to show (using TNF family receptors lacking a death domain) that cellular signals can protect cells against apoptotic signals from receptors possessing a death domain. Further studies are needed to identify the anti-apoptotic factor(s) activated and/or induced by LIGHT in hepatocytes. Studies using a GalN/LPS-induced liver injury model should be conducted to investigate the suitability of LIGHT for clinical trials.

## Figures

**Figure 2. Characterization of the human primary hepatocytes in this experiment**

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 (- - -).

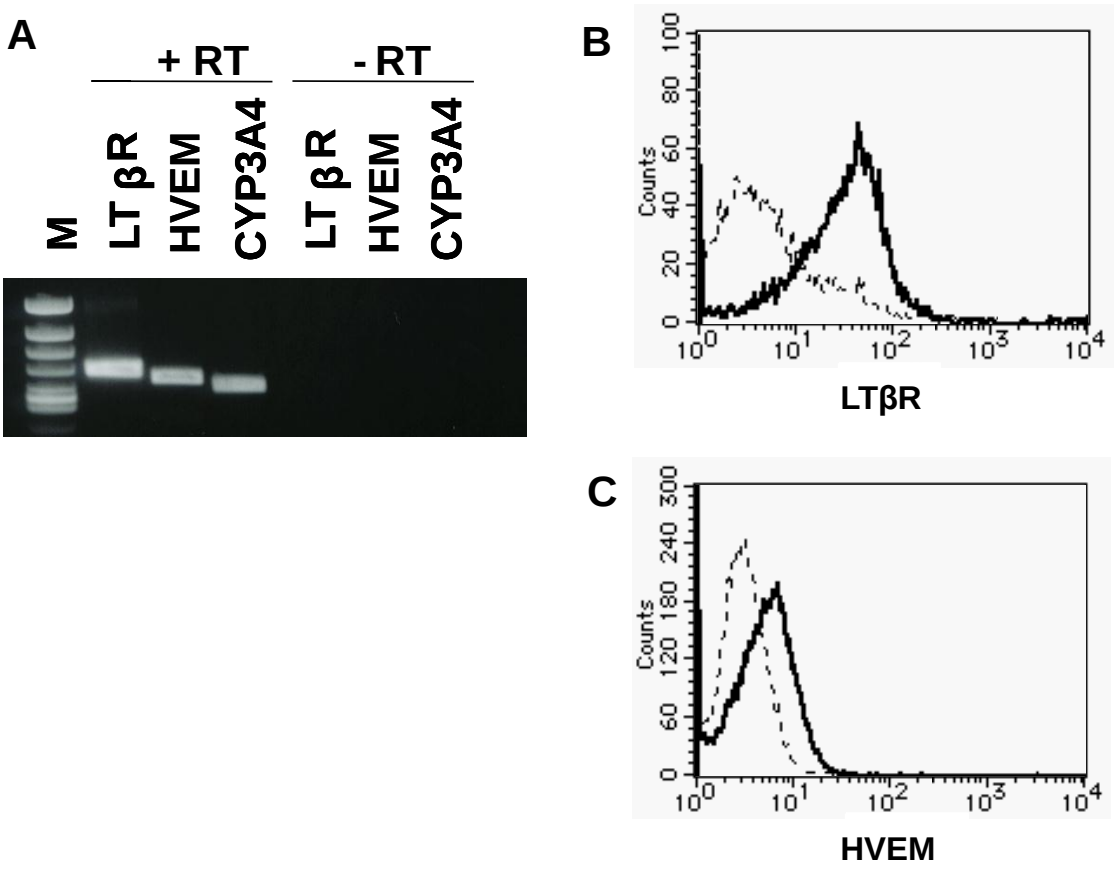
A
+ RT
- RT
M
LT βR
HVEM
CYP3A4
LT βR
HVEM
CYP3A4
B
Counts
LTβR
C
Counts
HVEM

Figure 2

**Figure 3. TNFα, LTα, and αFas, but not LIGHT, increase LDH release in the presence of ActD.**

Human primary hepatocytes were incubated in basal medium in the presence of various concentrations of LIGHT, TNFα, LTα, or αFas with or without 333 nM ActD for 18 h. The cytotoxicity of each well was determined by measuring LDH release. Data are expressed as mean ± S.E., n = 3.

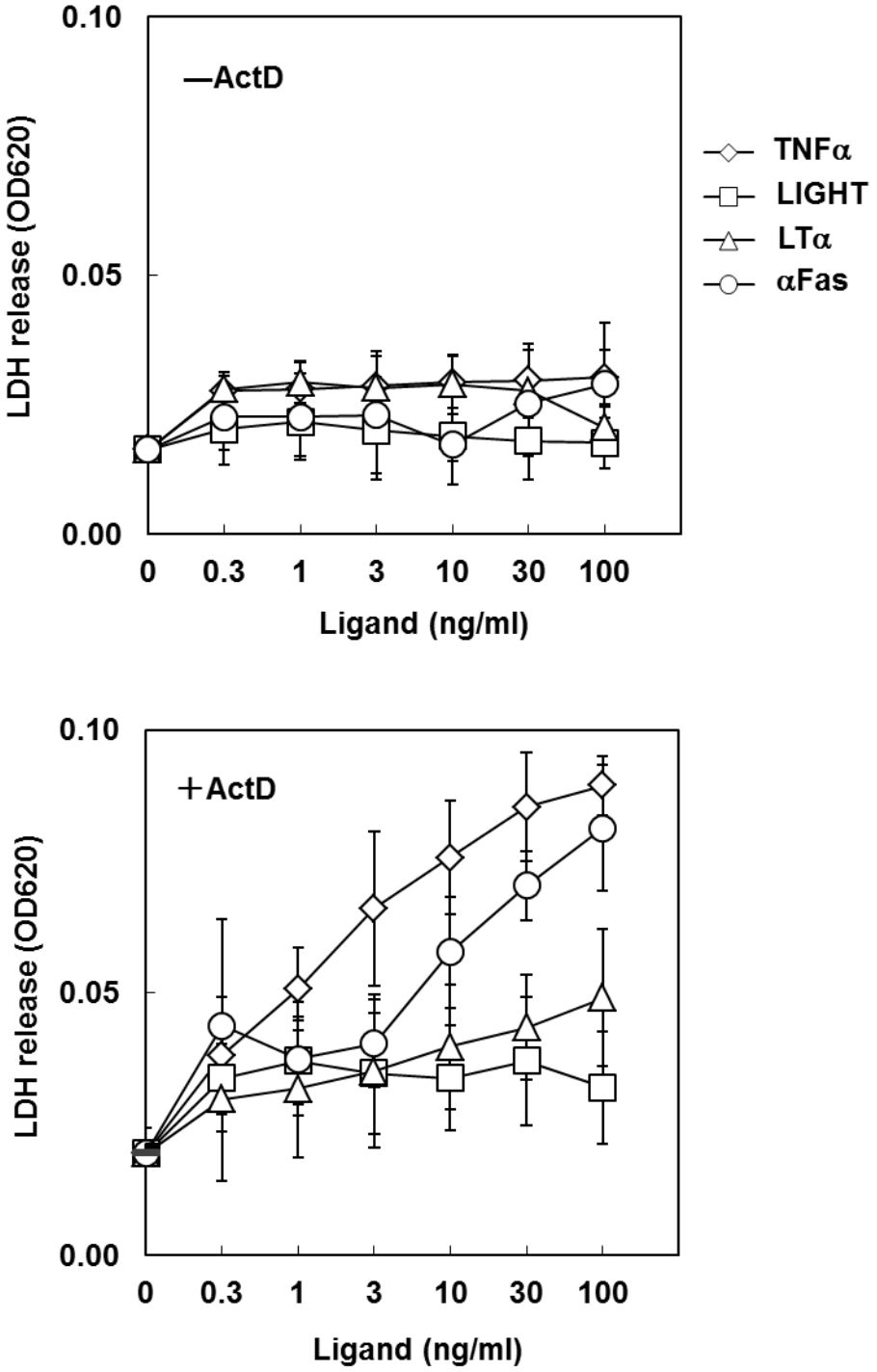
—ActD
+ActD
LDH release (OD620)
0.10
0.05
0.00
0
0.3
1
3
10
30
100
Ligand (ng/ml)
TNFα
LIGHT
LTα
αFas

Figure 3

**Figure 4. TNFα, LTα, and αFas, but not LIGHT, induce apoptosis in ActD-sensitized hepatocytes.**

Human primary hepatocytes, treated as described in experimental procedures, were stained with FITC-conjugated annexin-V or propidium iodide, and were analyzed by FACS analysis.

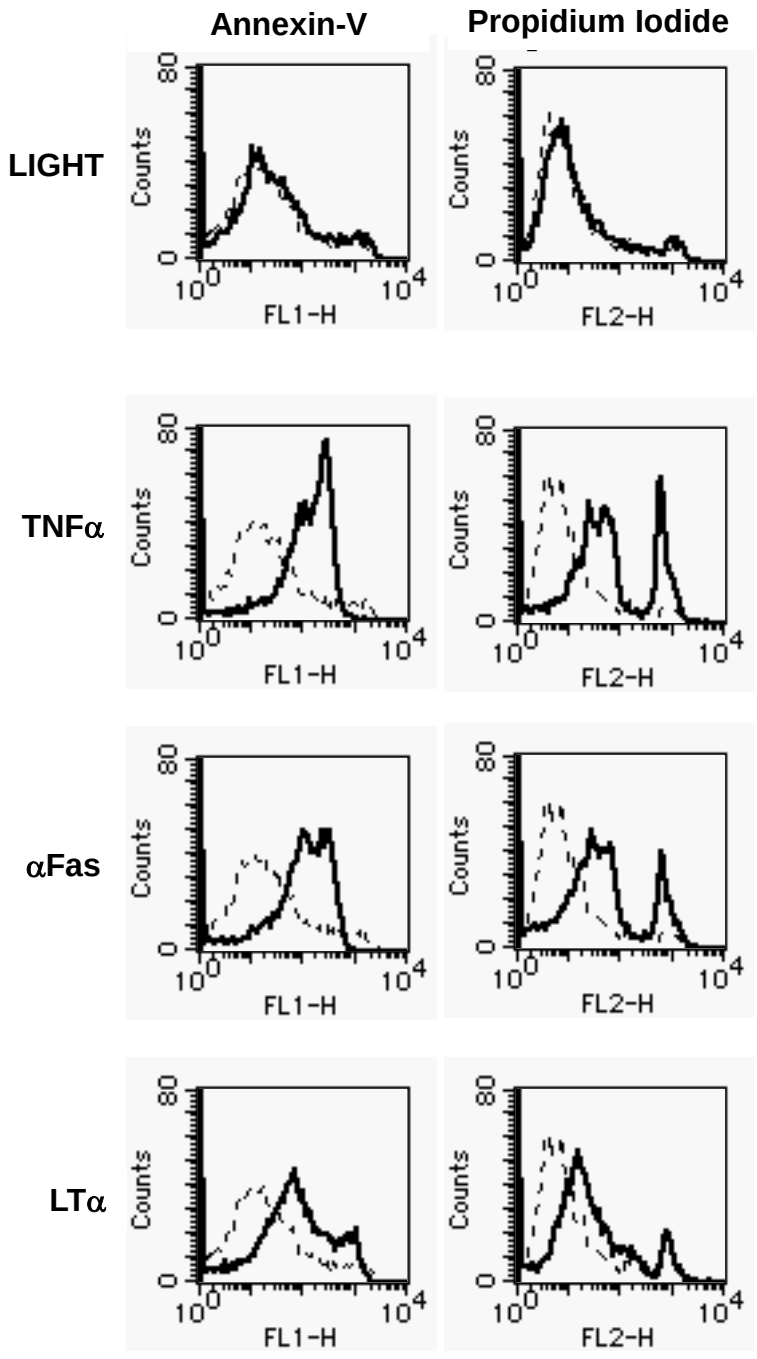
Annexin-V
Propidium Iodide
LIGHT
TNFα
αFas
LTα
Counts
80
0
10^0
10^4
FL1-H
FL2-H

Figure 4

**Figure 5. 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. 3. Data are expressed as mean ± S.E., n = 3.

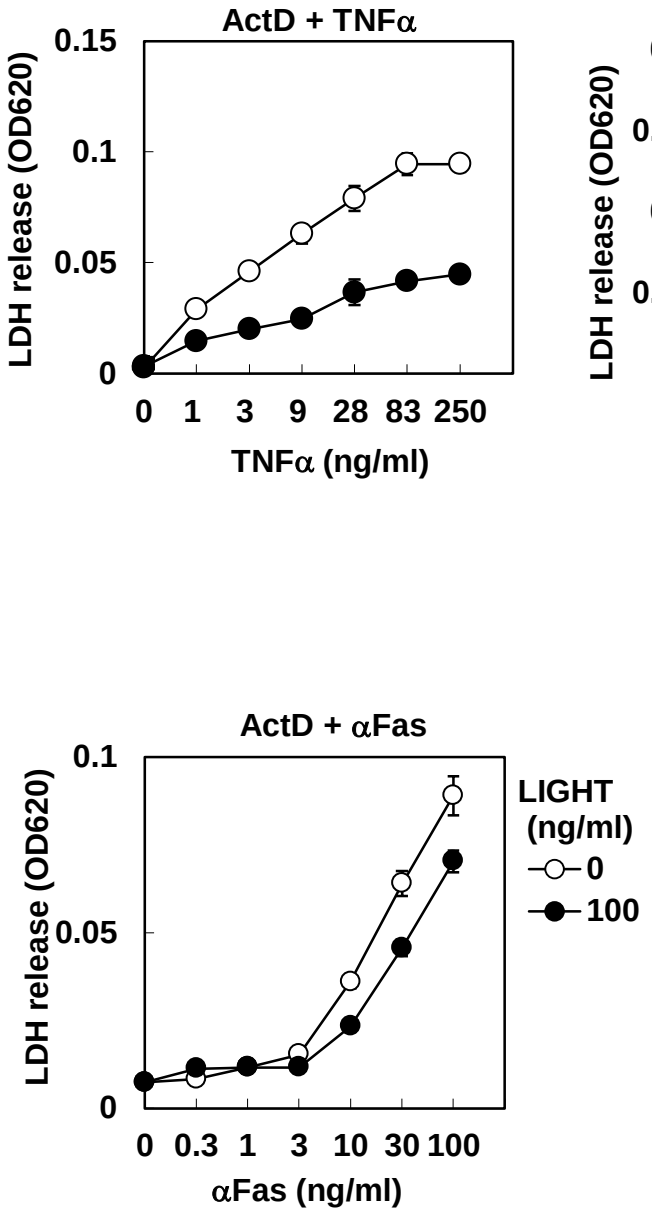
ActD + TNFα
LDH release (OD620)
0.15
0.1
0.05
0
0 1 3 9 28 83 250
TNFα (ng/ml)
ActD + TRAIL
LDH release (OD620)
0.2
0.15
0.1
0.05
0
0 0.3 1 3 10 30 100
TRAIL (ng/ml)
ActD + αFas
LDH release (OD620)
0.1
0.05
0
0 0.3 1 3 10 30 100
αFas (ng/ml)
LIGHT (ng/ml)
0
100

Figure 5

**Figure 6. The LIGHT-mediated anti-apoptotic effect requires LIGHT pretreatment at least 3 h prior to ActD administration in hepatocytes.**

Human primary hepatocytes were treated with 100 ng/ml LIGHT at different times as indicated before ActD administration, followed by treatment as described in the legend to Fig. 5. Data are expressed as mean ± S.E., n = 3.

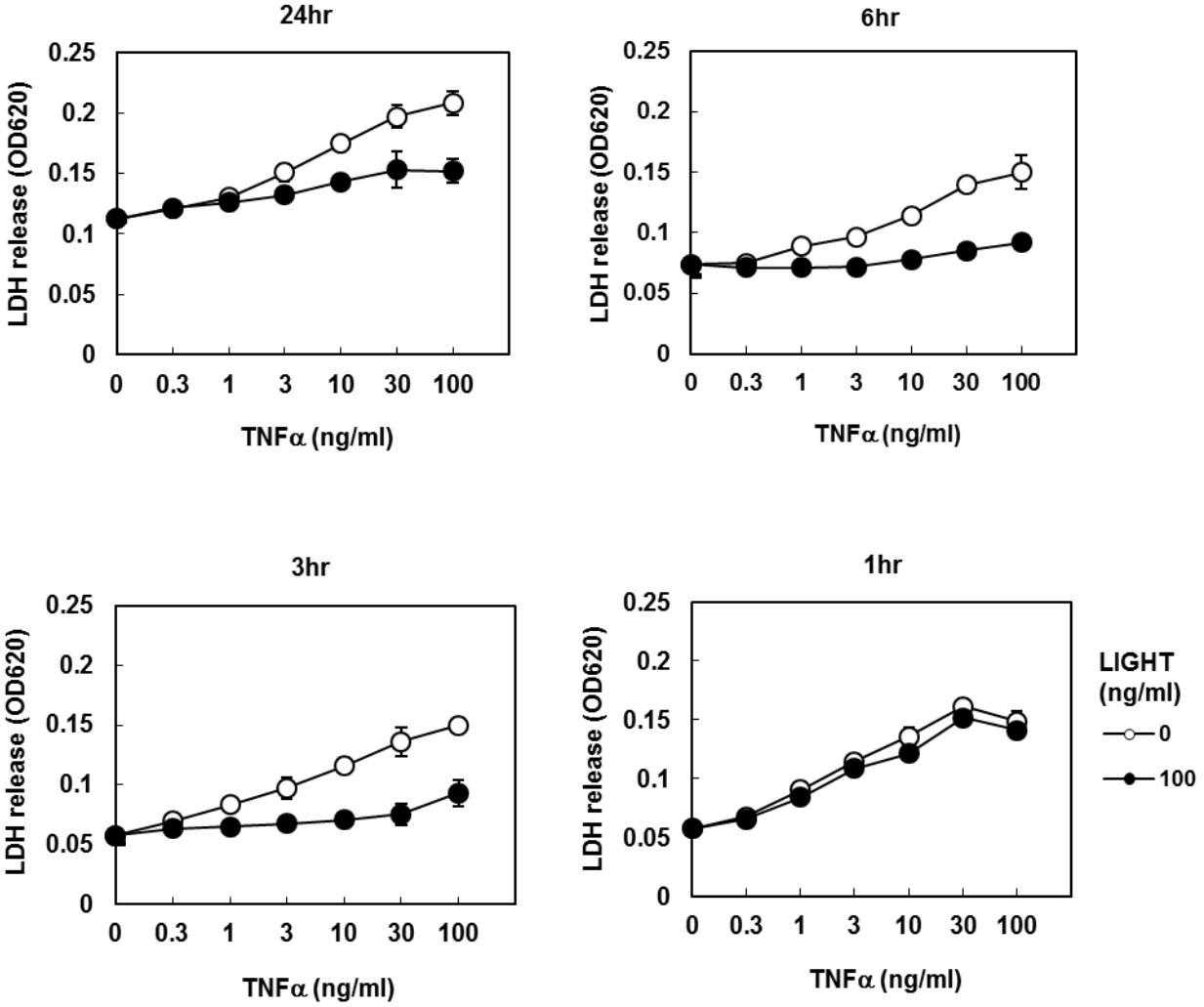
24hr
6hr
3hr
1hr
LDH release (OD620)
TNFα (ng/ml)
0
0.05
0.1
0.15
0.2
0.25
0.3
1
3
10
30
100
LIGHT
(ng/ml)
0
100

Figure 6

**Figure 7. LIGHT induces NFκB activation in human primary hepatocytes.**

Human primary hepatocytes transfected with NFκB dependent SEAP expression plasmids were stimulated with LIGHT, TNFα, or αFas. Culture supernatants were collected at each of the indicated time points, and NFκB activation was determined by measuring the SEAP activities. SEAP activities were expressed in chemiluminescent signal (CFU) detected on a plate luminometer, and the results are plotted as mean ± S.E., n = 4.

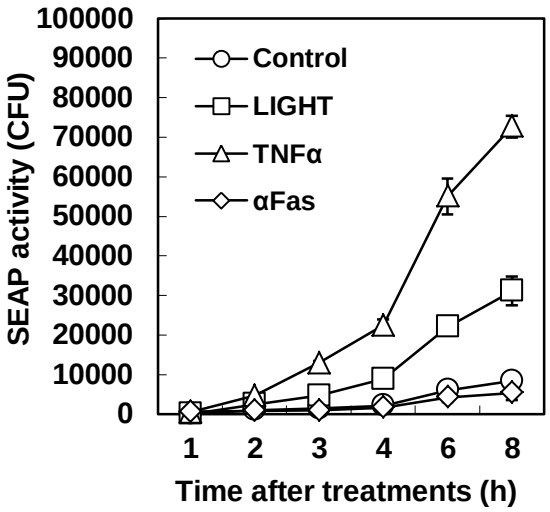
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
SEAP activity (CFU)
Control
LIGHT
TNFα
αFas
1
2
3
4
6
8
Time after treatments (h)

Figure 7

**Figure 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.

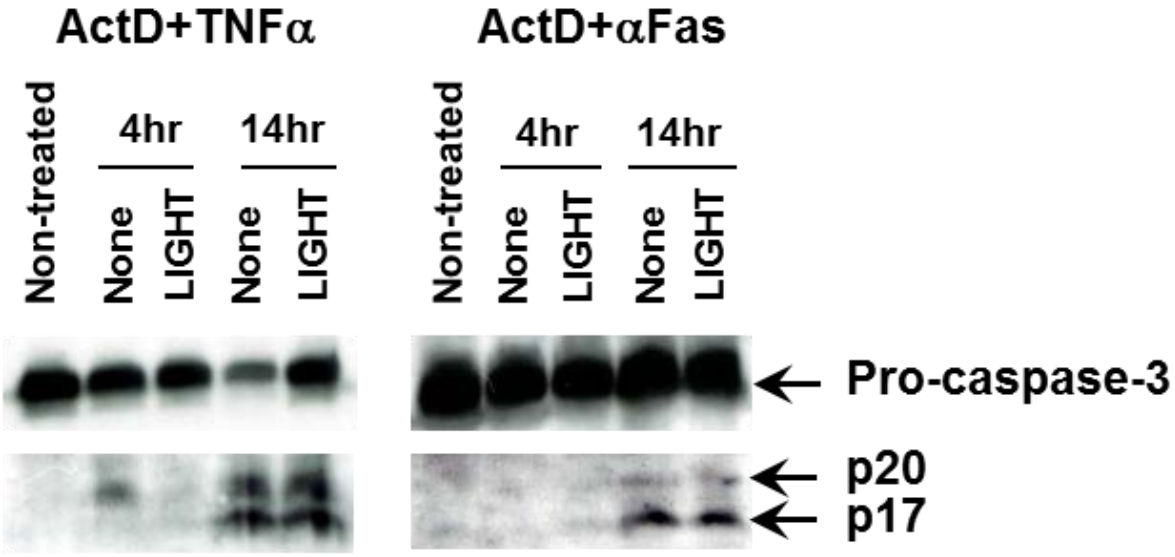
ActD+TNFα
ActD+αFas
Non-treated
4hr
14hr
None
LIGHT
None
LIGHT
Non-treated
4hr
14hr
None
LIGHT
None
LIGHT
Pro-caspase-3
p20
p17

Figure 8

## Part 2

## Expression of ICAM-1 on Glomeruli is Associated with Progression of Diabetic Nephropathy in a Genetically Obese Diabetic Rat, Wistar Fatty

## Abstract

To elucidate the relationship between TNFα and non-inflammatory disease (diabetic nephropathy), I focused on ICAM-1 expression as a marker for TNFα expression in tissues of interest. First, a genetically obese rat strain known as Wistar fatty was developed as an animal model of non-insulin-dependent diabetes mellitus. These rats show obesity-related features such as hyperinsulinemia and hyperlipemia. However only males develop diabetic features, including hyperglycemia, glucoseuria and polyuria, as they age. Histopathological study revealed a deposition of PAS-positive granules in epithelial cells, a diffuse thickening of the mesangial area, and moderate changes in the renal tubules. I found that ICAM-1 is expressed on the glomeruli of male Wistar fatty rats and that its expression is associated with the development of nephropathy. ICAM-1 expression is weak at 5 weeks, becomes markedly strong at 15 weeks, and progresses further at 29 weeks of age. I administered monoclonal anti-ICAM-1 *in vivo* (alone or in combination with anti-LFA-1) to male Wistar fatty rats during the period from 5 weeks to 17 weeks of age. The treatment,

however, could not prevent the development of nephropathy. ICAM-1 expressed on the glomeruli of Wistar fatty rats does not seem to play a key role in the development of nephropathy by mediating leukocyte infiltration; however it will be a useful marker for the development of the disease.

## Introduction

As described in part 1, the complexity of TNFα signaling enabled me to speculate about the existence of physiological roles for TNFα that are distinct from apoptosis and inflammatory reactions. It has been reported that regulation of expression of adhesion molecules is one of the inflammatory reaction by TNFα. Over 20 years ago, it was difficult to detect the amount of TNFα in specific tissues of interest, but the expression of adhesion molecules may be utilized as a surrogate marker for TNFα expression in those tissues.

Intercellular adhesion molecule-1 (ICAM-1), a 90-kDa adhesion molecule encoded by a member of an immunoglobulin supergene family, is a cell-surface ligand of integrins, lymphocyte function-associated antigen-1 (LFA-1), and Mac-1. ICAM-1 is known to play important roles in various cell-cell interactions in the immune system (Dustin et al., 1988; Wawryk et al., 1989). It is involved in the leukocyte-endothelial cell interaction, which controls the extravasation of leukocytes into connective tissues, particularly in inflammatory reactions. Recent studies have found that ICAM-1 is expressed in renal tissue in some human renal

diseases, including focal segmental glomerulosclerosis, rapidly progressive glomerulonephritis, and lupus nephritis (Chow et al, 1992; Lhotta et al, 1991; Canton et al, 1992). *In vivo* administration of anti-ICAM-1 antibody to model animals with renal dysfunction (in which infiltration of inflammatory cells are observed) demonstrated that ICAM-1 plays a key role in the pathogenesis of these diseases (Kawasaki et al., 1993; Nishikawa et al., 1993; Kelly et al., 1994; Harning et al., 1992). However, it is not known whether ICAM-1 is involved in the pathophysiology of renal diseases in which infiltration of the cells is considered unimportant, although the expression of ICAM-1 was reported to be increased in rat mesangial cells by TNFα (Brennan et al., 1990; Sterzel et al., 1993). Diabetic nephropathy is one of the renal diseases that proceed without infiltration of inflammatory cells into glomeruli. Pathological features of this disease are glomerulosclerosis, arteriosclerosis, and morphological changes of the glomerulus such as capillary basement membrane thickening, diffuse glomerulosclerosis, and nodular glomerulosclerosis (Cotran et al., 1989). The mechanisms of progression to diabetic nephropathy are not well understood. Therefore, it is important to establish an *in vivo* diabetic animal model to develop our understanding of the disease.

A genetically obese hyperglycemic rat strain, the Wistar fatty rat strain, was previously developed as a model for non-insulin-dependent diabetes mellitus (Ikeda et al., 1981). This strain was established by transferring the obesity-induced gene *fa* from the heterozygous Zucker rat to the Wistar Kyoto rat with blunted insulin sensitivity. Wistar fatty rats show obesity-related features such as hyperinsulinemia and hyperlipemia. Only males show diabetic features, including hyperglycemia, glucoseuria, and polyuria. Furthermore, Wistar fatty males develop diabetic nephropathy (Diani et al., 1988).

In the part 2, I studied the expression of ICAM-1 in the kidney section from Wistar fatty rats by immunostaining using a monoclonal antibody against rat ICAM-1 (1A29; Tamatani et al., 1990). I also examined the participation of ICAM-1 in the development of nephropathy by *in vivo* administration of anti-ICAM-1.

## Materials and Methods

### Animals

Wistar fatty rats and Wistar lean rats were bred by Takeda Pharmaceutical Company Ltd., and allowed free access to food and water.

### Analytical methods

Blood samples were taken from the tail vein under the fed condition at 08:30-09:30 h. Plasma glucose was determined by a glucose-oxidase assay kit using glucokinase (Iatron, Tokyo, Japan). Plasma triglyceride was determined by an enzymological assay kit using L-α-glycerophosphate oxidase (Iatron). Plasma cholesterol was determined by an enzymological assay kit using cholesterol-esterase and cholesterol-oxidase (Iatron), urinary samples (16 h) were collected in metabolic cages and their volumes were measured. Urinary protein excretion was measured by the Lowry method (Lowry et al., 1951). Urinary albumin was measured by an immunological method (Mohamed et al., 1984).

## Antibodies

A mouse IgG1 monoclonal antibody against rat ICAM-1 (1A29) and a mouse IgG2a monoclonal antibody against rat LFA-1 (WT.1) were generated and characterized as previously described (Tamatani et al., 1990; 1991). A mouse monoclonal antibody against human CR1 (57H, IgG1) was used as an isotype-matched control. Biotinylated $F(ab)'_2$ fragment of anti-mouse IgG was purchased from Amersham Laboratories Inc. (Buckinghamshire, UK). Fluorescein-conjugated (FITC) streptavidin was purchased from vector Labs Inc. (Burlingame, USA). Goat antibodies against rat Ig and rat C3 and FITC conjugated rabbit anti-goat Ig (H +L) were purchased from Cappel Laboratories (Durham, USA).

## Histology

After Wistar fatty and lean rats (six per group) at 17 weeks of age were sacrificed, the kidneys were removed, and fixed in 10% buffered formalin. The tissues were embedded in paraffin, 4 μm thick sections were taken and stained with hematoxylin and eosin, periodic acid-Schiff (PAS) reagent and periodic acid-methenamine-silver (PAM). The sections were examined on light microscopy.

## Immunohistochemical methods

Ten μm frozen sections were fixed in cold methanol for 10 min. After washing with PBS, the sections were incubated with PBS containing 2% bovine serum albumin (BSA, Fraction V; Wako Chem.Inc., Osaka, Japan) for 1 h at room temperature. They were washed three times with PBS containing 0.1% BSA and then treated with endogenous avidin biotin blocking kit (Nichirei Inc., Tokyo, Japan) followed by a wash. They were incubated either with 1A29 or 57H ascites at a dilution of 1:500 overnight at 4°C. After washing, they were incubated with $F(ab)'_2$ fragment of biotin-labeled anti-mouse IgG (1.7 μg/ml) for 1 h. They were washed and further incubated with FITC-streptavidin (25 μg/ ml) for 1 h at room temperature. Finally, the sections were mounted in glycerol-PBS solution (glycerol/PBS, 9:1) containing *p*-phenylenediamine to reduce fading (Platt et al., 1983) and examined with an Olympus microscope equipped with epifluorescence optics and appropriate filters. Pictures were taken of glomeruli, which showed a typical staining among several sections. Almost all glomeruli in one section showed similar degree of staining. The strength of ICAM-1 expression was classified as follows: ±, weak; +, moderate; ++, strong. In some experiments where specified, I used FITC-conjugated rabbit anti-mouse Ig (1:100) instead of

biotinylated F(ab)'$_2$ fragment of anti-mouse IgG to avoid the non-specific reaction to avidin.

### Administration of anti-ICAM-1 and anti-LFA-1 antibodies to Wistar fatty rats

Twenty Wistar fatty rats at 5 weeks of age were divided into four groups of five rats and treated either with PBS, anti-ICAM-1 (1A29), anti-LFA-1 (WT.1) or with both antibodies. Wistar lean rats (n=5) were treated with PBS as negative control. 1A29 and WT.1 were given intraperitoneously at a dose of 3 mg/kg twice a week during the period from 5 to 17 weeks of age. Urinary albumin excretion was measured at 6, 9, 12, 14, 16 and 18 weeks of age, and the immunostaining was performed at 19 weeks of age.

### Statistical analysis

Data are expressed as mean ± S.D. Differences of means between fatty rat groups and lean rat groups were statistically analysed by two-way ANOVA with Bonferroni's multiple comparison test.

## Results

### Development of diabetic nephropathy in Wistar fatty rats

The plasma glucose levels in Wistar fatty rats increased with age. They were 160 mg/dl at 6 weeks and 350 mg/dl at 17 weeks of age. In contrast, the plasma glucose levels in Wistar lean rats remained below 150 mg/dl throughout the observation period. The plasma triglyceride levels of Wistar fatty rats were 120 mg/dl, a significantly higher level than that seen in Wistar lean rats, 40 mg/dl at 6 weeks of age. Triglyceride levels reached 400 mg/dl at 18 weeks of age, whereas those of Wistar lean rats showed a slight increase to approximately 100 mg/dl. The difference in plasma cholesterol levels between Wistar fatty and Wistar lean rats became apparent at 12 weeks of age. The plasma cholesterol levels of Wistar fatty and Wistar lean rats at 18 weeks of age were 160 mg/dl and 90 mg/dl, respectively (Fig. 9).

I measured the daily urinary excretion of proteins and albumin to analyze the development of renal dysfunction. Urinary protein levels were low in both types of rat until 9 weeks of age. After 12 weeks of age, however, the urinary

protein levels of Wistar fatty rats started to increase, reaching 140 mg/day at 18 weeks, whereas the levels in Wistar lean rats remained low: 40 mg/day at 18 weeks of age. Excretion of large molecular weight proteins into urine was determined by measuring urinary albumin. Wistar fatty rats excreted a significant amount of albumin into their urine at 9 weeks of age, and its level reached 45 mg/day at 18 weeks of age. In contrast, Wistar lean rats did not excrete a detectable amount of albumin throughout the observation period (Fig. 10).

### Histopathological finding of the kidney

The renal glomeruli of Wistar fatty rats showed a mild to moderate deposition of PAS-positive granules in the epithelial cells and a diffuse thickening of the mesangial area (Fig. 11A). In the renal tubules, mild and moderate changes were observed, including glycogen vacuolization, basophilia, thickening of basement membrane, hyaline cast formation, microcalcification, dilatation, and appearance of eosinophilic bodies in the epithelial cells. In Wistar lean rats, however, no changes were observed in renal glomeruli and tubules (Fig. 11B).

**Expression of ICAM-1 in the kidneys of Wistar fatty rats**

I examined the expression of ICAM-1 in renal tissues of Wistar fatty rats and Wistar lean rats using immunohistochemical staining. As shown in Fig. 12, a diffuse, segmental, and strong staining of ICAM-1 was observed in the glomeruli of Wistar fatty rats, whereas only a faint staining was observed in Wistar lean rats at 17 weeks of age. I further studied the age-dependent expression of ICAM-1 from 5 to 29 weeks in the glomeruli of Wistar fatty and lean rats (Table 1). In both types of rat, ICAM-1 was weakly expressed at 5 weeks of age. After 15 weeks of age, however, expression of ICAM-1 became markedly stronger and diffused in Wistar fatty rats. The increase progressed further as they aged, whereas it remained weak in Wistar lean rats. I repeated the staining experiments at least three times and obtained similar results; thus, showing a marked increase of ICAM-1 staining in the glomeruli of Wistar fatty rats after 15 weeks of age. The elevated level of ICAM-1 expression in the glomeruli is associated with, or followed by, the increased excretion of urinary proteins. Because I could not demonstrated the specificity of the ICAM-1 staining in the renal tubules due to a non-specific staining with avidin, which is known to cross-react with kidney sections, I stained these tissues with FITC-conjugated rabbit anti-mouse Ig.

Specific expression of ICAM-1 was observed at the basement membrane and interstitium of the renal tubules, but not in the renal epithelial cells, in Wistar fatty and lean rats. Thus, the expression of ICAM-1 increased significantly with age only in glomeruli of Wistar fatty rats.

### In vivo administration of anti-ICAM-1 antibody

I administered anti-ICAM-1 antibody (alone or in combination with anti-LFA-1 antibody) to Wistar fatty rats. Rats treated with antibodies showed albuminuria comparable to that seen in control rats (Fig. 13). These treatments did not appear to prevent Wistar fatty rats from developing nephropathy. At 19 weeks of age, these rats were sacrificed and their kidney sections were examined. Frozen sections were stained with biotin-labeled anti-mouse IgG followed by FITC-streptavidin. Strong staining was observed in the glomeruli of the Wistar fatty rats administered with anti-ICAM-1 antibody; thus, showing that the antibodies had reached the glomeruli. Formalin-fixed sections were examined carefully for their histopathological features. I found no significant change in the sections of Wistar fatty rats regardless of the anti-ICAM-1 antibody treatment. A diffuse thickening of the mesangial area was observed in the glomeruli; hyaline

cast formation, dilatation, and basophilia were observed in the renal tubules of both groups of rats. Negative staining of the kidney section with anti-rat Ig and anti-rat C3 excluded the possibility that nephropathy developed in the antibody-treated rats due to the deposition of immune complexes. Thus, *in vivo* administration of anti-ICAM-1 antibodies failed to prevent the development of nephropathy in Wistar fatty rats.

## Discussion

I demonstrated that the level of ICAM-1 expression in the glomeruli of Wistar fatty rats increased in association with the development of diabetic nephropathy. Although I was not able to identify the cell type that showed a marked increase in the expression of ICAM-1, there are several lines of evidence suggesting that the mesangial cells are likely candidates. The mesangial region of diabetic Wistar fatty rats expands weakly and resembles the staining pattern with anti-ICAM-1. Primary cultured mesangial cells obtained from rat glomeruli showed an elevated expression of ICAM-1 upon stimulation with TNFα or IL-1 (Brennan et al., 1990; Sterzel et al., 1993).

ICAM-1 is one of the adhesion molecules that play important roles in the infiltration of leukocytes into inflammatory regions. However, infiltration of leukocytes into the glomeruli of Wistar fatty rats was not observed where ICAM-1 expression was highly elevated. I tried to administer anti-ICAM-1 antibody to Wistar fatty rats. Under the experimental conditions, there was no effect on the development of nephropathy. Strong staining by anti-mouse Ig antibody was

observed in the glomeruli of Wistar fatty rats treated with anti-ICAM-1 antibodies, which means the exposure of the antibodies in kidneys of the treated rats. Although anti-ICAM-1 antibodies reached the glomeruli of Wistar fatty rats, they did not prevent the development of nephropathy. Is the level of ICAM-1 expression related to the development of nephropathy? It has been shown that administration of anti-ICAM-1 antibody to rats with experimentally developed renal dysfunction associated with leukocyte invasion such as nephrotoxic serum nephritis and ischemic acute renal failure model rats prevents the development of proteinuria in these rats (Kawasaki et. al., 1993; Nishikawa et. al., 1993; Kelly et. al., 1994). With regard to no infiltration of leukocytes in kidney of Wistar fatty rats, it has been reported that T Lymphopenia was observed in Wistar fatty rats (Tanaka et al., 2000), suggesting that Wistar fatty rats might have some types of immune system deficits. These deficits could be one of causes in no infiltration of leukocytes in kidney of Wistar fatty rats, although the mechanism still remains unknown.

The pathogenesis of the non-insulin-dependent diabetic nephropathy is not yet fully understood. In our animal model, male Wistar fatty rats show high plasma glucose levels, whereas females failed to show similar levels. In addition,

both males and females show similar profiles in other parameters such as plasma triglycerides. Only Wistar fatty males develop proteinuria; thus, suggesting that high plasma glucose may cause the development of proteinuria in Wistar fatty rats. It has been reported that rat mesangial cells cultured under high glucose conditions show upregulation of fibronectin expression (Nahman et al., 1992). In male Wistar fatty rats, strong staining with PAS was observed in the mesangial region; thus, indicating expansion of the extracellular matrix.

The Wistar fatty rat was developed by transferring the *fa* gene from the Zucker fatty rat to the Wistar Kyoto rat (Ikeda et al., 1981). ICAM-1 expression was not observed in Wistar lean rats heterozygous for the *fa* gene (Fig. 12) or in female Wistar fatty rats who are homozygous for the *fa* gene but do not show diabetes (data not shown). These data excluded genetic background as the direct cause of the elevated expression of ICAM-1.

In conclusion, I found that ICAM-1 expression was elevated in the glomeruli of Wistar fatty rats. The increase is associated with the progression of nephropathy. These finding suggest that ICAM-1 is a very useful marker for the diabetic nephropathy although it is unlikely to play a key role in the development of the nephropathy with no infiltration of immune cells.

## Figures and a Table

**Figure 9. Change in plasma glucose, triglyceride and cholesterol in Wistar fatty rats.**

Plasma glucose (A), triglyceride (B) and cholesterol (C) in male Wistar fatty rats (black circle) and their lean littermates (white circle). mean ± S.D., n = 5. *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$ vs. the corresponding lean rats.

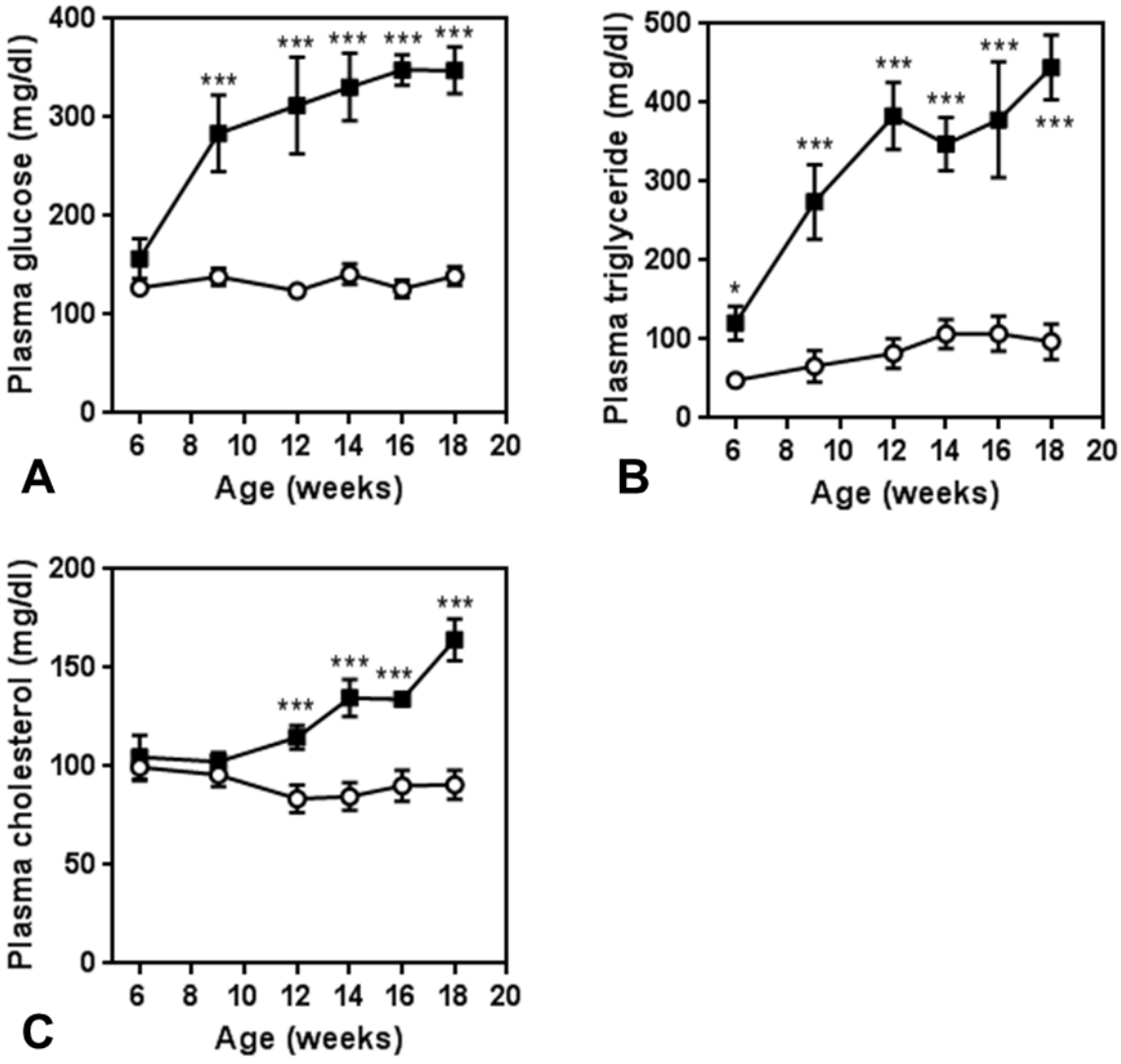
Plasma glucose (mg/dl)
400
300
200
100
0
***
***
***
***
***
6
8
10
12
14
16
18
20
Age (weeks)
A
Plasma triglyceride (mg/dl)
500
400
300
200
100
0
*
***
***
***
***
6
8
10
12
14
16
18
20
Age (weeks)
B
Plasma cholesterol (mg/dl)
200
150
100
50
0
***
***
***
***
6
8
10
12
14
16
18
20
Age (weeks)
C

Figure 9

**Figure 10. Change in urinary proteins and albumin in Wistar fatty rats.**

Urinary proteins (A) and albumin (B) in male Wistar fatty rats (black circle) and their lean littermates (white circle). mean ± S.D., n = 5. *p < 0.05, **p < 0.01, and *** p < 0.001 vs. the corresponding lean rats.

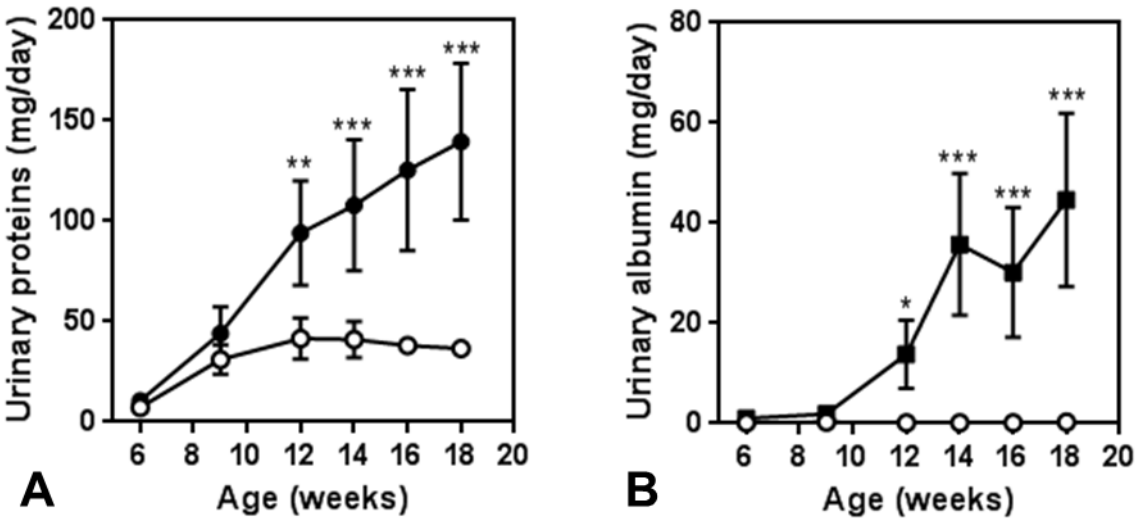
Urinary proteins (mg/day)
200
150
100
50
0
6 8 10 12 14 16 18 20
Age (weeks)
**
***
***
***
A
Urinary albumin (mg/day)
80
60
40
20
0
6 8 10 12 14 16 18 20
Age (weeks)
*
***
***
***
B

Figure 10

**Figure 11. Staining with periodic acid-methenamine-silver of glomeruli from Wistar fatty rat**

Wistar fatty rat (A), lean rat (B) at 17 weeks of age. Bars, 5 μm.

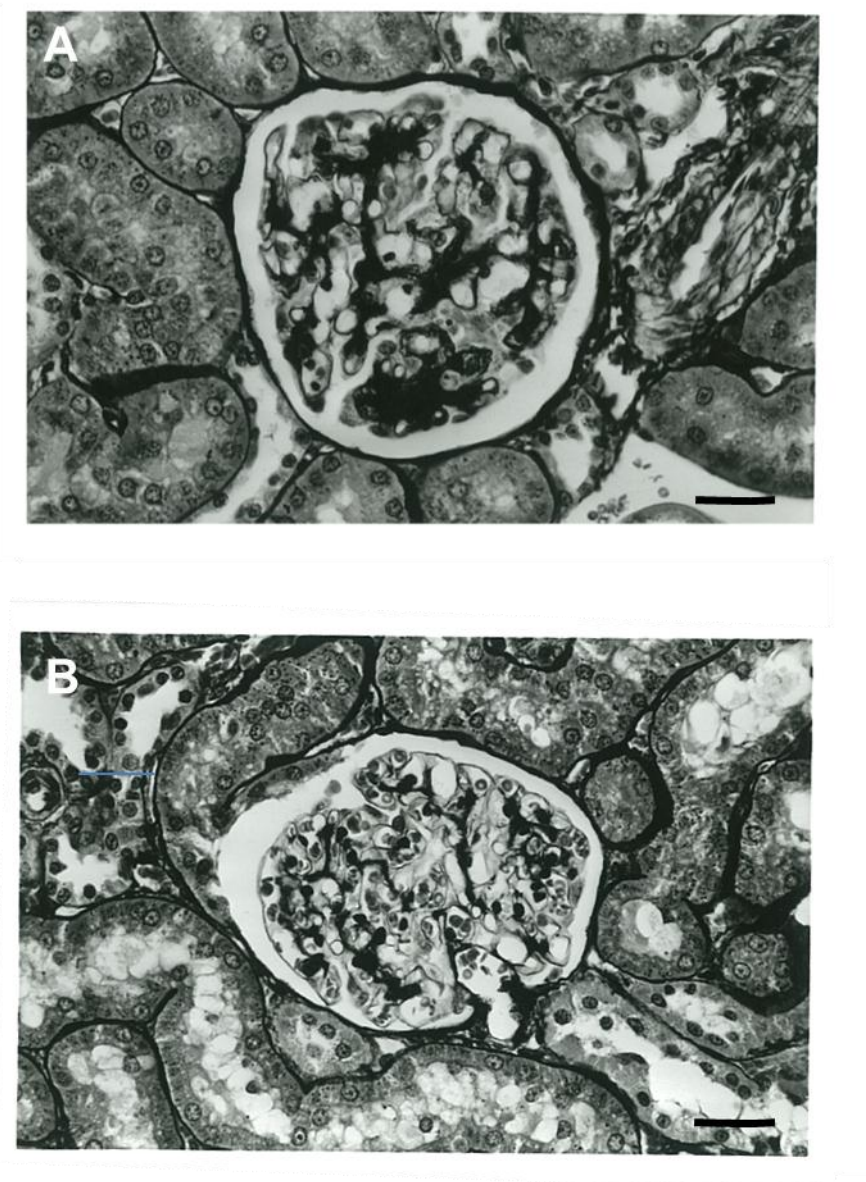


Figure 11

**Figure 12. Immunostaining with anti-ICAM-1 antibody in glomeruli.**

Wistar fatty rat (A) and lean rat (B) at 17 weeks of age. Bars, 2.5 μm.

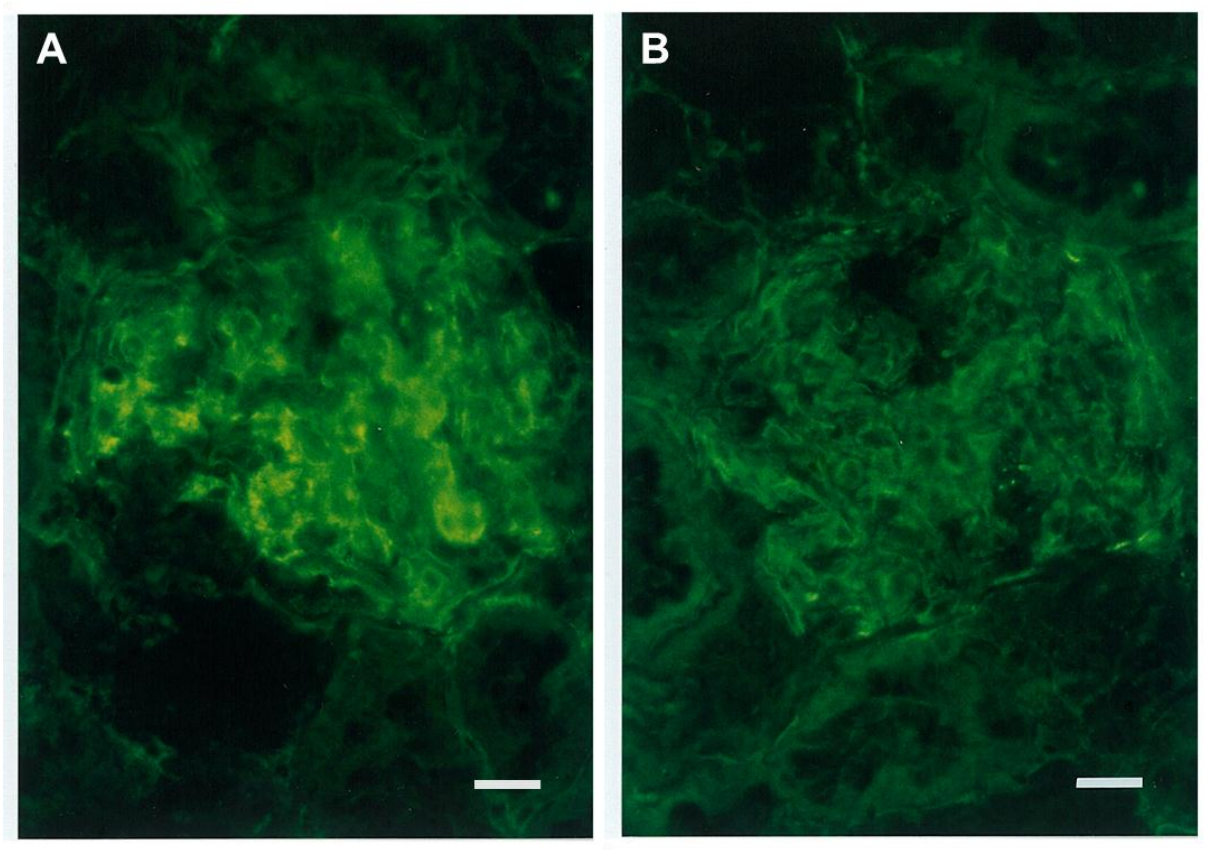


Figure 12

**Figure 13. Administration of anti-ICAM-1 and anti-LFA-1 antibodies to Wistar fatty rats.**

Wistar fatty rats were treated either with PBS (black circle), with 1A29 (triangle), with WT.1 (rectangle), or with 1A29 and WT.1 (diamond), and Wistar lean rats were treated with PBS (white circle). mean ± S.D., n = 5.

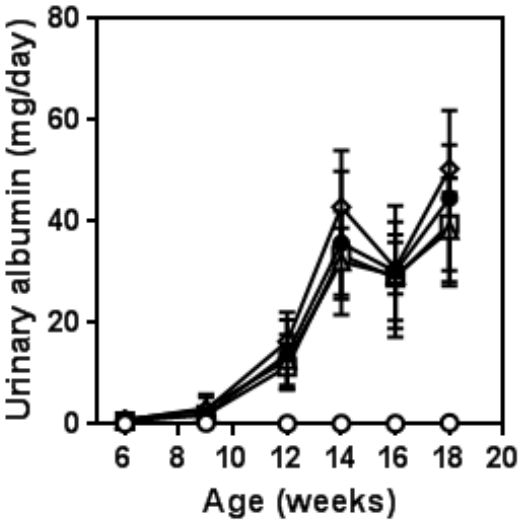
Urinary albumin (mg/day)
80
60
40
20
0
6
8
10
12
14
16
18
20
Age (weeks)

Figure 13

Table 1. Age-dependent expression of ICAM-1 from 5 to 29 weeks in glomeruli of Wistar fatty and lean rats.

| Rat | Age (weeks) | | | | |
|---|---|---|---|---|---|
| | 5 | 9 | 15 | 17 | 29 |
| Wistar fatty | ± | ± | + | ++ | ++ |
| Wistar lean | ± | ± | ± | ± | ± |

Expression coded as follows; ±, weak; +, moderate; ++, strong.

# General discussion

Members of the TNF superfamily have a large variety of physiological functions. This current study was designed not only to elucidate the mechanism that determine cell fate (survival or apoptosis) based on the resistance of several normal cells to TNFα apoptosis, but also to discover physiological functions other than those previously identified in non-inflammatory diseases. First, the anti-apoptotic effect of LIGHT, which binds non-DD bearing receptors, was evaluated in ActD- and TNFα-treated human normal hepatocytes (part 1). Second, ICAM-1 expression was evaluated in the kidney of Wistar fatty rats to investigate whether the expression of ICAM-1 could be utilized as a surrogate marker for TNFα expression (part 2).

In part 1, I first examined the apoptotic activity of several members of the TNF superfamily in human primary hepatocytes. LIGHT failed to induce apoptosis in ActD-sensitized hepatocytes, whereas TNFα, LTα, and αFas effectively induced the apoptosis, even though both LTβR and HVEM (specific receptors of LIGHT) which possess no authentic DD were expressed on the hepatocytes. In addition, LIGHT induced NFκB activation in the hepatocytes. These results suggest that LIGHT is able to activate an NFκB pathway, but not an apoptotic signaling pathway. Moreover, as expected, I also discovered that

LIGHT prevented ActD-sensitized hepatocytes from TNFα-mediated apoptosis. These results suggest that TNFα-mediated apoptosis is suppressed by the signaling from TNF receptors lacking DDs.

It seemed that these mechanisms could be an effective way to induce appropriate apoptosis along with changes of cell state and fate. I had no reason why LIGHT failed to rescue ActD/αFas- or TRAIL-treated cells from apoptosis in my studies. It was reported that TNFα- but not Fas-related apoptosis required sensitization of hepatocytes in mice (Nagaki et al., 1999). Fas signaling may stimulate apoptotic pathways different from those stimulated during TNFα-mediated apoptosis which were resistant to NFκB activation.

After my studies, a substantial amount of research on TNFα signaling has been conducted. First, Tchikov et al. (2011) focused on the compartmentalization of TNFRp55 and CD95 pro- and anti-apoptotic signaling. Recent findings reveal a regulatory role for receptor internalization and intracellular trafficking in selectively transmitting signals that lead either to apoptosis or to the survival of the cell; thus, providing a clue to the understanding of these contradictory phenomena. Second, RIP and TRADD are competitively bound to TNFRp55 after exposure of TNFα, which results in the switch between NFκB activation and

caspase processing. Dominant recruitment of TRADD induced NFκB activation whereas that of RIP did caspase activation. In the case of Fas and TRAIL receptors, the competitive binding was not identified. The difference might be one of the answers for the incompetent of NFκB on Fas or TRAIL-mediated apoptosis. Third, NFκB inhibition by an IKK mutant enhanced TNFα- but not TRAIL-mediated apoptosis in a keratinocytes cell line by increasing the level of caspase-8 activation (Diessenbacher et al., 2008). Thus, several findings have revealed the mechanisms behind the anti-apoptotic effects of TNFα, Fas and TRAIL signals could suppress this mechanism, which would result in their ability to induce apoptosis in LIGHT-pretreated cells.

Differences between the NFκB activation modalities of TNFα and LIGHT were reported (Madge et al., 2010). It was revealed that TNF-induced classical NFκB signaling upregulates RelB expression, which inhibits both the basal and non-canonical NFκB-dependent CXCL12 expression induced by LIGHT. This means that TNFα activates classical or canonical NFκB, whereas LIGHT activates only non-canonical NFκB. They also described several studies that indicated a role for p100 and RelB as positive regulators of the classical NFκB activation pathway; thus, suggesting an interplay between the non-canonical and

classical pathways. Therefore, it has been validated that two distinct NFκB pathways are activated by TNFα and LIGHT, respectively, while TNFα and LIGHT enable the prevention of apoptosis via NFκB activation in my studies.

Intracellular signaling via the TNF superfamily has been elucidated by numerous studies over the past 20 years. A range of results, summarized by Liedtke et al. (2012), have led to the identification of the role of the IKK family in TNFα intracellular signaling, as well as the threshold model for the induction of apoptosis by enhanced caspase-8 gene expression. Caspase-8 activation by Fas inhibits NFκB activation in Jurkat cells by the degradation of IKKγ/Nemo (Frelin et al., 2008). FADD inhibits NFκB activation in cardiomyocytes (Chao et al., 2005). Contrary to the results described above, LTβR signaling induced NFκB activation but failed to protect HT29-derived carcinoma cells from apoptosis (Vanarsdale et al 1997). Induction of NFκB by TNFα and IL-1β has a pro-apoptotic role in pancreatic beta cells (Ortis et al. 2008). These types of findings suggest that the regulation of apoptosis by the TNF superfamily is dependent on cell type, other stimuli, and the environment surrounding the cells.

Apoptosis is thought to play important roles in the processes of organogenesis and differentiation. Disruption of the RelA gene, which is a

member of the NFκB family, was reported to cause apoptosis of hepatocytes during ontogenesis. These cells can be rescued by crossbreeding of the mice with TNFα KO mice (Doi et al., 1999). Furthermore, Anders et al. (2005) reported that marked hyperplasia of the liver was observed in LIGHT transgenic mice, whereas mice deficient in LTβR signaling have a severe defect for survival from partial hepatectomy; thus, suggesting that the upregulation of NFκB signals derived from TNF superfamily ligands plays a considerable role in hepatocyte survival and liver regeneration.

In part 2, I tried to utilize ICAM-1 expression as a surrogate marker for TNFα expression based on a previous study that TNFα induced ICAM-1 expression in mesangial cells (Brennan et al., 1990; Sterzel et al., 1993). Our findings revealed that ICAM-1 expression in the kidney of a genetically obese diabetic rat: Wistar fatty rats increased along with an increase in urinary proteins; thus, suggesting that ICAM-1 expression is correlated with the progression of nephropathy. To elucidate the effects of ICAM-1 on the incidence of diabetes symptoms, chronic administration of anti-ICAM-1 antibodies was performed in Wistar fatty rats. The treatment failed to reduce the elevation of urinary albumin in Wistar fatty rats; thus, suggesting that ICAM-1 has a limited

role in disease progression, although ICAM-1 may serve as a surrogate marker for TNFα expression in the kidney during diabetic nephropathy.

As unresolved questions, our findings failed to achieve notable results that would enable me to understand the implication of ICAM-1 or the relationship of TNFα with diabetic nephropathy. These issues have been addressed by plenty of studies after my studies were completed. First, an increase in plasma TNFα was confirmed in the Wistar fatty rats used in part 2 (Murase et al. 1998). In addition, ICAM-1 expression has been identified in other diabetic nephropathy models such as streptozotocin-treated animals, which has resulted in the growing acceptance of the generality of ICAM-1 expression in diabetic nephropathy (Watanabe et al., 2011). On the other hand, infiltration of white blood cells was observed in the kidneys of other diabetic nephropathy models. This infiltration was suppressed by anti-ICAM-1 antibodies and in ICAM-1 knockout mice (Sugimoto et al., 1997; Miyatake et al., 1998; Okada et al., 2003; Chow et al., 2005). Therefore, a difference in filtration of immune cells between this model and the others was observed, whereas a similar ICAM-1 expression pattern was obtained among some diabetic models. The reason for this still remains unknown. However, a speculation was raised that it was due to the difference in genetic background

among them, considering that the Wistar fatty rat was generated by crossbreeding between Wistar Kyoto and Zucker fatty bearing *fa* gene, which might result in T Lymphopenia (Tanaka et al., 2000).

Many findings on relationship of TNFα and ICAM-1 with diabetic nephropathy have been described. It was reported that TNFα synthesis and the concentration of TNFα in the kidney of diabetic rats were correlated with urinary albumin, as well as with ICAM-1, as described above (Navarro et al., 2005; 2006). The soluble plasma ICAM-1 concentration was also correlated with urinary albumin in diabetic patients (Lenghel et al., 2012). Moreover, plasma and urinary TNFα were correlated with urinary albumin in patients with type 2 diabetes (Lu et al., 2011; Wu et al., 2013). These results indicate a relationship between TNFα and ICAM-1 associated with diabetic nephropathy. With respect to the mechanism of pathogenesis, Elmarakby et al. (2012) summarized a number of studies that TNFα induced by oxidative stress during hyperglycemia upregulates ICAM-1 expression. These findings and the studies presented in part 2 support the possibility that TNFα could play meaningful roles in positive feedback systems of oxidative stress and in induction of pro-inflammatory factors like ICAM-1, which may result in the infiltration of immune cells into kidneys during diabetic

nephropathy.

In conclusion, the findings described in part 1 reveal that TNFα-mediated apoptosis is effectively suppressed by pretreatment with LIGHT; thus, suggesting that some TNF superfamily ligands can negatively regulate TNFα-mediated apoptosis. As described in part 2, ICAM-1 expression, which is expected to serve as a surrogate marker for TNFα expression, was increased in the glomeruli of kidney in Wistar fatty rats; thus, suggesting that the pro-inflammatory cytokine TNFα has the potential to play pathophysiological roles in non-inflammatory diseases like diabetic nephropathy. These results obtained from my studies indicate a part of a wide variety of physiological implications for TNF superfamily.

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