Apoptosis Induced by Tumor Necrosis Factor in Cells Chronically Infected with Feline Immunodeficiency Virus

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Tumor necrosis factor alpha (TNF-α) induced morphologic changes such as chromatin condensation and cell shrinkage in a feline fibroblastic cell line (CRFK) chronically infected with feline immunodeficiency virus (FIV) but not in uninfected CRFK cells. DNA extracted from TNF-α-treated CRFK cells infected with FIV showed a ladder of nucleosomal DNA, indicating that this cytocidal effect by TNF-α was due to programmed cell death, or apoptosis. These findings may have implications for understanding the pathogenesis of FIV infection and for the design of specific therapeutic strategies for AIDS in humans as well as cats.

Feline immunodeficiency virus (FIV), as well as feline leukemia virus, is thought to be associated with feline AIDS (18, 27). The lymphocyte-proliferative response to mitogens is decreased at all stages in FIV infection (23), and selective depletion of the CD4$^+$ lymphocyte subset has been observed in experimentally infected specific-pathogen-free cats (1, 25). Recently, we reported that peripheral blood mononuclear cells from cats naturally infected with FIV were activated in vivo and that these cells were unresponsive to further stimuli in vitro (17).

Tumor necrosis factor alpha (TNF-α), also known as cachectin, is likely to play an important role in human immunodeficiency virus (HIV) infection. The serum level of TNF-α is increased in AIDS patients (12), and peripheral blood monocytes from HIV-infected patients spontaneously secrete more TNF-α than do monocytes isolated from uninfected individuals (13). TNF-α has been reported to activate replication of HIV in chronically infected cells (5, 15, 20), as do other factors such as interleukin-6 (19), granulocyte-macrophage colony-stimulating factor (10), and phorbol 12-myristate 13-acetate (5, 8, 20). Furthermore, TNF-α was found to have a direct cytocidal effect on chronically HIV-infected cells but not on uninfected cells in vitro (15). However the mechanism by which TNF-α exerts its cytotoxic effect on HIV-infected cells is not yet fully defined.

In this study, we demonstrated that recombinant human TNF-α induced programmed cell death (apoptosis) in cells chronically infected with FIV. The results reported here may provide useful information for understanding the pathogenesis of FIV infection.

MATERIALS AND METHODS

Cells and cell culture. The feline fibroblastic cell line CRFK was used as an uninfected control (2). CRFK cells chronically infected with a Petaluma strain of FIV (CRFK/FIV) (27) were kindly provided by T. Ishida, Nippon Veter-

inary and Animal Science University. These cells were maintained in RPMI 1640 containing gentamicin (20 μg/ml), L-glutamine (2 mM), and 10% heat-inactivated fetal calf serum. Both of these adherent cell lines were passaged with use of trypsin (0.05%) and EDTA (0.02%).

Cytokines. Human recombinant TNF-α and anti-TNF-α monoclonal antibody were obtained from Suntory Institute for Biochemical Research (Osaka, Japan). The purity of recombinant TNF-α (98.6%) was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The specific activity, confirmed by using L929 cells, was 2.8 × 10^6 U/mg.

Cytokine treatment. Cells were resuspended at 5 × 10^5/ml in RPMI 1640 supplemented with 10% fetal calf serum and cultured for 12 h to allow cell adhesion. Then the culture supernatants were removed, and the cells were cultured in fresh medium containing various concentrations of TNF-α. For the neutralization experiment, 10 ng of TNF-α was preincubated with 10 ng of anti-TNF-α antibody at 37°C for 1 h and applied to cultured cells. The supernatants were harvested 12 h later and tested for Mg^{2+}-dependent reverse transcriptase (RT) activity.

RT assay. The culture fluids (1 ml) were first centrifuged at 3,000 rpm for 10 min to remove cell debris and then recentrifuged at 15,000 rpm for 2 h. The resulting pellets were suspended in 10 μl of TNE buffer supplemented with 1% Nonidet NP-40 and then mixed with 90 μl of RT assay buffer containing 90 μg of poly(A) (Sigma) per ml, 20 μg of oligo(dT) (Pharmacia, Uppsala, Sweden) per ml, 10 mM MgCl$_2$, and 10 μCi of [^{3}H]dTTP (Amersham, Buckinghamshire, England). After incubation for 2 h at 37°C, the mixtures were spotted onto DE81 paper (Whatman International, Maidstone, England), air dried, and washed three times with 5% Na$_2$HPO$_4$ and twice with 99% ethanol. The paper was then dried, and incorporated radioactivity was measured in a scintillation counter.

Cell viability and morphology. Cells incubated with or without TNF-α were removed from the flasks by gentle scraping, and their viability was determined by the trypan blue dye exclusion test. Other samples were stained with Giemsa solution for morphologic examination. For transmission electron microscopy, CRFK/FIV cells cultured with or

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without TNF-α for 12 h were fixed with 2.5% glutaraldehyde in 0.1 μM cacodylate buffer (pH 7.0), postfixed with 1% osmium tetraoxide, and gradually dehydrated. These samples were embedded in Epon 812, sectioned, stained with uranyl acetate and citrate, and examined with a JEOL microscope.

DNA fragmentation assay. Cells (5 × 10⁶) incubated with or without TNF-α for 12 h were collected from the flasks by gentle scraping and centrifuged at 3,000 rpm. The resulting pellets were resuspended in 5 mM Tris-HCl (pH 7.4) containing 0.5% SDS, 2 mM EDTA, and 0.5 mg of proteinase K per ml and incubated at 50°C for 1 h. RNase (50 μg) was then added, and incubation at 50°C was continued for 1 h. DNA was extracted from the resulting viscous solution with phenol-chloroform, precipitated with ethanol, dried, and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The extracted DNA was mixed with the sample buffer containing 1 μg of ethidium bromide per ml and subjected to electrophoresis in 1.8% agarose gel.

Statistical analysis. The statistical significance of the differences between the means of the samples was determined by Student's unpaired t test. P values of less than 0.05 were considered significant.

RESULTS

Effects of TNF-α on FIV replication. For examination of the effect of TNF-α on replication of FIV, CRFK/FIV cells were cultured in the presence or absence of TNF-α for 12 h, and then the RT activity of the culture supernatant was determined. As shown in Fig. 1A, TNF-α suppressed FIV replication in a dose-dependent manner. Treatment with 100 U of TNF-α per ml induced 70% reduction in RT activity in CRFK/FIV cells. The suppression of FIV production was blocked by anti-TNF-α antibody (Fig. 1B), indicating that other impurities could be excluded in this experiment.

Morphologic change of CRFK/FIV cells cultured with TNF-α. As shown in Fig. 2A, after culture with TNF-α (100 U/ml) for 6 h, CRFK/FIV cells showed morphologic changes such as cell shrinkage and detachment from the culture flasks, whereas uninfected CRFK cells showed no morphologic changes. In the Giemsa-stained preparations, cell shrinkage and condensation of chromatin were apparent in most of these CRFK/FIV cells cultured with TNF-α (Fig. 2B). Transmission electron micrographs of TNF-α-treated CRFK/FIV cells also showed condensation of chromatin and cytoplasm together with disappearance of surface microvilli and enlargement of the endoplasmic reticulum (Fig. 2C), changes which are characteristics of apoptosis.

Cytocidal effect of TNF-α on CRFK/FIV cells. To determine the cytotoxic activity of TNF on these cells, we examined the viability of CRFK/FIV cells after treatment with TNF-α. The initial number of cells was adjusted to 2 × 10⁵/ml, and the cells were cultured for 12 h. TNF-α (1, 10, and 100 U/ml) was then added, and the viability of the cells was scored after 0, 24, 48, and 72 h of culture. As shown in Fig. 3, in the presence of 10 or 100 U of TNF-α per ml, the viability of CRFK/FIV cells markedly decreased after 48 h, whereas that of uninfected CRFK cells remained unchanged. After culture with TNF-α (100 U/ml) for 72 h, the viabilities of uninfected CRFK and CRFK/FIV cells were 84 and 19%, respectively. In the presence of TNF-α, the viability of CRFK/FIV cells did not change within 24 h of culture despite their morphologic changes.
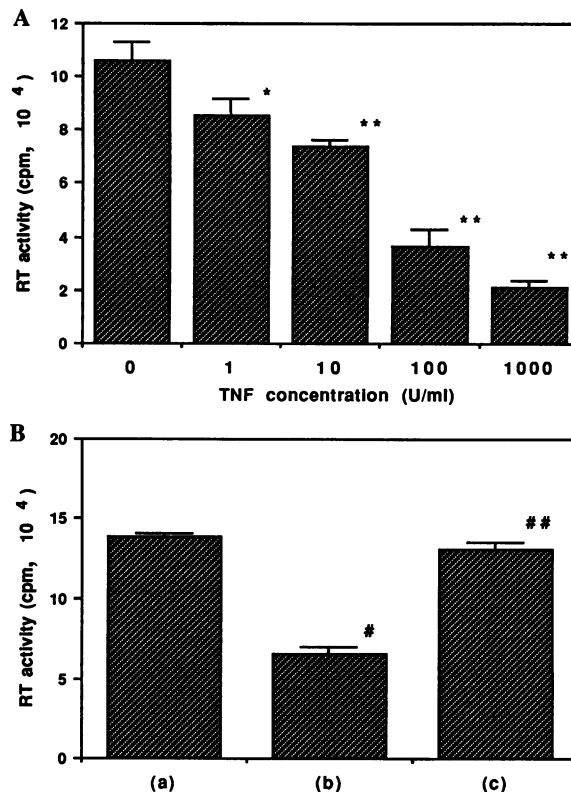

FIG. 1. Effect of TNF-α on FIV replication in CRFK/FIV cells. (A) CRFK/FIV cells (5 × 10⁵/ml) were cultured with TNF-α (0, 1, 10, 100, and 1,000 U/ml) for 12 h, and then the RT activity of the supernatants was assayed. Data are shown as mean values ± standard deviations of counts per minute determined in triplicate. Significant differences: ∗, $P < 0.05$; ∗∗, $P < 0.01$ (compared with the data obtained in the absence of TNF-α). (B) RT activity in the supernatant of CRFK/FIV cells (a), CRFK/FIV cells with TNF-α (10 ng/ml) (b), and CRFK/FIV cells with TNF-α (10 ng/ml) pretreated with anti-TNF-α antibody (10 ng/ml) at 37°C for 1 h (c). Each bar represent the mean ± standard deviation of counts per minute determined in triplicate. Significant differences: #, $P < 0.01$; ##, $P > 0.05$ (compared with the data for CRFK/FIV cells).

DNA fragmentation in TNF-α-treated CRFK/FIV cells. Morphologic changes such as cell shrinkage and chromatin condensation are characteristic of apoptosis, so we examined whether DNA fragmentation also occurred in CRFK/FIV cells cultured with TNF-α. As shown in Fig. 4, TNF-α-treated CRFK/FIV cells showed a ladder of multiples of 200 bp, indicating the fragmentation of nucleosomal DNA (lane 2), whereas that of untreated CRFK/FIV cells showed no fragmentation (lane 1).

DISCUSSION

A major immunologic abnormality in AIDS is considered to be selective depletion of CD4⁺ lymphocytes (4). HIV has a selective tropism for CD4⁺ cells, and active replication of virus usually induces host cell death (14). However, it is not reasonable to postulate that HIV alone is responsible for the reduction of CD4⁺ cells, and cofactors were postulated to act with HIV in the pathogenesis of AIDS. Among such cofactors, TNF-α has been reported to enhance HIV repli-
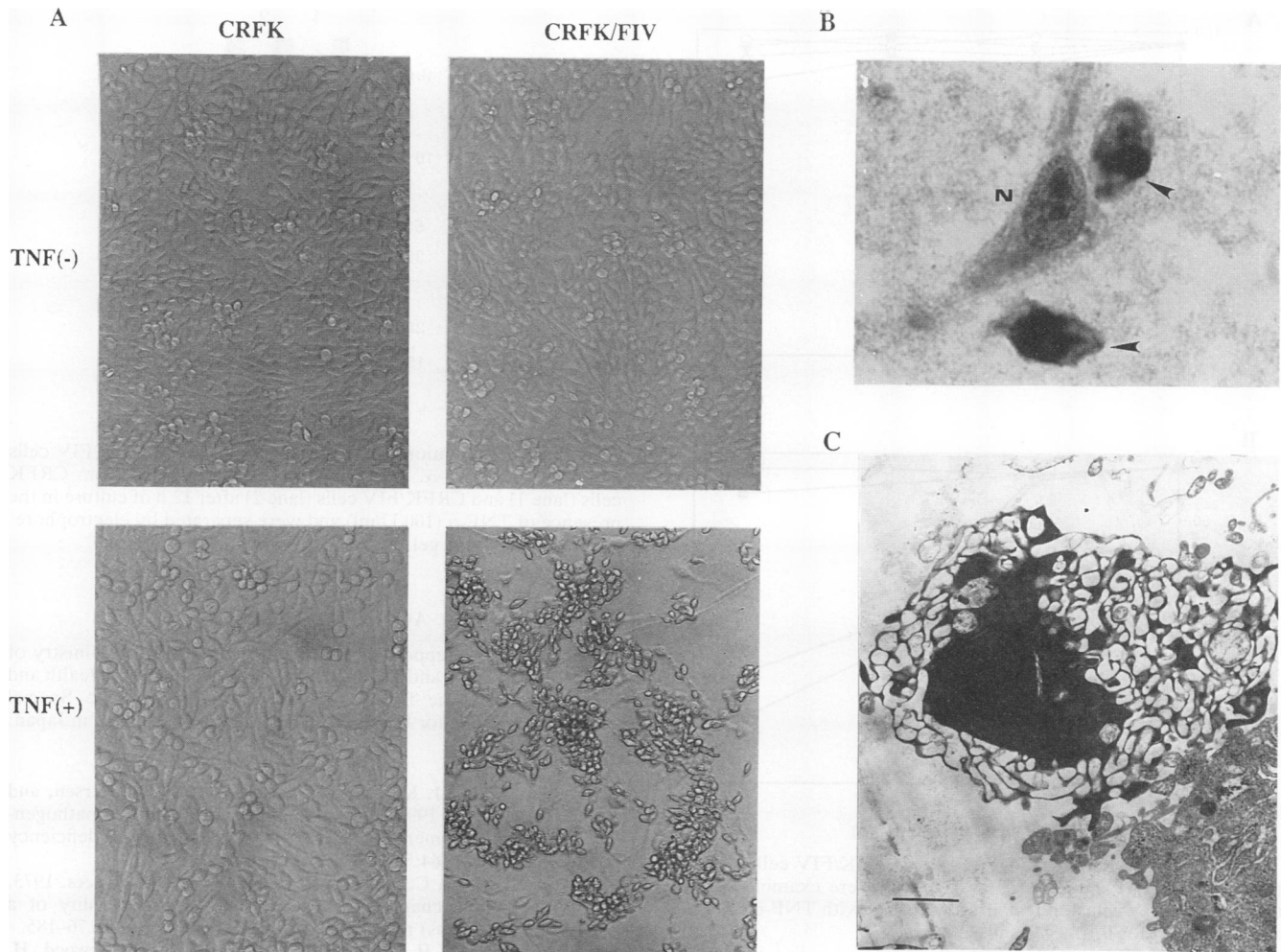
FIG. 2. Morphologic changes of TNF-α-treated CRFK/FIV cells. (A) CRFK and CRFK/FIV cells were cultured with or without 100 U of TNF-α per ml for 12 h, and then their morphology was examined by phase-contrast microscopy. TNF-α-treated CRFK/FIV cells showed cell shrinkage and detachment from the culture flasks. (B) Giemsa-stained preparation of TNF-α-treated CRFK/FIV cells, showing cell shrinkage and chromatin condensation (arrowheads). N, morphologically normal cells. (C) Transmission electron micrograph of TNF-α-treated CRFK/FIV cells, showing condensations of chromatin and the cytoplasm, disappearance of surface microvilli, and enlargement of the endoplasmic reticulum. Bar, 2 μm.

cation (5, 15, 20) and to kill HIV-infected cells (15), suggesting that TNF-α plays an important role in the pathogenesis of AIDS.

In this study, TNF-α was found to destroy CRFK/FIV cells but not uninfected CRFK cells. This result is consistent with the observation that TNF-α has a cytocidal effect on cells chronically infected with HIV (15). TNF-α-induced morphologic changes of CRFK/FIV cells were detected as early as within 3 h of cultures. The viability of these cells showing cell shrinkage and chromatin condensation did not alter within 24 h of culture, as judged by the trypan blue dye exclusion test. However, further cultivation with TNF-α caused cell death (after 48 h of culture). The cytopathic changes observed in TNF-α-treated CRFK/FIV cells were similar to those observed during programmed cell death (apoptosis) (22). Apoptosis is an active suicide process in which an endogenous endonuclease cuts DNA in nucleosomal linker regions while the cells are still viable. Some investigators have reported that TNF-α kills certain cell lines

by apoptosis (11, 21). Moreover, by a DNA fragmentation assay, we found that DNA extracted from TNF-α-treated CRFK/FIV cells showed a ladder of nucleosomal DNA, strongly suggesting that TNF-α induced apoptosis of CRFK/FIV cells. Apoptosis-mediated cell death has also been observed in the ligation of CD4 before signaling through the T-cell receptor (16) and acute HIV-1 infection (24). Therefore, apoptosis may account at least in part for the cytopathic effects in FIV infection as well as in HIV infection.

It is not clear how TNF-α selectively destroys FIV-infected cells by apoptosis without affecting uninfected cells. In preliminary experiments, we found that uninfected CRFK cells became sensitive to TNF-α cyotoxicity upon treatment with actinomycin D, suggesting the existence of a TNF-α receptor on both uninfected CRFK cells and CRFK/FIV cells. Recent findings indicate that several cellular genes such as bcl-2 (9), p53 (28), and c-myc (3) are associated with programmed cell death, suggesting that the sensitivity to
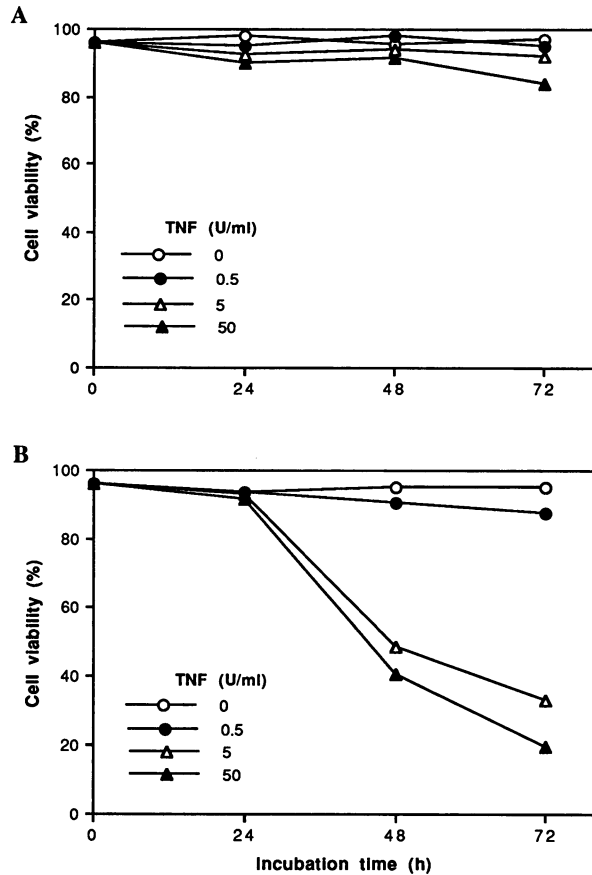
A

B

FIG. 3. Cytocidal effect of TNF-α on CRFK/FIV cells. Viabilities of CRFK (A) and CRFK/FIV (B) cells were examined by the trypan blue dye exclusion test after treatment with TNF-α (0, 1, 10, and 100 U/ml) for 0, 24, 48, and 72 h.

FIG. 4. Examination of DNA fragmentation in CRFK/FIV cells cultured with TNF-α. DNA samples were extracted from CRFK cells (lane 1) and CRFK/FIV cells (lane 2) after 12 h of culture in the presence of TNF-α (100 U/ml) and were separated by electrophoresis in 1.8% agarose gel.

TNF-α of CRFK/FIV cells is associated with modulations of such genes by FIV infection. Furthermore, double-stranded RNA is reported to enhance the sensitivity of cells to TNF-α-mediated cell death (7). Thus, conceivably the FIV RNA is associated with the sensitivity of CRFK/FIV cells to TNF-α.

In this study, in contrast to the accumulated findings observed in HIV-infected cell lines (5, 15, 20), recombinant human TNF-α suppressed viral replication in CRFK/FIV cells in a dose-dependent manner. Although the mechanism of the suppression of FIV replication is not clear, the programmed cell death that occurred in TNF-α-treated CRFK/FIV cells may be associated with this phenomenon. Recently, Wong et al. (26) reported that TNF-α and gamma interferon exerted synergistic blockade on the replication of HIV and killed HIV-infected cells. It might be also necessary to examine whether gamma interferon participates in the suppression of FIV replication by TNF-α.

TNF-α is one of the earliest products by the host response to many infections (6), so its induction by other viral or bacterial infections is likely to induce programmed cell death in FIV-infected cells. Thus, it is highly conceivable that TNF-α is associated with the pathogenesis of the immunodeficiency-like syndrome in FIV infection through the induction of apoptosis in the virus-infected cells. These findings may also have implications for the design of specific therapeutic strategies for AIDS in humans as well as cats.
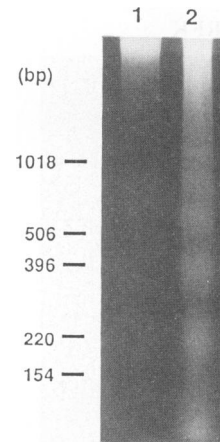
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