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Evidence that Genetic Deletion of the TNF Receptor p60 or p80 Inhibits Fas Mediated Apoptosis in Macrophages

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Abstract

Almost nineteen members of the tumor necrosis factor (TNF) superfamily have been identified that interact with 29 different receptors. Whether these receptors communicate with each other is not understood. Recently, we have shown that receptor activator of NF- κ B ligand signaling is modulated by genetic deletion of the TNF receptor. In the current report, we investigated the possibility of a cross-talk between Fas and TNF- α signaling pathway in macrophage cell lines derived from wild-type (WT) mice and from mice with genetic deletion of the type 1 TNF receptor (p60^{-/-}), the type 2 TNF receptor (p80^{-/-}), or both receptors (p60^{-/-}p80^{-/-}). We found that the macrophages expressing TNF receptors were highly sensitive to apoptosis induced by anti-Fas. The genetic deletion of TNF receptors, however, made the cells resistance to anti-Fas-induced apoptosis. Anti-Fas induced activation of caspase-3 and PARP cleavage in WT cells but not in TNF receptor-deleted cells. This difference was found to be independent of the expression of Fas, Fas-associated protein with death domain (FADD) or TNF receptor-associated death domain (TRADD). We found that anti-Fas induced recruitment of TNFR1 into Fas-complex. We also found that TRADD, which mediates TNF signaling, was constitutively bound to Fas receptor in TNF receptor-deleted cells but not in wild-type cells. Transient transfection of TNFR1 in TNFR1-deleted cells sensitized them to anti-Fas-induced apoptosis. Overall our results demonstrate that Fas signaling is modulated by the TNF receptors and thus providing the evidence of cross-talk between the receptors of two cytokines.

Keywords

Fas; TNFR; apoptosis; NF- κ B; macrophages

1. Introduction

The TNF superfamily now includes 19 different members that interact with 29 different receptors. Among these, TNF, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) induce apoptosis in different cells. TNF mediates its effects through TNF receptor 1 (also called p55 or p60) and TNF receptor 2 (also called p75 or p80). TNFR1 is characterized by the presence of death domain (DD) in its cytoplasmic portion, TNFR2, however, lacks DD.

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TNFR1, through its DD, sequentially recruits TNFR associated death domain (TRADD), Fas-associated protein with death domain (FADD) and FADD-like interleukin-1 β -converting enzyme (FLICE), which leads to activation of apoptotic pathway. Fas was first identified on the surface of human fibroblasts (fibroblast-associated cell surface antigen). Like TNFR1, it also contains DD that directly recruits FADD and FLICE, which leads to apoptosis.

There are many reports that suggest that TNF receptor and Fas may be linked. Yonehara *et al*, in their original report on the discovery of Fas, suggested that cytolytic activity mediated by Fas-antigen was associated with TNF receptor [1]. Subsequently, it was found that FADD and receptor-interacting protein (RIP) bind to both TNFR1 and Fas [2,3]. Spanaus *et. al* showed that TNF rendered microglia cells sensitive to Fas ligand-induced apoptosis through induction of Fas [4]. Teh *et. al* found that TNFR2-deficient CD8 T cells were resistant to FasL-induced cell death [5]. Another most compelling evidence of cross-talk between these two receptors, is a recent report that mice lacking TNFR1 and TNFR2 are resistant to death and liver-injury induced by agonistic anti-Fas antibody [6].

While most reports suggest that TNF mediates its signaling primarily through TNFR1, our recent report using TNFR-deleted cells suggests that both receptors are needed for optimum signaling [7]. Using TNFR-deleted cells, we have also recently provided evidence for cross-talks between TNF and LPS signaling, and between TNF and RANKL signaling [8,9]. The cell signaling induced by both LPS and RANKL require the recruitment of TNF receptor-associated factor (TRAF) 6. In spite of reports suggesting existence of a cross-talk between TNF and Fas signaling, there is no direct demonstration of such a link. To investigate this, we used macrophages derived from mice in which either TNFR1 or TNFR2 or both receptors had been genetically deleted. We provide evidence that Fas-induced apoptosis requires the presence of TNF receptor, and that Fas can recruit TNFR1 into Fas-complex.

2. Materials and Methods

2.1. Materials

Agonistic anti-Fas/CD95 (Jo2) antibody was purchased from BD Biosciences (San Diego, CA). Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Invitrogen (Grand Island, NY). Antibodies against PARP, JNK1, FADD, and TRADD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cleaved-caspase 3 and Src were obtained from Cell Signaling Technology (Danvers, MA). Antibody against uPA was obtained from Calbiochem (San Diego, CA). Cycloheximide and anti- β -actin antibody were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell lines and culture

Production of mice with genetic deletions of p60, p80, or both have been described [10,11]. Briefly, immortalized macrophage cell lines were established from the bone marrow of wt C57BL/6J mice and its TNFR knockout homozygous mice (p60^{-/-}, p80^{-/-}, and p60^{-/-}p80^{-/-}) as previously described [7–9]. By using RT-PCR, FACS analysis, and Western blot analysis, the cells were shown to lack expression of TNF receptors [7]. All cell lines were cultured in RPMI 1640 medium supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Cytotoxicity assay—The cytotoxic effects of Fas were determined by the MTT uptake method as described [12]. Briefly, 1×10^4 or 5×10^3 cells were incubated in triplicate in 96-well plates with the indicated concentrations of Fas for 72 h or with 1 ng/ml Fas for the indicated times at 37 °C. Thereafter, MTT solution was added to each well. After a 2 h incubation at 37 °C, lysis buffer (20 % SDS, 50 % dimethylformamide) was added, the mixture incubated

overnight at 37°C, and the optical density measured at 570 nm using a 96-well multiscanner (Dynex Tech., MRX Revelation; Chantilly, VA).

2.3. Western blot analysis

Thirty to fifty micrograms of whole-cell extracts were prepared as described [13], and resolved by SDS-PAGE. Then the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5 % nonfat dry milk, and probed with primary antibodies for 2 h at 4°C. The blotting membrane was washed, exposed to horse radish peroxidase-conjugated secondary antibodies for 1 h, and then detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL).

2.4. NF-κB assay

NF-κB activation was analyzed by electrophoretic mobility shift assay (EMSA) as described previously [14]. In brief, 15 μg nuclear extracts prepared from TNF-treated or untreated cells were incubated with ³²P end-labeled 45-mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5'-TTGTTACAAGGGACTTTCCTGGGGACTTTCAGGGAGGCGTGG-3'; underlined sequence is binding site) for 30 min at 37 °C, and the DNA-protein complex resolved in a 6.6 % native polyacrylamide gel. The specificity of binding was examined by competition with unlabeled 100-fold excess oligonucleotide and with mutant oligonucleotide. The composition and specificity of binding was also determined by supershift of the DNA-protein complex using specific and irrelevant antibodies. For supershift experiments, the antibody-treated samples of NF-κB were resolved by 5.0 % native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by PhosphorImager using ImageQuant software (Amersham).

2.5. JNK assay

The JNK kinase assay was performed by a method described previously [15]. Briefly, 200 μg of whole-cell extracts were incubated with 0.2 μg antibody against JNK1, and precipitated with protein A/G-agarose beads (Pierce, Rockford, IL). The beads were washed with lysis buffer, and then assayed in kinase assay mixture containing 2 μg of substrate GST-c-Jun (aa1-79). After incubation at 30 °C for 30 min, boiling with SDS sample buffer for 5 min terminated the reaction. Finally, the protein was resolved on 10 % SDS-PAGE, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of JNK in each sample, same whole-cell extracts were resolved by 10 % SDS-PAGE, electrotransferred to nitrocellulose membrane, and then blotted with anti-JNK1 antibody.

2.6. Immunoprecipitation

Four million cells were washed with ice-cold PBS and lysed in a buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10 mM NaF, 10 % glycerol, 0.2 % Triton X-100, 2 mM sodium orthovanadate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. The whole-cell extracts were incubated with 1 μg of anti-Fas antibody for 2 h and precipitated using protein A/G-sepharose beads. After 1 h incubation, the immunocomplex was washed with lysis buffer, boiled with SDS sample buffer for 5 min, resolved on SDS-PAGE, and then subjected to Western blot analysis.

2.7. Transfection

Cells were transiently transfected with the expression vectors for TNFR1 p60 (pCMV-p60-Flag) plasmids by the FuGENE 6 method (Roche Applied Science). After 24 h, cells were treated with indicated concentrations of anti-Fas and then analyzed for cell viability by MTT method as described above.

3. Results

The aim of the present study was to investigate the role of TNF receptor in Fas-induced cell signaling. To understand the role of each type of TNF receptor, we used macrophage cell lines isolated from mice in which the genes for either one or both the TNF receptors were deleted. We have recently reported the characterization of these cells [7–9].

3.1. Deletion of TNF receptors prevent Fas-induced apoptosis

Whether Fas-induced apoptosis is modulated by individual TNF receptors was investigated. We treated wild-type (WT) macrophages and its TNF receptor-deficient variants ($p60^{-/-}$, $p80^{-/-}$, and $p60^{-/-}p80^{-/-}$) with anti-Fas antibody, and analyzed for cell viability by the MTT method. We found that anti-Fas decreased cell viability in a dose-dependent manner (Fig. 1A) and a time-dependent manner (Fig. 1B) only in WT cells, not in TNF receptor-deficient variants.

3.2. Deletion of TNF receptors prevent Fas-induced caspase-3 activation and PARP cleavage

Whether the cell-killing effects of anti-Fas correlate with apoptosis of the cells, we examined the expression of cleaved-caspase-3, and PARP cleavage. We found that treatment with anti-Fas caused enhanced expression of cleaved caspase-3 (Fig. 2A) and PARP (Fig. 2B) only in WT, and not in $p60^{-/-}p80^{-/-}$ cells. These results indicate that the deletion of TNF receptors protect the cells from anti-Fas-induced apoptosis.

3.3. Deletion of TNF receptors has no effect on the expression of Fas, FADD and TRADD

To investigate why TNF receptor deleted cells did not respond to anti-Fas-induced apoptosis, we examined the expression of Fas, FADD and TRADD. Western blot analysis revealed that deletion of either one or both TNF receptors had no effect on the expression of Fas, FADD and TRADD (Fig. 3A).

3.4. Deletion of TNF receptors has no effect on activation of NF- κ B and JNK

Both TNF and Fas are potent activators of JNK and activation of JNK has been linked to Fas-induced apoptosis [16]. Also, TNF is known to activate NF- κ B in most cells, whereas Fas-induced NF- κ B activation is cell-type specific [17]. NF- κ B activation has been shown to suppress apoptosis [17]. Whether the inability of TNFR-deleted cells to respond to anti-Fas, was due to difference in activation of NF- κ B or JNK, was examined. Cells were treated with anti-Fas, nuclear extract prepared, and analyzed for NF- κ B activation. As shown in Fig. 3B, anti-Fas did not induce NF- κ B activation in any of the four cell lines.

Fas has also been known to induce JNK activation [16]. To determine whether anti-Fas could induce JNK activation in WT and TNF receptor deficient macrophages, cells were treated with anti-Fas, whole-cell extracts were prepared, immunoprecipitated with anti-JNK1 antibody, and immunocomplex kinase assay was performed. As shown in Fig. 3C (left panel), JNK was constitutively active in all four cell types, and anti-Fas failed to induce JNK activation in any of the four cell lines. For confirming specificity of JNK kinase assay, anti-Src antibody was used as a negative control (Fig 3D, lower panel).

3.5. Resistance to anti-Fas induced apoptosis in TNF receptor deleted cells can not be abrogated by protein synthesis inhibitor

Antiapoptotic proteins, such as Bcl-2, Bcl-x_L, XIAP, IAP1/2 and survivin, are newly synthesized upon stimulation of cells with TNF, and consequently inhibit apoptosis [17]. Whether failure of apoptosis induction by anti-Fas in TNF receptor-deficient cells was caused by induction of antiapoptotic proteins was determined by using protein synthesis inhibitor

cycloheximide. Cells were exposed to anti-Fas in the presence or absence of cycloheximide, and then analyzed cell viability by MTT method. Cycloheximide had no effect on anti-Fas mediated apoptosis in TNF-receptor deleted cells (Fig. 4).

Inducible NO synthase (iNOS) in macrophages plays an important role in apoptosis-induced by Fas [18]. To determine whether Fas could regulate NO production was determined. Cells were treated with Fas, and assayed for NO production by using Griess reagent. Fas treatment caused no significant difference on NO production in all the four cell lines. (data not shown).

3.6. Anti-Fas-induces recruitment of TNF receptor1 into Fas-complex only in wild-type macrophages

It has been suggested that cytotoxic activity of TNF is mediated by association of Fas antigen with TNF receptor [1]. Whether, in our system, anti-Fas could induce association of Fas with the TNF receptor was investigated. WT macrophage and its TNF receptor-deficient variants were treated with anti-Fas, whole-cell extracts were prepared, immunoprecipitated with anti-Fas antibody, and Western blotting was performed using anti-p60 antibody. As shown in Fig. 5A, anti-Fas induced recruitment of TNF receptor1 into Fas-complex in WT cells, but not in $p60^{-/-}p80^{-/-}$ cells.

3.7. Deletion of TNF receptors potentiate recruitment of TRADD into Fas-complex in TNF receptor-deficient variants

The TNF receptor-deletion could have altered the interaction between adaptor molecule and receptor, such as found in association of RANK with TRAF6 [9]. Whether TNF receptor-deletion modulated the association between adaptor molecule TRADD, and Fas-complex was determined. Cells were treated with anti-Fas, and then immunoprecipitated with anti-Fas antibody, and Western blotted using anti-TRADD antibodies. Fig. 5B showed that the deletion of TNF-receptors caused constitutive association of TRADD with Fas. In WT cells Fas was not associated with TRADD.

3.8. Overexpression of TNF receptor 1 potentiates anti-Fas-induced apoptosis in TNF receptor-deficient cells

Whether exogenous expression of TNF receptor 1 into TNF receptor-deficient cells could reverse resistance to anti-Fas-mediated apoptosis, was determined. Cells were transiently transfected with TNF receptor 1 p60 cDNA expression vector into WT and $p60^{-/-}$ cells, treated with anti-Fas, and cell viability was analyzed by MTT method. As shown in Fig. 6, overexpression of exogenous p60 potentiated anti-Fas-induced apoptosis in $p60^{-/-}$ cells but had no effect on WT cells. These results suggest that the TNF receptor 1 is needed for anti-Fas-induced apoptosis.

4. Discussion

The aim of the current study was to determine whether there exist a cross-talk between Fas and TNF signaling. For this we used macrophage cell lines derived from wild-type (WT) mice and from mice with genetically-deleted type 1 TNF receptor ($p60^{-/-}$), the type 2 TNF receptor ($p80^{-/-}$) or both receptors ($p60^{-/-}p80^{-/-}$). We demonstrate that in spite of similar expression level of Fas, the four cell lines differed with respect to Fas-induced apoptosis. The cells, in which either of the TNF receptor was deleted, were resistant to anti-Fas-induced apoptosis. The lack of ability of TNFR-deleted cells to respond to anti-Fas, was not due to the difference in their ability to activate NF- κ B or JNK, or production of NO. The resistance to anti-Fas-induced apoptosis in TNF receptors-deficient variants was also due to the difference in *de novo* protein synthesis. Fas induced recruitment of TNFR1 into Fas-complex in WT cells but not in TNFR-deficient variants. We found that TRADD, which mediates TNF signaling, was

constitutively bound to Fas in TNF receptor-deleted cells but not in WT cells. The resistance to Fas-induced apoptosis in TNFR-deleted cells was restored by overexpressing exogenous TNFR1.

Costelli *et. al* reported that mice lacking TNFR1 and TNFR2 are protected from anti-Fas-induced lethality, as compared to the control mice [6]. However, how TNFR deleted mice were protected, was not explained in that study. Our results indicate that TNFR is needed for Fas-induced apoptosis. FasL activates apoptosis through sequential interaction with Fas, FADD, FLICE and leading to caspase-3 activation. FasL can also activate NF- κ B through sequential interactions with FasL, FADD, RIP, and IKK in certain cell lines [19]. In contrast TNF activates NF- κ B through sequential interactions with TNFR1, TRADD, RIP, and IKK [20–23].

In our study, we demonstrated that anti-Fas-induced interaction between Fas and TNFR1 p60 in WT cells. We also showed the reversal of anti-Fas-induced apoptosis reconstituted by p60 overexpression in p60^{-/-} cells. These results are in agreement with Yonehara *et. al* who reported that cell killing induced by TNF and anti-Fas, are linked; suggested the possibility of association of Fas with TNFR1 [1]. The potential mechanism of cross-talk between TNFR1 and Fas through the death domain motif present in signal-sharing adaptor molecules FADD, TRADD and RIP, has also been suggested [24,25]. Hu *et. al* reported that *Molluscum contagiosum* virus proteins MC159 and MC160 and the equine herpesvirus 2 protein E8, can inhibit both TNFR1- and Fas-induced apoptosis, suggesting similarity between the two pathways [26]. These report are in agreement with our study. Our results clearly show an anti-Fas recruitment of TNFR1 to the Fas complex, however delineation of the domain in TNFR1 that might interact with Fas requires further investigation.

Our results indicate that deletion of either of the TNFR is sufficient to induce resistance to anti-Fas. TNFR1, which contains DD, has been found to be essential for TNF induced apoptosis [17]. In spite of similar level of expression of TRADD in all the four cell lines, we found the constitutive recruitment of TRADD into Fas-complex in TNFR-deficient variants, but not in WT cells. This constitutive TRADD binding to Fas in TNF receptor deficient cells might interfere with the normal pathway of anti-Fas induced apoptosis mediated via FADD and caspase-8. Whether FADD and caspase-8 recruitment to Fas-complex is altered when TRADD associates with the Fas complex or whether caspase-8 activation is altered under these conditions remains to be investigated. However, lack of TNFR1 in TNFR-deficient cells would constitute the primary reason for the failure of Fas-induced apoptosis. It is equally possible that additional mechanism, such as aberrant recruitment of TRADD may also contribute.

Our results clearly show that the TNF receptors play an important role in Fas signaling. The desensitization of Fas signaling by deletion of TNFR may be mediated through the association of receptor themselves and also through sharing of the adaptor molecule TRADD. Both TNFR1 and Fas are known to have the DD and exhibit structural homology not only in their extracellular domain but also in their intracellular domain. Therefore, it is not surprising that two receptors can interact. Our results, however, for the first time demonstrate the functional consequence of this interaction. Overall our studies provide a strong evidence for a cross-talk between TNF and Fas signaling.

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Abbreviations used

TNF	tumor necrosis factor
TNFR	TNF receptor
NF-κB	nuclear factor- κ B
RANK	receptor activator of NF- κ B, RANKL, receptor activator of NF- κ B ligand
IκB	inhibitory subunit of NF- κ B
IKK	I κ B α kinase
TRAF	TNF receptor-associated factor
TRADD	TNF receptor-associated death domain
FADD	Fas associated protein with death domain
NIK	NF- κ B-inducing kinase
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase

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A. **B.**

Fig. 1. Deletion of TNF receptors prevents anti-Fas-induced apoptosis

A. Dose-dependent cell death induced by anti-Fas in wild-type and TNF receptor-deleted macrophages. Ten thousand cells were treated with the indicated concentrations of anti-Fas for 72 h, and then analyzed cell viability by the MTT method. **B.** Time-dependent cell death induced by anti-Fas in wild-type and TNF receptor-deleted macrophages. Ten thousand cells were treated with 1 ng/ml anti-Fas for the indicated times, and then analyzed cell viability by the MTT method.

A. **B.**

Fig. 2. Cleaved caspase-3 expression (A) and PARP cleavage (B) induced by anti-Fas in wild-type but not in TNF receptors-deleted macrophages

One million cells were treated with 1 ng/ml anti-Fas for the indicated times, prepared whole-cell extracts, resolved by 15% and 7.5% SDS-PAGE, electrotransferred to nitro cellulose membrane, and then Western blot analysis was performed using cleaved caspase-3 and PARP antibodies.

Fig. 3. A. Deletion of TNF receptors have no effect on the expression of Fas, FADD, and TRADD Whole-cell extracts were prepared from wild-type and TNF receptor-deleted macrophages, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane. Western blot analysis was performed using the indicated antibodies. The western blotting of β -actin was used as loading control. **B. Deletion of TNF receptors has no effect on NF- κ B activation by anti-Fas.** One million cells were treated with the indicated concentrations of anti-Fas for 30 min, prepared nuclear extracts, and analyzed NF- κ B activity by EMSA. KBM-5 cells were also treated with 0.1 nM TNF for 30 min, prepared nuclear extracts, and analyzed NF- κ B activity by EMSA as a positive control. **C. Deletion of TNF receptors has no effect on JNK activation by anti-Fas.** One million cells were treated with 10 ng/ml anti-Fas for the indicated

times. Whole-cell extracts were prepared, incubated with anti-JNK1 antibody, and then immunoprecipitated with protein A/G-Sepharose beads. The beads were washed and subjected to immuno-complex kinase assay as described in Materials and Methods. To examine the effect of anti-Fas on the level of expression of JNK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-JNK antibodies. Whole-cell extracts were also precipitated with anti-Src antibody, and analyzed for JNK activation by kinase assay as a negative control.

Fig. 4. *De novo* protein synthesis is not required for the function of anti-Fas

Five thousand cells were treated with 0.01 μ g/ml cycloheximide for 2 h, treated with 1 ng/ml anti-Fas for 12 and 24 h, and then analyzed cell viability by the MTT method.

Fig. 5. A. Anti-Fas induces recruitment of TNF receptor1 into the Fas-complex only in wild-type macrophages

Fifty million cells of wild-type macrophages and macrophages with both TNFRs deleted were treated with 1 ng/ml anti-Fas for the indicated times. Whole-cell extracts were prepared, immunoprecipitated with anti-Fas antibody, and subjected to SDS-PAGE, and then Western blot analysis was performed using anti-TNFR1 p60 antibody. IgG heavy chain is shown as loading control. To examine the effect of anti-Fas on the level of expression of p60 proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-p60 antibodies. The whole-cell extracts were also precipitated with anti-uPA antibody, and analyzed for TNFR1 as a negative control.

B. Deletion of TNF receptors potentiates recruitment of TRADD into the Fas-complex in TNF receptor-deficient variants. Fifty million cells of wild-type macrophages and macrophages with both TNFRs deleted were treated with 1 ng/ml anti-Fas for the indicated times. Whole-cell extracts were prepared, immunoprecipitated with anti-Fas antibody, and subjected to SDS-PAGE, and then Western blot analysis was performed using anti-TRADD antibodies. To examine the basal expression of TRADD and Fas proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis.

Fig. 6. Overexpression of TNFR1 p60 potentiates anti-Fas-induced cell death in TNF receptor1-knockout macrophages

Ten thousand cells of wild-type and TNF receptor1-deleted macrophages were seeded in a 96-well plate, transiently transfected with 0.5 μ g of TNFR1 p60-expressing plasmid for 24 h, treated with 1 ng/ml anti-Fas for 72 h, and then analyzed cell viability by the MTT method. Whole-cell extract was fractionated on SDS-PAGE and examined by Western blot analysis using anti-p60 and anti-Flag antibodies.