

Regulation of Fas (CD95)-induced apoptosis by nuclear factor- κ B and tumor necrosis factor- α in macrophages

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The APO-1/Fas ligand (FasL) and tumor necrosis factor- α (TNF- α) are two functionally related molecules that induce apoptosis of susceptible cells. Although the two molecules have been reported to induce apoptosis via distinct signaling pathways, we have shown that FasL can also upregulate the expression of TNF- α , raising the possibility that TNF- α may be involved in FasL-induced apoptosis. Because TNF- α gene expression is under the control of nuclear factor- κ B (NF- κ B), we investigated whether FasL can induce NF- κ B activation and whether such activation plays a role in FasL-mediated cell death in macrophages. Gene transfection studies using NF- κ B-dependent reporter plasmid showed that FasL did activate NF- κ B promoter activity. Gel shift studies also revealed that FasL mobilized the p50/p65 heterodimeric form of NF- κ B. Inhibition of NF- κ B by a specific NF- κ B inhibitor, caffeic acid phenylethyl ester, or by dominant expression of the NF- κ B inhibitory subunit I κ B caused an increase in FasL-induced apoptosis and a reduction in TNF- α expression. However, neutralization of TNF- α by specific anti-TNF- α antibody had no effect on FasL-induced apoptosis. These results indicate that FasL-mediated cell death in macrophages is regulated through NF- κ B and is independent of TNF- α activation, suggesting the antiapoptotic role of NF- κ B and a separate death signaling pathway mediated by FasL.

tumor necrosis factor- α receptor; caspase-activated deoxyribonuclease

THE MAINTENANCE OF CELL homeostasis by apoptosis is a critical regulatory mechanism in the normal immune system. The Fas/APO-1 (CD95) and tumor necrosis factor receptor (TNF-R) are members of the TNF/nerve growth factor receptor superfamily involved in various forms of physiological and pathological cell death. The Fas and TNF-R are homologous proteins, and both have death domains in their cytoplasmic regions.

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These domains have the ability to initiate cell death through interaction with intracellular proteins, leading to the activation of a series of enzymes, some of which cause protein degradation (caspases; see Ref. 27) while others cause DNA cleavage [caspase-activated DNase (CAD); see Ref. 15]. The critical roles of these death receptors have been demonstrated in the mouse and in humans. For example, mice carrying naturally occurring mutations in the Fas gene (*lpr* mice) or in Fas ligand (FasL; *gld* mice) suffer from lymphadenopathy and autoimmune diseases (10). In humans, mutations of the Fas gene that render it nonfunctional cause similar effects, producing a condition termed autoimmune lymphoproliferative syndrome (16, 37).

Increasing evidence indicates that the nuclear factor (NF)- κ B plays an important role in the regulation of apoptosis by serving as a pro- or antiapoptotic signal (1). NF- κ B belongs to a superfamily of protein dimers frequently composed of two DNA-binding subunits, NF- κ B₁ (p50) and RelA (p65), which are normally kept inactive in the cytoplasm by an attachment of the inhibitory subunit inhibitory factor κ B (I κ B; see Refs. 3 and 4). Activation of NF- κ B is accomplished by phosphorylation of the I κ B by a specific I κ B kinase (IKK) complex, which triggers a complete degradation of the inhibitor (39). The activation of NF- κ B has been reported by a wide variety of apoptosis-inducing stimuli, including oxidative stress, cytotoxic agents, and ionizing radiations (30, 35). Additional evidence for the proapoptotic role of NF- κ B is provided by studies showing that the expression of FasL and its induction of apoptosis in T lymphocytes require NF- κ B activation (23). Similarly, the activation of Fas death receptor in fibroblasts is dependent on NF- κ B activation (29). The evidence that supports the antiapoptotic role of NF- κ B has mainly derived from gene knockout studies (6, 19, 24). NF- κ B (p65^{-/-})-deficient mice die during embryonic development through liver cell apoptosis (6). IKK β knockout mice die as embryos and show massive liver cell apoptosis, a response similar to that of the p65^{-/-}

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knockout mice (24). Mice with an inactivated X-linked gene encoding IKK γ /NEMO, a key regulator of the IKK complex for NF- κ B activation, die at midgestation because of massive lymphocyte apoptosis in the thymus in addition to liver degeneration (25, 34). Thus NF- κ B plays both pro- and antiapoptotic roles in regulating apoptotic cell death depending on cell types and physiological conditions.

Most studies investigating the role of NF- κ B in apoptotic cell death have been focused on TNF- α -mediated cell death. These studies indicate that death induced by TNF- α is negatively regulated by NF- κ B (5, 22, 41, 42). Cells that are unable to appropriately activate NF- κ B are significantly more susceptible to TNF- α -induced apoptosis. Unlike TNF- α , the role of NF- κ B in FasL-induced apoptosis has not been consistently demonstrated. Activation of NF- κ B by Fas stimulation has been reported to occur in some cells but not in others (7). In TNF- α -sensitive cells, NF- κ B could be activated by TNF- α but not by Fas ligation (36). Likewise, activation of NF- κ B by TNF- α could not prevent cell death induced by FasL but by TNF- α (41). In Jurkat cells Fas causes proteolysis of NF- κ B subcomponents and prevent its activation (32). However, Fas-induced NF- κ B activation has been reported (31, 32), and this activation could protect cells against Fas-mediated cell death (13). Interestingly, we have found that Fas stimulation can also induce TNF- α production, suggesting the possible involvement of this molecule in Fas-induced apoptosis. The objective of this study is to clarify the involvement of NF- κ B and TNF- α in FasL-induced apoptosis and to determine whether NF- κ B plays a protective or promoting role in this process.

MATERIALS AND METHODS

Cells and reagents. The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO Life Technologies, Gaithersburg, MD) supplemented with 5% FBS, 2 mM glutamine, and 100 U/ml penicillin-streptomycin. Specific antibodies against NF- κ B p50 and p65 subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used in the supershift assay. The liposomal transfecting agent LipofectAMINE was obtained from GIBCO Life Technologies. Anti-TNF- α and anti-Fas antibodies and their isotype-matched control antibodies were purchased from PharMingen (San Diego, CA). The NF- κ B inhibitor caffeic acid phenylethyl ester (CAPE) and recombinant FasL (*SuperFasL*) were purchased from Alexis Biochemicals (San Diego, CA). The reporter plasmid containing a luciferase gene under the control of NF- κ B promoter was a kind gift from Dr. Peter Johnson (National Cancer Institute, Frederick, MD). The I κ B plasmid was obtained from Dr. Chuanshu Huang (Nelson Institute of Environmental Medicine, New York University).

Detection of apoptosis. Analysis of cell apoptosis was performed by using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay kit (Boehringer Mannheim, Indianapolis, IN) and ELISA-based DNA fragmentation assay kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturers' instructions. For the TUNEL assay, cytospin preparations were fixed in 4% paraformaldehyde at room temperature for 5 min. After

incubation with the buffer provided, the slides were immersed in terminal deoxynucleotidyl transferase (TdT) buffer, and TdT and fluorescein-dUTP were added and allowed to incubate for 60 min at 37°C. After being washed with PBS, the slides were counterstained with propidium iodide and examined under a fluorescence microscope. For ELISA assay, cells were lysed with 200 μ l of lysis buffer at room temperature, and the cell lysate (20 μ l) was mixed with an antibody solution (80 μ l) at room temperature for 2 h. The substrate was then added after the wells were washed three times with a washing buffer. After incubation for 10 min at 37°C, the reaction was stopped, and optical density was measured using a microplate reader at a wavelength of 405 nm.

Gene transfection. Approximately 1×10^6 cells were plated on a 12-well plate and allowed to grow for 24 h before the transfection. The plasmid DNA NF- κ B luciferase (1 μ g/ml) or I κ B (1 μ g/ml) was diluted in 200 μ l of DMEM. The liposomal agent LipofectAMINE (12 μ g/ml; GIBCO Life Technologies) was diluted in 200 μ l of DMEM. The diluted DNA and liposome samples were combined and incubated at room temperature for 15–20 min. Cells with transfection reagents were incubated for 4 h. Transfection medium was then replaced with growth medium containing 10% FBS. The transfected cells were maintained in the growth medium at 37°C for 24 h before use.

Assays of luciferase activity and TNF- α protein expression. Luciferase activity was measured by enzyme-dependent light production using a luciferase assay kit (Promega, Madison, WI). After each experiment, cells were washed and incubated at room temperature for 10 min in 250 μ l of lysis buffer (Promega). Ten-microliter samples were then taken and loaded in an automated luminometer (Bio-Rad, Hercules, CA). At the time of measurement, 100 μ l of luciferase substrate were automatically injected in each sample, and total luminescence was measured over a 20-s time interval. Output is quantitated as relative light units per microgram protein of the sample. For analysis of TNF- α protein, cell-free supernatants were used. TNF- α levels were determined using a TNF- α ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

Flow cytometry. Cells were harvested and suspended in PBS containing 1% BSA. They were fixed with ice-cold 4% formaldehyde for 15 min and stained with a rabbit anti-Fas antibody or isotype-matched control antibody, followed by phycoerythrin-conjugated rat anti-rabbit secondary antibody. Subsequently, the cells were subjected to flow cytometric analysis with a gate set for examining a total of 10^4 cells.

Electrophoretic mobility shift assay. To detect NF- κ B binding activity, nuclear protein extracts were first prepared as follows: cells were treated with 500 μ l of lysis buffer [50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, 20 μ l/ml aprotinin, and 100 mM dithiothreitol (DTT)] on ice for 4 min. Nuclei were pelleted by centrifugation at 14,000 rpm for 1 min and were resuspended in 300 μ l of extraction buffer (500 mM KCl, 10% glycerol, 25 mM HEPES, 1 mM PMSF, 1 μ l/ml leupeptin, 20 μ g/ml aprotinin, and 100 μ M DTT). After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested and stored at -70°C . The protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

The DNA-protein binding reaction was conducted in a 24- μ l reaction mixture including 3 μ g nuclear protein extract, 1 μ g poly-(dI·dC) (Sigma), 3 μ g BSA, and 4×10^4 counts/min of ^{32}P -labeled oligonucleotide (ON) probe. The ON probe contained a NF- κ B binding sequence of IL-6 gene

promoter (-74-TGGGATTTTCCCATGAGTCT--54) and was used as a standard NF- κ B probe. The ON probes were denatured at 80°C for 5 min and annealed with their complementary sequence at room temperature. The double-stranded probes were labeled with [³²P]ATP (Amersham, Arlington Heights, IL) using T4 kinase (BRL, Gaithersburgh, MD). The reaction mixture was incubated on ice for 10 min with or without antibody in the absence of radiolabeled probe and then for 20 min at room temperature in the presence of radiolabeled probe. In supershift assays, antibody specific to NF- κ B p50 or p65 subunit (200 μ g) was also added to the reaction mixture. The mixture was resolved on a 5% polyacrylamide gel that had been prerun at 200 V for 30 min with 0.5 \times Tris-borate-EDTA buffer. The loaded gel was run at 200 V for 90 min and then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

RESULTS

FasL induces apoptotic cell death in macrophage RAW cells. Apoptosis triggered by Fas/FasL signaling is an important process in the homeostasis of the immune system (8). Macrophages are key cellular effec-

tors of the immune system; however, their regulation of apoptosis induction by Fas/FasL is largely unknown. In the present study, we investigated the induction of apoptosis by FasL in macrophage RAW 264.7 cells. Treatment of the cells with FasL (0–400 ng/ml) caused a dose-dependent increase in the level of apoptosis, as analyzed by DNA fragmentation enzyme-linked immunosorbent assay (ELISA; Fig. 1A). Peak response time for the apoptosis induction was ~16 h posttreatment (Fig. 1B). Morphological analysis of cell apoptosis by TUNEL assay showed an increasing number of apoptotic cells (green fluorescent signals) with time after FasL treatment (Fig. 1, D–F). In contrast, no or very few apoptotic cells were observed in the control samples (Fig. 1C). These results indicate that FasL was able to induce apoptosis in macrophage RAW cells.

Fas expression in macrophage RAW cells. Fas expression has been reported in a variety of cells from different tissues (43, 44). To determine whether Fas is expressed in RAW cells, we performed flow cytometric

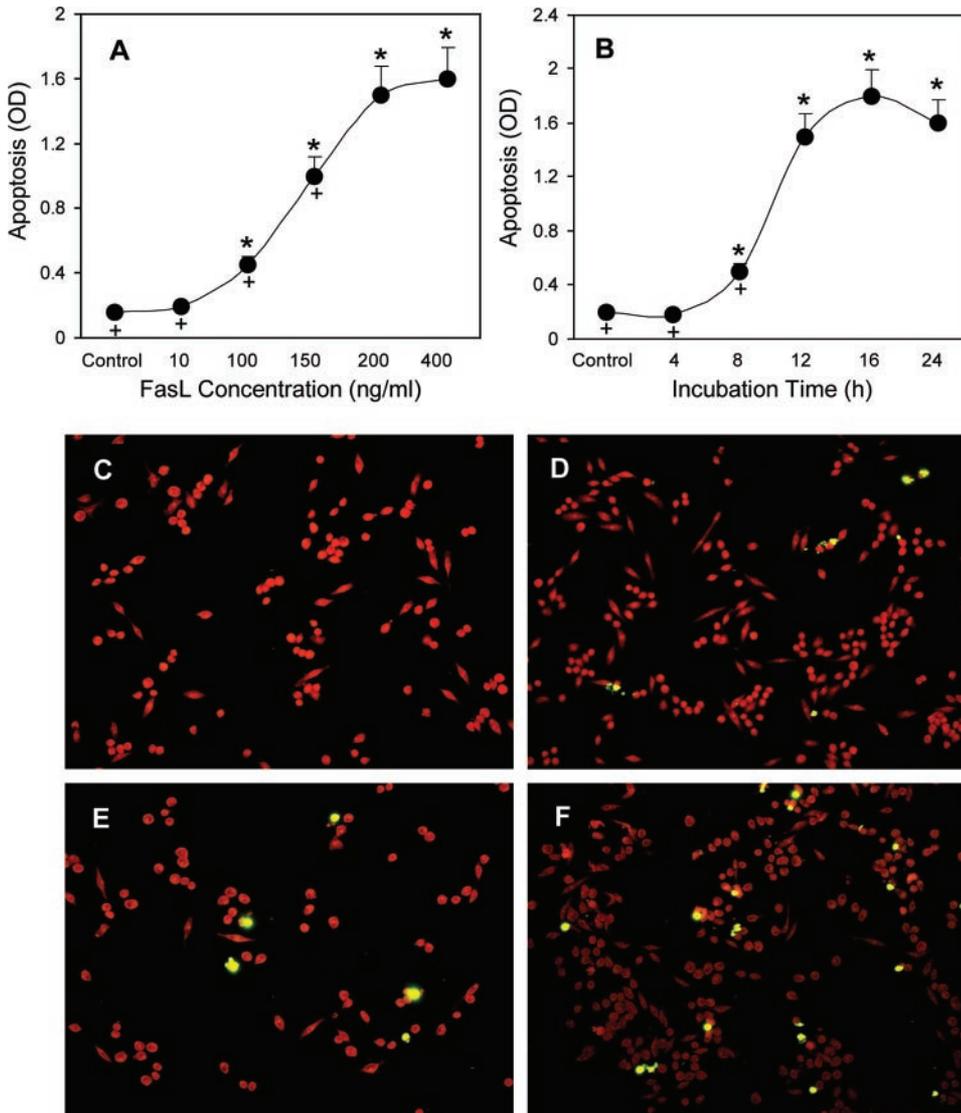


Fig. 1. FasL-induced apoptosis. Cells were treated with ligand (FasL; 0–400 ng/ml) at 37°C for up to 24 h. A: effect of FasL concentration on apoptosis determined at 12 h by enzyme-linked immunosorbent assay (ELISA). B: time course of apoptosis induced by FasL (200 ng/ml). Values are means \pm SD; $n = 4$. * $P < 0.05$ vs. untreated control. + $P < 0.05$ vs. maximum response at 400 ng/ml in A or at 16 h in B. C–F: microscopic detection of apoptotic cells by dUTP nick end-labeling (TUNEL) at 0, 8, 12, and 16 h, respectively, after FasL treatment (200 ng/ml). Apoptotic cells are indicated by bright yellow/green fluorescence signal. The red fluorescence signal indicates propidium iodide counterstain. (Original magnification, $\times 200$.) OD, optical density.

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analysis of Fas expression. The results indicated that cells stained with the anti-Fas antibody showed a strong positive signal (Fig. 2B), whereas those stained with the control isotype-matched antibody showed a weak signal (Fig. 2A). These results indicated the expression of Fas in RAW cells.

FasL-induced NF- κ B activation. The nuclear transcription factor NF- κ B has been shown to play a role in regulating Fas-induced apoptosis in some cells (13, 31, 33) but not in others (7, 36, 41). To investigate the role of this transcription factor in RAW cell apoptosis induced by FasL, electrophoretic gel shift and gene transfection studies were carried out. The results from the gel shift study showed that FasL was able to induce DNA binding activity of NF- κ B at the concentration shown to induce cell apoptosis (200 ng/ml; Fig. 3A, lanes 1 and 2). To determine the specificity of NF- κ B binding in this assay, a nonlabeled NF- κ B probe was used as competitor for NF- κ B binding. Figure 3A, lane 3, shows that the nonlabeled probe was able to compete for the binding, whereas a nonspecific activator protein-1 probe could not (lane 4). Supershift assays using antibodies specific to the p50 and p65 subunits of NF- κ B also showed a band shift of the NF- κ B complexes (lanes 5 and 6). Together, these results indicated the specificity of NF- κ B binding and the formation of p65/p50 and p50/p50 complexes in FasL-treated cells.

In gene transfection studies, cells were transiently transfected with the NF- κ B-luciferase reporter plasmid. The cells were then treated with FasL, and the luciferase expression was determined. Figure 3B shows that FasL treatment caused an increase in cel-

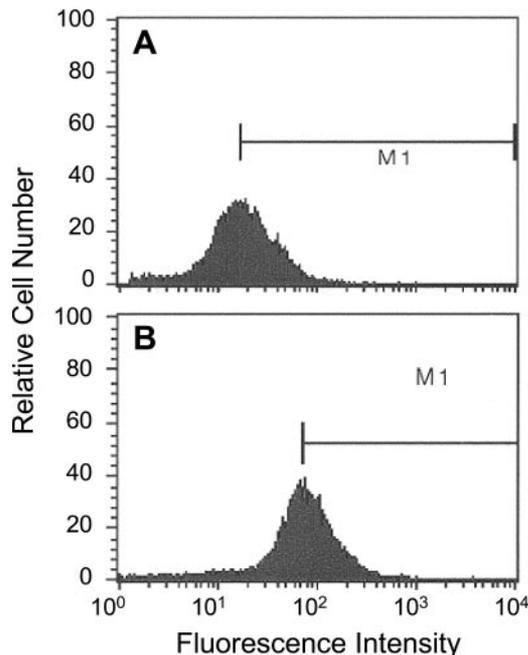


Fig. 2. Expression of Fas in macrophage RAW 264.7 cells. Flow cytometric analysis of Fas expression using isotype-matched control antibody (A) or monoclonal antibody directed against Fas (B). Surface Fas expression was detected by indirect immunofluorescence using phycoerythrin-labeled secondary antibody.

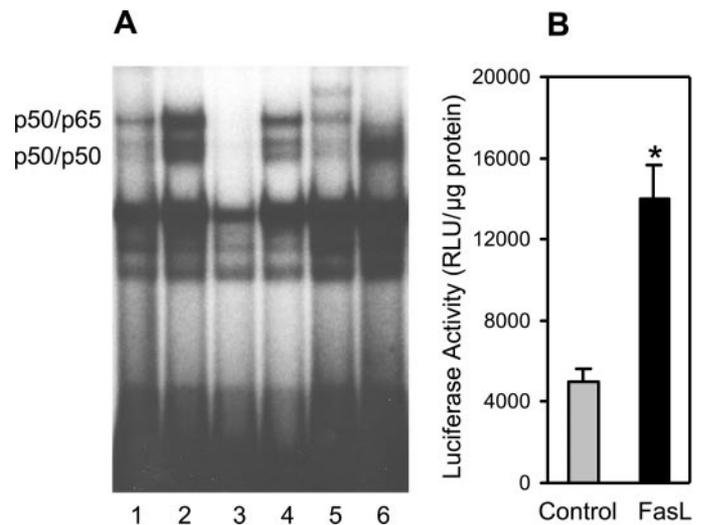


Fig. 3. FasL-induced nuclear factor (NF)- κ B activation. A: electrophoretic mobility shift assay (EMSA) studies of cells treated with FasL (200 ng/ml) for 2 h (peak response time). Lane 1, control cells; lane 2, FasL-treated cells; lane 3, cold NF- κ B probe; lane 4, cold activator protein-1 probe; lane 5, p50 antibody; lane 6, p65 antibody. B: transcriptional activity of NF- κ B-dependent luciferase gene. Cells were transfected with NF- κ B-luciferase plasmid (1 mg/ml) for 24 h and then treated with FasL (200 ng/ml) for 12 h. RLU, relative light units. Values are means \pm SD; $n = 4$. * $P < 0.05$ vs. untreated control.

lular luciferase activity, further substantiating the activation of NF- κ B by FasL.

FasL-induced TNF- α expression and its effect on apoptosis. NF- κ B has been shown to play an essential role in regulating TNF- α expression (11, 47). Because our results showed that FasL was able to induce NF- κ B activation, we therefore investigated whether FasL could induce TNF- α expression in RAW cells. Treatment of the cells with FasL caused a strong induction in TNF- α protein expression, as analyzed by ELISA (Fig. 4A). This effect was both dose and time dependent with a peak response time of \sim 8 h (Fig. 4B). Gene transfection assay with the use of a TNF-luciferase reporter plasmid containing NF- κ B-binding sites similarly showed the activation of the TNF- α gene promoter by the FasL treatment (results not shown). The observation that FasL-induced TNF- α expression raised the possibility that TNF- α may be involved in FasL-mediated cell death. To test this possibility, cells were treated with FasL in the presence or absence of neutralizing anti-TNF- α antibody or control isotype-matched antibody. Figure 4C shows that treatment of the cells with either the anti-TNF- α or control antibody had no significant effect on FasL-induced apoptosis. Varying the concentration of the antibodies from 1 to 500 ng/ml gave similar results. These results indicated that TNF- α was not involved in FasL-induced apoptosis in RAW cells. To further confirm the observed absence of effect of TNF- α , we directly exposed the cells to purified TNF- α and measured its effect on cell apoptosis. Figure 4D shows that the exogenously administered TNF- α had no effect on cell apoptosis when used at the concentration range of 0.1–10 ng/ml. This con-

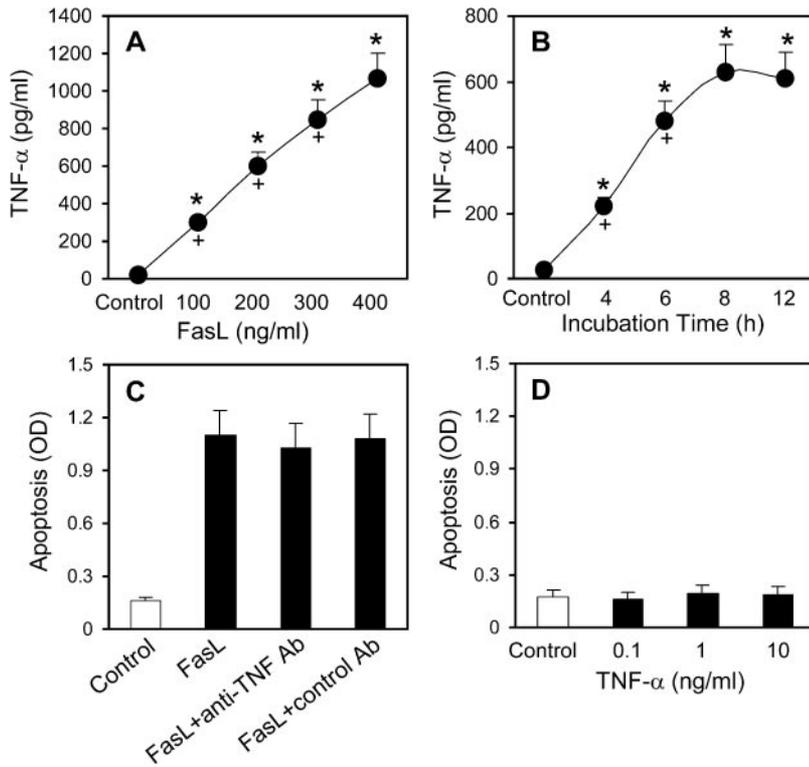


Fig. 4. FasL-induced TNF- α expression and its effect on apoptosis. *A*: dose effect of FasL on TNF- α protein expression. Cells were treated with FasL (0–400 ng/ml) for 12 h and analyzed for TNF- α by ELISA. *B*: time course of TNF- α induction by FasL (200 ng/ml). *C*: effect of neutralizing anti-TNF- α antibody (Ab) on FasL-induced apoptosis. Cells were treated for 12 h with FasL (200 ng/ml) with or without antibody (200 ng/ml) and analyzed for apoptosis by ELISA. *D*: effect of TNF- α on apoptosis in the absence of FasL. Values are means \pm SD; $n = 4$. * $P < 0.05$ vs. untreated control. + $P < 0.05$ vs. maximum response at 400 ng/ml in *A* or at 8 h in *B*. No significant difference was observed among the FasL-treated groups with or without antibody (*C*) and between the TNF-treated and control group (*D*); $P < 0.05$.

centration range was used on the basis of the TNF- α produced during FasL treatment (Fig. 4A). These results confirmed the finding from antibody experiments and indicated that TNF- α was not involved in FasL-induced apoptosis.

Role of NF- κ B in FasL-induced apoptosis. To investigate the role of NF- κ B in FasL-induced apoptosis, cells were treated with a specific NF- κ B inhibitor, CAPE, or transfected with a dominant form of the NF- κ B, I κ B, and their effects on FasL-induced apoptosis were examined. Figure 5A shows that treatment of the cells with CAPE caused an inhibition of FasL-induced NF- κ B activation. Such treatment also resulted in an increase in cell apoptosis induced by FasL (Fig. 5B). CAPE by itself had a partial apoptosis-inducing effect on the cells, suggesting the role of NF- κ B in the normal maintenance of cell apoptosis. Morphological analysis of the cells by TUNEL (Fig. 5, C–F) similarly indicated the antiapoptotic role of NF- κ B and its inhibition by CAPE.

The role of NF- κ B in regulating FasL-induced apoptosis was further examined using I κ B-transfected cells. A significant increase in cell apoptosis was observed in these cells in response to FasL stimulation (Fig. 6A). Inhibition of NF- κ B by I κ B expression was also shown to result in a decrease in TNF- α expression induced by FasL (Fig. 6B), indicating that TNF- α induction by FasL is dependent on NF- κ B activation. The observed inhibition of TNF- α expression along with the increased level of apoptosis in I κ B-transfected cells argues against the role of TNF- α in FasL-induced apoptosis and supports our earlier findings.

DISCUSSION

FasL binding to the Fas receptor initiates apoptosis in a variety of cell types. However, not all cell types that express Fas are susceptible to Fas-induced apoptosis (28). For example, cells of the lymphoid origin need to be stimulated to become sensitive to Fas-induced cell death (2, 45). Such activation is required to increase Fas expression and consequently its competency to deliver the death signal. In this study, we have shown that the macrophage RAW 264.7 cells constitutively and abundantly express a functional Fas receptor. Activation of the cells by FasL induces dose- and time-dependent apoptosis (Fig. 1). Such induction is also associated with NF- κ B activation, the process that was shown to negatively regulate the FasL-induced apoptosis. This notion is supported by the following observations: 1) FasL increases the DNA-binding activity of NF- κ B and mobilizes its p50/p65 heterodimeric form (Fig. 2A), 2) it also increases NF- κ B promoter activity of the luciferase reporter gene (Fig. 2B), and 3) inhibition of NF- κ B by the specific NF- κ B inhibitor CAPE or by dominant expression of I κ B makes the cells more susceptible to FasL-induced apoptosis (Figs. 5 and 6).

The observed activation of NF- κ B and its suppressive effect on FasL-induced apoptosis are consistent with previous findings (13, 31, 33) but are contradictory to others (7, 36, 41). Because of the diverse functions of different cell types and their variable susceptibilities to apoptosis induction, it is likely that the observed discrepancies between these test results are

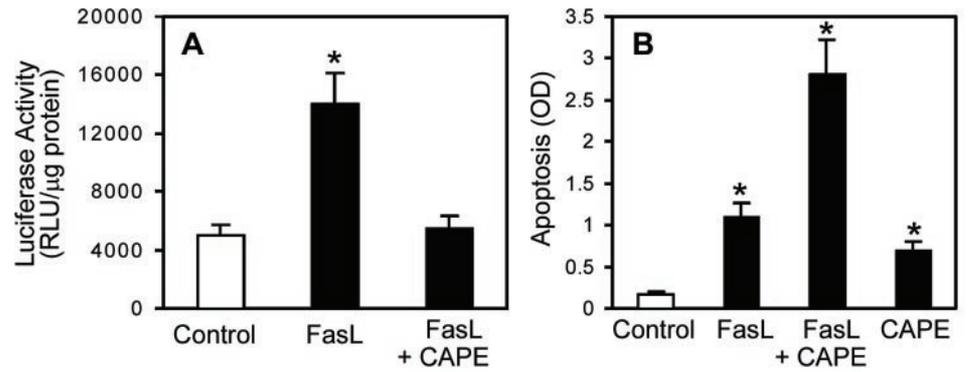
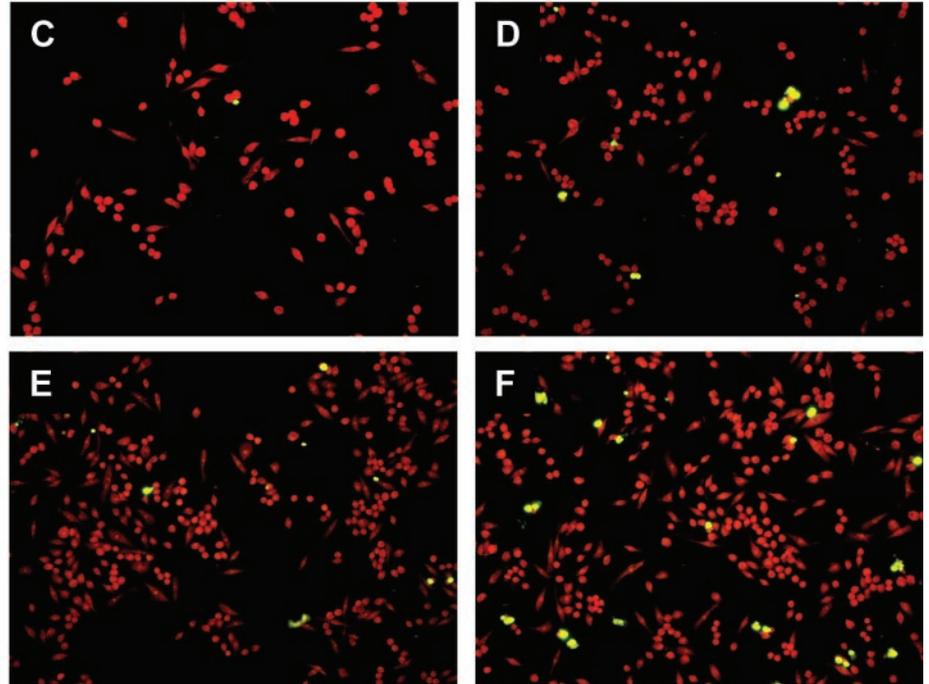


Fig. 5. Effect of NF- κ B inhibitor [caffeic acid phenylethyl ester (CAPE)] on FasL-induced apoptosis. *A*: cells were treated with FasL (200 ng/ml) with or without CAPE (0.5 μ g/ml) for 8 h. *B*: after the treatment, cells were analyzed for apoptosis by TUNEL using cytospin preparations. *C*: control cells. *D*: FasL-treated cells. *E*: CAPE-treated cells. *F*: cells treated with both FasL and CAPE. (Original magnification, $\times 200$.)



cell type specific. Some cell types express Fas, whereas others do not (14, 17). Additionally, some Fas-bearing cells need to be stimulated, as described above, to become sensitive to Fas-induced cell death. In sensitive cells, the activation of NF- κ B should lead to an increased expression of NF- κ B-dependent genes. One of the prototype genes that is under the dominant control of NF- κ B is the TNF- α gene (11, 47). TNF- α is produced principally by macrophages; therefore, activation of NF- κ B by FasL should lead to an increased expression of TNF- α . Our results showed that activation of the cells by FasL did indeed result in an increase in TNF- α expression (Fig. 4); the effect was shown to be dependent on NF- κ B activation, since inhibition of NF- κ B by I κ B inhibited such expression (Fig. 6B).

Because TNF- α is known to be a positive regulator of NF- κ B, its induction during FasL stimulation suggests its possible involvement in NF- κ B activation. Our results, however, showed that NF- κ B activation occurred relatively early and peaked at ~ 2 h (Fig. 3A), whereas TNF- α expression occurred at later times and peaked at ~ 8 h (Fig. 4B). These results, along with the observed low level of TNF- α expression relative to the

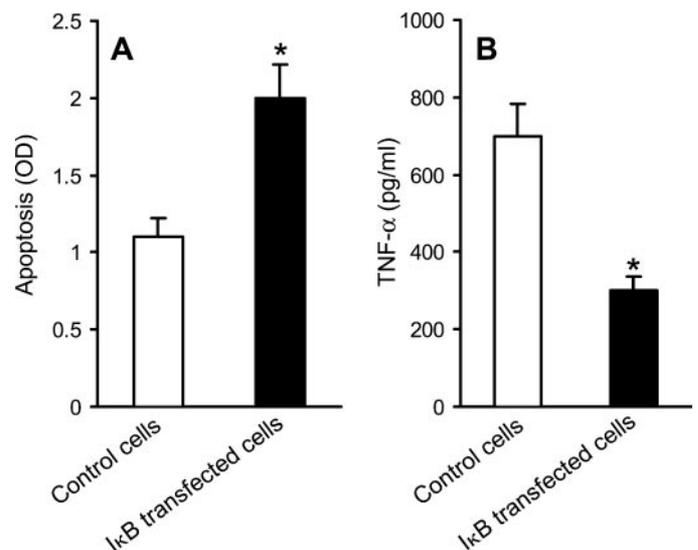


Fig. 6. Effect of inhibitory factor κ B (I κ B) transfection on FasL-induced apoptosis and TNF- α expression. I κ B-transfected cells were treated with FasL (200 ng/ml), and after 12 h they were analyzed for apoptosis (*A*) and TNF- α (*B*) by ELISA. Values are means \pm SD; $n = 4$. * $P < 0.05$ vs. control cells.

level of FasL in the system, suggest that the activation of NF- κ B during FasL treatment is mediated primarily by FasL. The mechanism by which FasL induces NF- κ B activation is unclear, but previous studies suggest that reactive oxygen species (ROS) may be involved (12, 18, 21, 40). Gulbins et al. (18) implicated superoxide anion as a functional mediator of Fas-induced cell death in Jurkat cells, whereas others (12, 21, 40) suggested the role of multiple ROS in various cell systems. However, Hug et al. (20) found no requirement of ROS in L929 murine fibroblasts stably expressing Fas. Because different cell types produce varying levels of ROS, their susceptibility to ROS-mediated NF- κ B activation is likely to be different. Supporting this notion is the observation that the ROS-producing macrophage is highly responsive to NF- κ B activation, as demonstrated in this study.

Because TNF- α is also known to be an apoptosis inducer, its activation during FasL treatment suggests its possible involvement in FasL-induced apoptosis. Furthermore, because Fas and TNF-R share common structural homologies and the activation of Fas, like TNF- α (33), causes mobilization of the p50/p65 heterodimer, it is therefore possible that the two ligands may mediate the death signal via a common signaling pathway. Using neutralizing anti-TNF- α antibody, we showed that inhibition of TNF- α by the antibody had no effect on FasL-induced cell death (Fig. 4C). Furthermore, direct exposure of the cells to TNF- α at the concentrations shown to be produced during FasL treatment failed to induce cell apoptosis (Fig. 4D). These results demonstrated that TNF- α was not involved in FasL-induced apoptosis under the experimental conditions. The inability of TNF- α to induce apoptosis may be a result of its relatively low level of expression during FasL stimulation. The activation of NF- κ B by FasL may also provide a survival signal that suppresses the potential apoptosis-inducing effect of TNF- α . These results are consistent with previous findings (4, 26, 36) and suggest that apoptosis mediated by FasL occurs via a separate signaling pathway independent of TNF- α . This finding is further supported by previous observations that FasL does not bind to TNF-R and that apoptosis induced by FasL is mediated by the Fas receptor (38).

In conclusion, we have shown that FasL can induce apoptosis of macrophage RAW 264.7 cells and activate the NF- κ B p50/p65 complex. Such activation negatively regulates FasL-induced cell death, supporting the antiapoptotic role of NF- κ B in this cell system. NF- κ B may mediate its antiapoptotic effect through the activation of protective proteins such as cIAP, TRAF, and IEX-1L, which has been previously reported (9, 42, 46). FasL activation of RAW cells is also associated with an increased expression of TNF- α , the process that is dependent on NF- κ B activation. Despite the structural similarities between FasL and TNF- α , and their receptors, FasL-induced apoptosis is independent of TNF- α , suggesting separate death signaling pathways. The ability of FasL to activate NF- κ B, a key transcription factor of various important genes, sug-

gests that this molecule may serve a much broader range of biological functions in addition to its role as an apoptosis inducer.

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