TNF-α-Induced Cell Death in Feline Immunodeficiency Virus-Infected Cells
Is Mediated by the Caspase Cascade

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INTRODUCTION

Feline immunodeficiency virus (FIV) was first isolated in 1987 by Pedersen et al. (1987) and belongs to the genus lentivirus, like human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). Cats infected with FIV show variable clinical signs according to the progression of the disease stage. At the AIDS stage, infected cats show debilitating symptoms such as profound anemia (Shelton et al., 1991), opportunistic infections (Ishida et al., 1992), and tumors (Beatty et al., 1995). The pathogenesis of FIV infection is not fully understood; however, it has been shown that the immune system is progressively impaired according to the advancement of the disease stage (Torten et al., 1991). FIV infection in cats results in nonspecific polyclonal activation of B cells which is mediated by increased production of IL-6 (Flynn et al., 1994; Lawrence et al., 1995; Ohashi et al., 1992). PBMC from symptomatic FIV-infected cats showed depressed IL-2 production in response to mitogens (Lawrence et al., 1992), accompanied by significant increases in IL-1, IL-6, and TNF-α production (Lawrence et al., 1995).

TNF-α is known as a proinflammatory cytokine produced in response to infection and inflammation. In FIV infection, many studies on the production of TNF-α have been reported. Serum TNF-α levels were shown to be increased in cats experimentally infected with FIV (Lehmann et al., 1992; Lawrence et al., 1992) as well as in those naturally infected with the virus (Lawrence et al., 1995). Kraus et al. reported increased TNF-α mRNA level intimately linked to the expression of FIV p26 in lymph nodes of FIV-infected cats (Kraus et al., 1996). In vitro culture, infection with one strain of FIV, FIV-Bl22, induced a significant increase in the mRNA level of TNF-α (Lin-enberger and Deng, 1999). Recently, it was reported that a positive correlation was found between viral RNA load and TNF-α mRNA expression in the central nervous system of cats acutely infected with FIV (Poli et al., 1999). These studies indicate that TNF-α is closely associated with the pathogenesis of FIV infection.

Two cell surface receptors, TNF receptor type I (TNFR I) and TNF receptor type II (TNFR II), have been identified as cellular receptors for TNF-α. It has been shown that both receptors are expressed in a wide variety of cell types, but TNFR I and TNFR II were shown to transduce signals in different manners (Wallach et al., 1999). TNFR I transduces death signals as well as survival signals, whereas TNFR II transduces only survival signals. TNFR I has an 80-amino-acid domain in the intracellular region, called the death domain, which is required for the transduction of the death signal. In general, induction of apoptosis by TNF-α is mediated by a family of interleukin...
1β-converting enzyme (ICE)-related proteases, the caspases. On reception of a death signal, a cascade of proteolytic cleavages results in the activation of the pre-existing inactive caspases, which finally destroy the cell. Caspases have been classified into two groups, upstream caspases, which act as initiators of the death signal from the cell surface receptor, and downstream caspases, which act as executioners of apoptosis. The downstream caspases cleave and inactivate proteins crucial for the maintenance of cytoskeleton, DNA repair, signal transduction, and cell cycle control and finally induce cell death. In addition to the death signal, TNFR I can also transduce a survival signal or an antiapoptotic signal, through the activation of NF-κB (Beg and Baltimore, 1996). The NF-κB family plays a key role in the regulation of cell growth and survival (May and Ghosh, 1998). In quiescent cells, NF-κB is inactive because of its localization in the cytoplasm, which is regulated by inhibitory proteins, the Iκ-B family. Following exposure of the cells to TNF-α, activation of NF-κB is initiated. Dissociation of Iκ-B from NF-κB leads to the generation of the nuclear localization signal in NF-κB, which allows NF-κB to translocate to the nucleus, where it serves as a transcriptional regulator. Unlike TNFR I, the signaling through TNFR II is unclear. Signaling through TNFR II requires its intracellular domain, which has no death domain. TNF-α usually induces cell death when active gene expression is inhibited, suggesting that the death signal is mediated by molecules present in a quiescent phase.

FIV infection in cats is associated with various immunological abnormalities and induces lymphopenia in the AIDS stage (Sparkes et al., 1993). Lymphoid depletion is observed at the terminal stage in both HIV infection in humans and FIV infection in cats (Kohmoto et al., 1998; Matsumura et al., 1994). In HIV infection, it has been suggested that the decrease of CD4+ T cells is mediated by the apoptotic mechanism (Jaroworski and Crowe, 1999). We and others reported that lymphocytes from FIV-infected cats are prone to die in short-term culture (Bishop et al., 1993; Guiot et al., 1993; Holznagel et al., 1998; Johnson et al., 1996; Momoi et al., 1996). In feline T-lymphoblastoid cell lines, FIV infection rapidly induces obvious apoptosis (Ohno et al., 1994). Moreover, we demonstrated that a feline fibroblastic cell line (CRFK) chronically infected with FIV underwent apoptosis after treatment with TNF-α (Ohno et al., 1993).

For cats, there are several reports describing the apoptotic mechanism and genes associated with apoptosis (Haagmans et al., 1996; Hofmann-Lehmann et al., 1998; Holznagel et al., 1996; Ikeda et al., 1997, 1998; Rojko et al., 1992, 1996). We have carried out molecular cloning of the feline Fas antigen, Fas ligand (Mizuno et al., 1998), and TNFR I (GenBank/EMBL/DDBJ Accession No. AB051103). Sequence of feline TNFR II gene is also available from the nucleotide sequence data base (GenBank/EMBL/DDBJ Accession No. AB051103). In this study, to identify the mechanism of apoptosis in FIV infection, we examined the expression of TNFRs and activation of the caspase and NF-κB pathways in CRFK cells infected with FIV that underwent apoptosis by treatment with TNF-α.

RESULTS

TNFRs were expressed in both CRFK and CRFK/FIV cells

To examine the difference of susceptibility to TNF-α in the induction of apoptosis between CRFK and CRFK/FIV cells, we first examined the expression of TNFR mRNAs by reverse transcriptase (RT)-PCR. Figure 1 shows the results of gel electrophoresis after RT-PCR of 15, 20, 25, and 30 cycles for TNFR I and TNFR II mRNAs. Amplified fragments of feline TNFR I and TNFR II were 215 and 244 bp, respectively, the sizes expected from the feline TNFR I and TNFR II cDNA sequences. The bands amplified from TNFR I mRNA became visible after 25 cycles of PCR and were distinct after the 30 cycles in both CRFK and CRFK/FIV cells. The bands amplified from TNFR II mRNA were also similar between CRFK and CRFK/FIV cells, although the bands in CRFK/FIV cells were slightly less intense than those in CRFK cells after 25 and 30 cycles. RT-PCR analysis carried out in this study indicated that the expression levels of TNFR I and TNFR II mRNAs were similar between uninfected CRFK cells and CRFK/FIV cells.

CRFK cells were sensitive to TNF-α-induced apoptosis in the presence of Actinomycin D (ActD) and Cycloheximide (CHX)

The TNFRs on CRFK/FIV cells were considered to be functional, because we previously showed that CRFK/FIV cells underwent apoptosis after treatment with TNF-α (Ohno et al., 1993). Many studies on apoptosis induced by TNF-α have shown that inhibition of RNA or protein synthesis sensitizes cells to apoptosis (Hill et al., 1995;
Kull and Cuatrecasas, 1981; Nio et al., 1990; Utasinsincharoen et al., 1999). To examine whether TNFRs on uninfected CRFK cells are functional, we examined whether treatments with ActD and CHX influence TNF-α-induced cell death in CRFK cells. As shown in Fig. 2, in the absence of ActD and CHX, cell death of up to 30% of the cells was observed in uninfected CRFK cells at a high concentration of TNF-α (10 ng/ml). Pretreatment with ActD and CHX resulted in a marked increase in cytotoxicity even at low concentrations of TNF-α (0.1 and 1 ng/ml). This result indicated that TNFR on uninfected CRFK cells was also functional, as shown in CRFK/FIV cells.

Caspase inhibitor blocked TNF-α-induced apoptosis in CRFK/FIV cells

To examine whether TNF-α-induced cell death in CRFK/FIV cells is inhibited by caspase inhibitors, three kinds of caspase inhibitors, Z-VAD-FMK, Z-DEVD-FMK, and Ac-YVAD-FMK, were added 2 h before treatment with TNF-α. Figure 3 shows the percentages of cytotoxicity measured by the release of LDH into the culture supernatants. The percentages of cytotoxicity were decreased by addition of Z-VAD-FMK or Z-DEVD-FMK in a dose-dependent manner in CRFK/FIV cells, but Ac-YVAD-CMK showed only partial inhibition of cell death in CRFK/FIV cells. These results indicate that TNF-α-induced cell death in CRFK/FIV cells is mediated through the activation of the caspase cascade.

We also examined the morphological changes in CRFK/FIV cells treated with TNF-α and the caspase inhibitors. Most of the CRFK/FIV cells underwent apoptosis after treatment with TNF-α (5 ng/ml) for 12 h, but the

FIG. 2. Induction of cell death in uninfected CRFK cells by treatment with TNF-α. Uninfected CRFK cells were treated with different concentrations of TNF-α 2 h after pretreatment with 0.1 µg of ActD per milliliter (open circles) or 0.1 µg of CHX per milliliter (open squares) or without either (closed circles). After treatment with TNF-α for 12 h, the percentages of cytotoxicity were determined by measurement of LDH activity released from the cells. Results are expressed as means ± standard deviation (SD) of triplicate assays.

FIG. 3. Inhibition of TNF-α-induced cell death in CRFK/FIV cells by three different caspase inhibitors. CRFK/FIV cells were pretreated with the indicated concentrations of caspase inhibitors Z-VAD-FMK (A), Z-DEVD-FMK (B), and Ac-YVAD-CMK (C) for 2 h and then cultured for 12 h in the presence of TNF-α (5 ng/ml) (open circles) or in its absence (open squares). After treatment with TNF-α for 12 h, the percentages of cytotoxicity were determined by measurement of LDH activity released from the cells. Results are expressed as means ± SD of triplicate assays.
apoptosis observed in CRFK/FIV cells was almost completely inhibited by the addition of Z-VAD-FMK (10 μM).

Treatment with a caspase inhibitor, Z-VAD-FMK, increased the production of FIV

Previously, we reported that RT activity declined in the culture supernatant of CRFK/FIV cells that underwent apoptosis after treatment with TNF-α (Ohno et al., 1993). To examine the effect of caspase inhibitor on virus production in CRFK/FIV cells, we examined RT activity in CRFK/FIV cells treated with TNF-α and/or Z-VAD-FMK. As shown in Fig. 4, RT activity in the culture supernatant of CRFK/FIV cells that underwent apoptosis induced by TNF-α treatment was significantly decreased. The decrease of RT activity induced by TNF-α treatment was almost completely restored, by addition of Z-VAD-FMK, to the level of the culture supernatant of CRFK/FIV cells before the treatment with TNF-α.

Cleavage of Poly(ADP-ribose) Polymerase (PARP) was detected in CRFK/FIV cells but not in uninfected CRFK cells after treatment with TNF-α

PARP is known as a main substrate for caspase 3, a caspase that functions at the final step of signal transduction of apoptosis (Nagata, 1997). It has been shown that activation of caspase 3 cleaves 113-kDa PARP into two fragments of 89 and 24 kDa. To confirm the activation of caspase 3 during TNF-α-induced cell death in CRFK/FIV cells, cell extracts prepared at different times after addition of TNF-α were analyzed by Western blotting using anti-PARP polyclonal antibody. As shown in Fig. 5, cleavage of 113-kDa PARP into the 89-kDa fragment was observed in CRFK/FIV cells from 3 h after the initiation of the treatment with TNF-α. No cleavage of PARP was found in uninfected CRFK cells during treatment with TNF-α for 12 h. The result indicated that caspase 3 was activated when TNF-α induced cell death in CRFK/FIV cells.

Activation of NF-κB was detected in both CRFK and CRFK/FIV cells treated with TNF-α

Nuclear extracts of CRFK and CRFK/FIV cells before and after addition of TNF-α (final concentration, 5 ng/ml) were analyzed by electrophoretic mobility shift assay (EMSA) (Fig. 6). In the cell extracts of uninfected CRFK cells, two upper bands became more intense 1 and 3 h after addition of TNF-α, and these bands were abolished by competition with unlabeled NF-κB motif oligonucleotide. Moreover, when the nuclear extracts were incubated with anti-p65 antibody prior to the addition of labeled NF-κB motif oligonucleotide, a supershift band larger than the NF-κB-DNA complex was generated, indicating the presence of p65 in the complex. The results of EMSA using the NF-κB motif oligonucleotide in CRFK/FIV cells were similar to those in uninfected CRFK cells. NF-κB was shown to be similarly activated by the treatment with TNF-α in both CRFK cells and CRFK/FIV cells, indicating that the NF-κB activation pathway was not associated with the difference in sensitivity to TNF-α for the induction of apoptosis between CRFK/FIV cells and uninfected CRFK cells.

The NF-κB activity in the uninfected CRFK cells and CRFK/FIV cells before and after treatment with TNF-α was also examined by an experiment using a reporter plasmid, pkBtkLuc construct, which contains three copies of NF-κB-binding sites and the firefly luciferase reporter gene (Fig. 7). In both the uninfected CRFK cells and the CRFK/FIV cells, the relative luciferase activity was increased 1 and 3 h after addition of TNF-α, indicating that the activity of NF-κB was increased after addition of TNF-α. Moreover, the activity of NF-κB as shown by the luciferase activity in CRFK/FIV cells was higher than that in uninfected CRFK cells before and after treatment of TNF-α, indicating that FIV infection did not inhibit RNA or protein synthesis in CRFK cells.
carried out for the samples treated with TNF-α. The supershift assay using anti-p65 NF-κB antibody was carried out for the samples treated with TNF-α for 3 h.

![FIG. 6. Activation of NF-κB induced by TNF-α in uninfected CRFK and CRFK/FIV cells. Nuclear extracts were prepared from uninfected CRFK (A) and CRFK/FIV (B) cells after treatment with TNF-α (5 ng/ml) for 1 or 3 h and then subjected to an electrophoretic mobility shift assay with radiolabeled NF-κB consensus oligonucleotide in the presence or absence of the unlabeled consensus competitor oligonucleotide. For supershift assay, before incubation with radiolabeled NF-κB oligonucleotide, the nuclear extracts were incubated with anti-p65 NF-κB antibody. The supershift assay using anti-p65 NF-κB antibody was carried out for the samples treated with TNF-α for 3 h.](image)

**DISCUSSION**

In this study, we investigated the molecular mechanism of TNF-α-induced apoptosis in CRFK/FIV cells. TNF-α rarely induces apoptosis even if TNF-α binds to TNFR unless RNA or protein synthesis is blocked, indicating that there may be some cellular factors synthesized in the cell which suppress the apoptotic signal generated by TNF-α. Thus, to examine whether TNFR on the cell surface of uninfected CRFK cells is functional, the CRFK cells were pretreated with ActD or CHX in addition to TNF-α. Low concentrations of TNF-α induced apoptosis in the uninfected CRFK cells in the presence of ActD or CHX, suggesting that the TNFR I was functional on the cell surface of CRFK cells.

TNFR I and TNFR II are known to exist as soluble forms, in the culture supernatant or serum, which are generated by proteolytic cleavage of authentic TNFR I and TNFR II. These soluble forms can block the binding of TNF-α to TNFRs. Thus, the expression of TNFR I and TNFR II mRNAs does not directly correlate with the level of expression of the membrane-bound molecules. Although we detected the expression of TNFR I and TNFR II mRNAs in both CRFK and CRFK/FIV cells in this study, we could not detect the proteins of TNFR I and TNFR II because specific antibodies directed to these molecules were not available. As mentioned above, however, cell death was observed in uninfected CRFK cells when ActD or CHX was added to the culture, suggesting that TNFRs were constitutively expressed on the cell surface of uninfected CRFK cells, as well as CRFK/FIV cells, in which apoptosis was easily induced by TNF-α even in the absence of ActD or CHX.

It has been shown that binding of TNF-α to TNFR stimulates not only the caspase cascade but also NF-κB nuclear translocation. We examined the activation of the caspase cascade and NF-κB translocation to identify the mechanism of difference in the sensitivity to TNF-α for induction of apoptosis between CRFK and CRFK/FIV cells. Fourteen molecules belonging to the caspase family have been found in mammalian cells and each of them participates in the caspase cascade to induce apoptotic cell death (Kumar, 1998). Based on their substrate specificity, the caspase family is divided into three groups. Group I, group II, and group III are composed of caspases 2, 3, 6, 7, 8, 9, and 10, caspases 1, 4, 5, and 11, and caspases 12, 13, and 14, respectively. The three caspase inhibitors used in this study, Z-VAD-FMK, Z-DEVD-FMK, and Ac-YVAD-CMK, inhibit caspases 1, 3, 4, and 7, caspases 3, 6, 7, 8, and 10, and caspases 1 and 4, respectively. Z-VAD-FMK and Z-DEVD-FMK efficiently blocked TNF-α-induced cell death in CRFK/FIV cells in this study, whereas Ac-YVAD-CMK showed only a partial effect. The results suggested that ICE protease and caspase 3 were involved in the TNF-α-induced cell death in CRFK/FIV cells. The partial effect of Ac-YVAD-CMK on the inhibition of apoptosis was possibly due to inefficient cellular uptake of this agent or the incomplete inhibition of caspases 1 and 4 by Ac-YVAD-CMK. Several caspase inhibitors have been shown to block apoptosis in various viral infections. Z-VAD-FMK blocked transmissible gastroenteritis coronavirus-induced apoptosis, which was mediated by the oxidative stress pathway, but not by inhibition of NF-κB (Sirinarumit et al., 1998). In bovine herpesvirus 1 infection, activation of caspase and p53 resulted in apoptosis (Devireddy and Jones, 1999). Parvovirus H-1 induced apoptosis in U937 cells, accompanied with activation of caspase and downregulation of c-Myc protein (Ray et al., 1998). It was also reported that hepatitis C virus core protein was bound to the cytoplasmic domain of TNFR I and enhanced TNF-α-induced apoptosis through blocking of NF-κB activation (Zhu et al., 1998). In HIV infection, Z-VAD-FMK was shown to inhibit Fas-induced apoptosis in peripheral blood CD4+ and CD8+ T cells of asymptomatic HIV carriers (Katsikis et al., 1997) and in a human T cell line infected with HIV.

We identified the cleavage of PARP in CRFK/FIV cells which underwent apoptosis induced by TNF-α treatment.
PARP, which is involved in DNA repair, is one of the substrates of caspase 3 during apoptosis. In this study, TNF-α was shown to trigger the cleavage of PARP and a broad caspase inhibitor could inhibit apoptosis induced by TNF-α, indicating that caspase 3 was activated when CRFK/FIV cells underwent apoptosis after treatment with TNF-α.

We previously reported that TNF-α treatment reduced virus production from CRFK/FIV cells (Ohno et al., 1993). The decrease of the virus production in the cells that underwent apoptosis induced by TNF-α treatment was shown to be restored to the level of the untreated cells in this study. It was shown that Z-VAD-FMK itself did not enhance the virus production in CRFK/FIV cells because RT activity in the CRFK/FIV cells did not change after treatment with Z-VAD-FMK alone. In HIV-1 infection, T-cell leukemia cells and peripheral blood mononuclear cells exposed to HIV-1 also showed enhanced viral production in the presence of Z-VAD-FMK (Chinnaian et al., 1997). It is conceivable that the increase of viable cells by inhibition of apoptosis leads to the enhancement of virus production. Because inhibition of cell death was shown to apparently enhance the virus production, inhibition of apoptosis with caspase inhibitors in vivo may not be appropriate for therapy in cats infected with FIV and humans infected with HIV-1.

Moreover, we examined NF-κB activation during TNF-α-induced apoptosis and found that activation of NF-κB occurred after treatment with TNF-α in both uninfected and FIV-infected CRFK cells. In both the EMSA and the NF-κB–luciferase reporter assay, the degree of NF-κB activation after treatment with TNF-α was apparently similar between the uninfected and the FIV-infected CRFK cells. From these results, it is conceivable that the different susceptibility to TNF-α in the induction of apoptosis between the uninfected and the FIV-infected CRFK cells cannot be explained by the difference in the activation of the NF-κB pathway. Prior to treatment with TNF-α, the band corresponding to the NF-κB–DNA complex in CRFK/FIV cells was less intense than that in uninfected CRFK cells in EMSA; however, luciferase activity derived from the pkBtkLuc reporter construct in CRFK/FIV cells was higher than that in uninfected CRFK cells in the NF-κB luciferase reporter assay. There seemed to be a contradiction between the results in these two different assays for the measurement of NF-κB activity. But, the results in the NF-κB luciferase reporter assay were conceivably more reliable because this assay reflects not only DNA binding of NF-κB but also the succeeding RNA and protein synthesis. The data obtained from the NF-κB luciferase reporter assay in this study also support the notion that FIV infection does not inhibit RNA or protein synthesis; therefore, the apoptosis induced in CRFK/FIV cells by TNF-α treatment can be interpreted as a phenomenon different from the apoptosis induced in TNF-α-mediated apoptosis in other cells.

FIG. 7. NF-κB luciferase reporter assay showing the activation of NF-κB induced by TNF-α in the uninfected CRFK and CRFK/FIV cells. Uninfected CRFK (A) and CRFK/FIV (B) cells transfected with the pkBtkLuc construct, which contains three copies of NF-κB-binding sites and the luciferase reporter gene, were treated with TNF-α (5 ng/ml) for 1 or 3 h and then subjected to the assay for the reporter luciferase activity, which is dependent on the NF-κB activity. Results are expressed as means ± SD of triplicate assays.
sis induced by chemical inhibitors such as ActD and CHX.

Binding of NF-κB to the long terminal repeat (LTR) of HIV-1 was shown to lead to the enhancement of HIV-1 replication. In this study, RT activity was reduced in CRFK/FIV cells after treatment with TNF-α. This result seems to conflict with the phenomenon in HIV-1-infected cells treated with TNF-α. Unlike HIV-1, there is no NF-κB binding site in the FIV LTR; therefore, virus replication was suppressed possibly due to the decrease of viable cells because of TNF-α-induced apoptosis.

Recently, membrane-bound TNF-α-mediated apoptosis was reported in HIV infection. Membrane-bound TNF-α expressed on macrophages was shown to induce apoptosis in CD8+ T cells expressing TNFR II (Herbein et al., 1998), but the mechanism involved in TNFR II-induced cell death was not clear. Lazdins et al. reported that membrane-bound TNF induced cooperative signaling of TNFRI and TNFRII, which resulted in the induction of cell death rather than virus production in the cells latently infected with HIV (Lazdins et al., 1997). In this study, we detected the expression of TNFR II mRNA in both CRFK and CRFK/FIV cells but the involvement of TNFRII in apoptosis was not analyzed. The mechanism of apoptosis mediated by the membrane-bound TNF will be investigated in further studies.

In this study, we showed that TNF-α-induced apoptosis in CRFK/FIV cells was mediated by activation of the caspase cascade but not by the increased expression of TNFRs or inhibition of NF-κB. Because the activation of caspase directly associated with the induction of apoptosis was clearly different between the uninfected CRFK cells and the CRFK/FIV cells, further investigation of the mechanism responsible for the activation of caspase by FIV infection would clarify the molecular basis for virus-induced apoptosis.

MATERIALS AND METHODS

Cells and cell cultures

A feline fibroblastic cell line derived from feline kidney (CRFK) and CRFK chronically infected with the Petaluma strain of FIV (CRFK/FIV) were used in this study. These cell lines were maintained in DMEM containing penicillin/streptomycin and 10% heat-inactivated fetal calf serum (FCS).

Caspase inhibitors

Three different classes of caspase inhibitors, Z-VAD-FMK (broad-spectrum inhibitor of ICE-like proteases), Z-DEVD-FMK (inhibitor of caspase 3-like enzymes), and Ac-YVAD-CMK (inhibitor of ICE) (Calbiochem, La Jolla, CA), were used in this study. All of the caspase inhibitors were dissolved in dimethyl sulfoxide and then diluted (25–200 μM) in the culture media.

Reverse transcriptase assay

Culture supernatants (1 ml per sample) were first centrifuged at 750 g for 10 min to remove cell debris and then recentrifuged at 20,000 g for 2 h. The resulting pellets were suspended in 10 μl of TNE buffer (0.1 M NaCl, 0.01 M Tris–HCl, pH 7.6, and 0.001 M EDTA) supplemented with 1% Nonidet P-40 and then mixed with 90 μl of RT assay buffer containing 90 μg of poly(A) (Sigma Chemical, St. Louis, MO) per milliliter, 20 μg of oligo(dT) (Amersham Pharmacia Biotech, Buckinghamshire, UK) per milliliter, 10 μM MgCl₂, and 10 μCi of [³H]dUTP (Moravek Biochemicals, CA). After incubation at 37°C for 1 h, the mixtures were spotted onto DE81 filter papers (Whatman International, Maidstone, England), air-dried, and washed three times with 5% Na₂HPO₄ and twice with 99% ethanol. The filter papers were then dried, and the incorporated radioactivity on the filter paper was measured with a scintillation counter. Group mean data were statistically compared by t tests. Differences between the groups were considered to be significant at P < 0.05.

Reverse transcription-PCR analysis

Total RNA was extracted from the cell lines using the acid guanidium–phenol–chloroform method with RNAzol (Biotex, Houston, TX). After removal of DNA contamination using DNase I (Life Technologies, Rockville, MD), 0.5 μg of total RNA was reverse-transcribed using the RNA PCR Kit (PE Biosystems, Foster City, CA) in the presence of oligo(dT). PCR amplification of TNFRI and TNFRII genes was performed as per the manufacturer’s instruction. For RT-PCR analysis, the reaction for the housekeeping β-actin gene was used as an internal control. The PCR consisted of 15, 20, 25, or 30 cycles of denaturation at 95°C for 1 min and annealing and polymerization at 60°C for 1 min followed by final extension at 72°C for 7 min. The amplified products were electrophoretically separated through a 3% agarose gel and visualized by staining with ethidium bromide. Each primer pair was designed to amplify partial fragments in the coding region of feline TNFRI, TNFRII, and β-actin genes. The sequences of the PCR primers were as follows: sense primer for feline TNFRI, 5′-CGA AGT GCC ACA AAG GGA CCT AC-3′ (nt 388–411 in the feline TNFRI gene, GenBank/EMBL/DDJB Accession No. AB051103), reverse primer for feline TNFRI, 5′-CTG TTT TCC TTC CTG CAG CCA CAC AC-3′ (nt 601–579); sense primer for feline TNFRII, 5′-CTC AGG CAG CAC GGC AGA CGG-3′ (nt 1–21 in the feline TNFRII gene, GenBank/EMBL/DDJB Accession No. U51429), reverse primer for feline TNFRII, 5′-GCC GGA GGA GCT GGC ATC G-3′ (nt 247–226); sense primer for β-actin, 5′-GGG TGC ACC CAC ACT GTG CCC ATC-3′ (nt 284–307 in the feline β-actin gene, GenBank/EMBL/DDJB Accession No. AB051104), reverse
primer for β-actin, 5′-ACG TCA CAC TTC ATG ATG GAG TTG-3′ (nt 672–649).

**Cytotoxicity assay**

Cytotoxicity induced by TNF-α was examined using the Cytotoxicity Detection Kit (LDH) (Roche Molecular Biochemicals, Mannheim, Germany). Prior to the assay, the cells (4 × 10⁶ cells/well) were cultured for 12 h. Then the culture supernatants were removed, and the cells were incubated in fresh serum-free medium (OPTI-MEM) (Life Technologies) containing 2% FCS with or without caspase inhibitors, 0.1 μg of ActD per milliliter (Sigma Chemical), and 0.1 μg of CHX per milliliter (MBL, Nagoya, Japan). Two hours later, the medium containing TNF-α was added to a final concentration of 0.1–10 ng/ml. The supernatants were harvested after treatment with TNF-α for 12 h and tested for cytotoxicity according to the manufacturer’s instructions. LDH activity estimated by the absorbance at 490 nm was measured with a spectrophotometer. To determine the percentage cytotoxicity, from the LDH activity of the sample, LDH activity in the background control was subtracted. The percentage of cytotoxicity was expressed as (value of sample – minimum control value) × 100/(maximum control value – minimum control value). Maximum control value corresponds to LDH activity obtained after complete lysing of untreated control cultures with 0.2% Triton X-100. Minimum control value corresponds to the LDH activity released from the untreated normal cells.

**Western blot analysis**

For Western blot analysis, cells were washed with PBS and then lysed in 100 μl of ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) (Boehringer Mannheim). The protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA). The protein preparations from the cell extracts were separated by SDS–PAGE and subjected to Western blot analysis using rabbit anti-pARP polyclonal antibody (Pharminingen, San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as a secondary antibody and the reaction was visualized using an ECL chemiluminescence system (Amersham Pharmacia Bio-Tech).

**Electrophoretic mobility shift assay**

Activated NF-κB was assayed using a gel shift assay kit (Promega, Madison, WI). Nuclear extracts were prepared according to the method by Schreiber et al. (1989). Briefly, 2 × 10⁶ cells were washed with cold PBS and suspended in 0.4 ml of hypotonic lysis buffer containing a protease inhibitors cocktail (Sigma Chemical). The cells were then lysed by the addition of 25 μl of 10% Nonidet P-40. The homogenates were centrifuged, and the resultant pellet was resuspended in 100 μl of ice-cold nuclear extraction buffer. After intermittent mixing for 30 min, the tubes were centrifuged at 20,000 g at 4°C for 5 min, and the supernatant (nuclear extract) was used for assay immediately or stored at −80°C for later use. The protein content was measured by the Bradford method. EMSA was performed according to the manufacturer’s instructions. Briefly, 10 μg of the nuclear extract was incubated with 17.5 pmol of 32P-end-labeled 21-mer double-stranded NF-κB motif oligonucleotide (Promega) at 37°C for 15 min. The incubation mixture included 2–3 μg of poly(dI–dC) in a binding buffer. The DNA–protein complex formed was separated from free oligonucleotide through native 6.6% polyacrylamide gel electrophoresis, and then the gel was dried. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, the nuclear extracts were incubated with 2 μg of anti-p65 NF-κB antibody (Oncogene Research Product, Cambridge, MA) for 1 h and then subjected to EMSA.

**NF-κB promoter assay**

Cells (1.0 × 10⁶) were cultured for 12 h and then transfected with the NF-κB-dependent luciferase reporter plasmid, pBlkLuc (Kashiwada et al., 1998), using the Effectene reagent (Qiagen, Chatsworth, CA) according to the manufacturer’s instruction. The pBlkLuc construct contains the firefly luciferase reporter gene together with three copies of the NF-κB-binding site derived from immunoglobulin κ light chain enhancer and thymidine kinase promoter. The pRL-TK construct (Promega), containing the Renilla luciferase gene, was co-transfected with the pBlkLuc construct to normalize the transfection efficiency by dual-promoter assay. Twenty-four hours after transfection, the cells were further cultured for 1 to 3 h in the presence of 5 ng of TNF-α per milliliter. After treatment with TNF-α, the cells were lysed and the luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega). Cell debris was removed by centrifugation at 20,000 g for 30 s, and the luciferase activity in 20 μl of the supernatant was measured in triplicate with an Autolumat LB953 luminometer (EG&G, Bethold, Germany). Values of firefly luciferase activity derived from pBlkLuc construct were standardized by values of Renilla luciferase activity derived from the pRL-TK construct. The values of the standardized luciferase activity were divided by the values of the luciferase activity derived from the pGL3Basic construct (Promega), which does not contain the NF-κB-binding site, and then the fold increases were calculated to show the activation of NF-κB in the cells.

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