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Function of Nitric Oxide in Activation-Induced Cell Death of T Lymphocytes

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Using a murine T cell hybridoma, activation-induced cell death (AICD) was studied. As an *in vitro* model system for the AICD, T cell hybridoma expressing TCR/CD3 complex was incubated onto the immobilized purified anti-CD3 antibody. The immobilized anti-CD3 antibody induced AICD effectively up to 40%. At 1-100 μM range of SNP, an exogenous source of nitric oxide (NO), the cell proliferation was not affected, but at 1 mM SNP, cell proliferation was significantly reduced. The AICD of T cell hybridoma was inhibited by exogenous NO at non-cytotoxic concentration. In the cells undergoing AICD, the expressions of caspase-3 and FasL were detected, but not iNOS. Similar result was recognized in the apoptosis induced by dexamethasone, an apoptosis-inducing agent. However, the conversion from the inactive form of caspase-3 (32 kDa) to the active form (17 kDa) was significantly reduced in the cells in AICD induced by anti-CD3 antibody. With the result of increased PARP cleavage in the cells, we propose that another PARP cleavage pathway not involving caspase-3 may function in the anti-CD3 antibody induced AICD in the T cell hybridoma.

Apoptosis, or programmed cell death, is a cell-intrinsic process essential for animal development and tissue homeostatic balance. Mature resting T lymphocytes are activated when triggered via their antigen-specific T cell receptor (TCR) and co-stimulatory signals, to elicit an appropriate immune response. Subsequently, the activated T cells may undergo activation-induced cell death (AICD) in response to the same signals, which leads to termination of the cellular immune responses. Malfunction of this tightly controlled mechanism of cell death may result in cancer and various pathological conditions (Li and Yuan, 1999). The cell death pathway can be initiated in primed mature T lymphocytes by T cell receptor (TCR)-ligation with antigenic peptide, superantigen, or monoclonal antibody against the TCR/CD3 complex.

T cell apoptosis and AICD are controlled by survival cytokines such as interleukin-2 and by death factors such as tumor necrosis factor and CD95 ligand. Although it is not evident what events control TCR-induced expressions of death receptor and ligand, it requires TCR-stimulated signals from calcineurin (Shi et al., 1989; Williams et al., 1998; Sanzenbacher et al., 1999) and tyrosine kinases (Shi et al., 1989; Sanzenbacher et al., 1999), ZAP-70 (Anel et al., 1994; Eischen et al., 1997), and *lck* (Gonzalez-Garcia et al., 1997; Sanzenbacher., 1999). Inhibition of AICD is associated with a diminished TCR-induced tyrosine phosphorylation, reduced levels of CD3-inducible FasL mRNA and FasL surface expression and suppression of TCR-stimulated production of IFN-γ and IL-2 (Sanzenbacher et al., 1999).

Nitric oxide (NO) is produced by three different forms of nitric oxide synthases (NOS) (Nathan and Xie, 1994). Among the three isoforms, iNOS is usually not found in resting cells, but is transcriptionally induced by activating cytokines (e.g. IFN-γ) and microbial products (e.g. LPS) leading to sustained production of large amounts of NO (Nathan and Xie, 1994; Thuring et al., 1995; Brito et al., 1999). NO regulates T cell proliferation, cytokine production, apoptosis, and signaling activity *in vitro* (Duhe et al., 1998; Huang et al., 1998; Bogdan et al., 2000) and *in vivo* (Diefenbach et al., 1998; Hierholzer et al., 1998; Tarrant et al., 1999).

Recently, NO was reported to regulate the activity of caspase-3, which serves as a component of apoptotic signaling pathway, by nitrosylation. Since macrophages activated by antigen produce a large amount of NO, it is natural that NO affects the antigen-specific T cells interacting with the macrophages as antigen presenting cells. In this report, we have studied the effect of NO in cell death induced with immobilized anti-CD3 antibody in T cell hybridoma. Also biochemical signaling pathway with respect to caspase-3 activation and poly(ADP-ribose)

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Table 1. Nucleotide sequences of PCR primers

Primers	Sequences
iNOS	
Sense	5'- ACG-GGC-ATT-GCT-CCC-TTC-CGA-AGT-3'
Antisense	5'- ACC-GAA-GAT-ATC-TTC-ATG-ATA-ACG-3'
CPP32	
Sense	5'-AGT-CAG-TGG-ACT-CTG-GGA-TC-3'
Antisense	5'-GTA-CAG-TTC-TTT-CGT-GAG-CA-3'
β-actin	
Sense	5'-GTG-GGG-CGC-CCC-AGG-CAC-CA-3'
Antisense	5'-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC-3'

Table 2. PCR conditions

Reaction step	Temperature(℃)		Reaction time(sec)	
	I	II	I	II
Predenaturation	95	94	60	120
Denaturation	95	94	10	60
Annealing	55	55	60	60
Elongation	72	72	30	60
Postelongation	72	72	40	300
total cycles	30	30		

Symbol I indicates the RT-PCR condition for iNOS and II for caspase-3.

polymerase (PARP) cleavage in the AICD was studied.

Materials and Methods

Cell culture and reagents

SNP, propidium iodide, dexamethasone and S-methylisothiourea were purchased from Sigma (St. Louis, MO). [^{3}H]-thymidine was obtained from Amersham (Aylesbury, U.K.). The polyclonal antibodies against caspase-3 and PARP were purchased from Upstate Biotech (Lake Placid, NY) and Pharmingen (San Diego, CA), respectively. The 11-2D10 cells, a murine T cell hybridoma, and Jurkat cells, a human T cell line, were cultured in RPMI 1640 (Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) and 5% FBS (Life Technologies/BRL, Gaithburg, MD), and were maintained at 37℃ in a humidified incubator containing 5% CO_2. A hamster hybridoma secreting monoclonal antibody specific to mouse CD3ε (clone 145-2C11) was maintained in RPMI-1640 media.

Induction of activation-induced cell death

Anti-CD3 monoclonal antibody purified through Protein G column was allowed to adhere to flat-bottomed 96-well plates in 100 μl sodium carbonate buffer at 4℃ for 20 h. The 11-2D10 cells were plated onto the plate coated with various concentrations of the anti-CD3 antibody. After 3 days in culture, approximately 40-50% of cell death could be recognized by trypan blue exclusion. As a control, dexamethasone, a steroid, was added to the cells to induce apoptosis.

Nuclear DNA fragmentation

Cells incubated with the immobilized anti-CD3 antibody were harvested and washed with PBS. The cell pellets were resuspended in the extraction buffer (10 mM Tris-Cl, pH 8.0, 0.1 M EDTA, and 0.5% SDS), gently mixed by pipetting, and treated with RNase A (10 μg/ml) for 1 h at 37 ℃. After treatment with proteinase K (20 μg/ml), proteins were removed by phenol/chloroform extraction. The purified genomic DNA was run on 2% agarose gel and monitored for nucleosomal fragmentation.

Detection of apoptosis by subgenomic DNA contents

T cells incubated with immobilized anti-CD3 antibody were harvested and cell pellets were fixed with 75% ethanol on ice for 30 min. Cells were then stained with propidium iodide (50 mg/ml) and RNase A (50 mg/ml) for 30 min in the dark. The proportion of cells with subgenomic DNA content was determined using FACS Calibur (Beckton Dickinson, USA).

Cell proliferation assay

T cells (5×10^3 cells/well) were incubated with the immobilized anti-CD3 antibody for 3 days and pulse-labeled with [^{3}H]-thymidine (1 μCi/well) for 12 h. The cells were harvested onto glass wool papers using a cell harvester (PHD cell harvester, Skatron, Norway). The radioactivity incorporated into cells was determined using a liquid scintillation counter.

RT-PCR

Total RNA was isolated from activated T cells using RNA zol^B (Tel-Test, Inc). The isolated total RNA (3.0 μg) was reverse transcribed using M-MLV RT enzyme (Promega WSI, USA) and 1/20th of the reaction was subjected to PCR. The primers used and PCR conditions are illustrated in Table 1 and 2.

Western blot analysis

Cells were harvested and washed with PBS. Cell pellets were resuspended in an ice-cold hypotonic lysis buffer containing 50 mM NaCl, 0.02% NaN_3, 25 mM PMSF, 10 mM Tris-Cl [pH 7.2] and 0.5% NP-40. Approximately 50-100 μg of the cell lysates were run on SDS-PAGE and proteins were electrotransferred onto polyvinylidine difluoride membrane (Immobilon-P). The blots were probed with either anti-caspase 3 antibody (Upstate Biotech, Lake Placid, NY) or anti-PARP antibody (Pharmingen, San Diego, CA), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody as secondary antibody. Specific interaction with the primary and secondary antibodies was visualized using the enhanced chemiluminescent detection system (ECL^{TM}) from Amersham (Aylesbury, UK).

Results

Before inducing the AICD, we purified anti-CD3 monoclonal antibody from culture supernatant of the 145-2C11 hybridoma and tested its reactivity to CD3 molecules

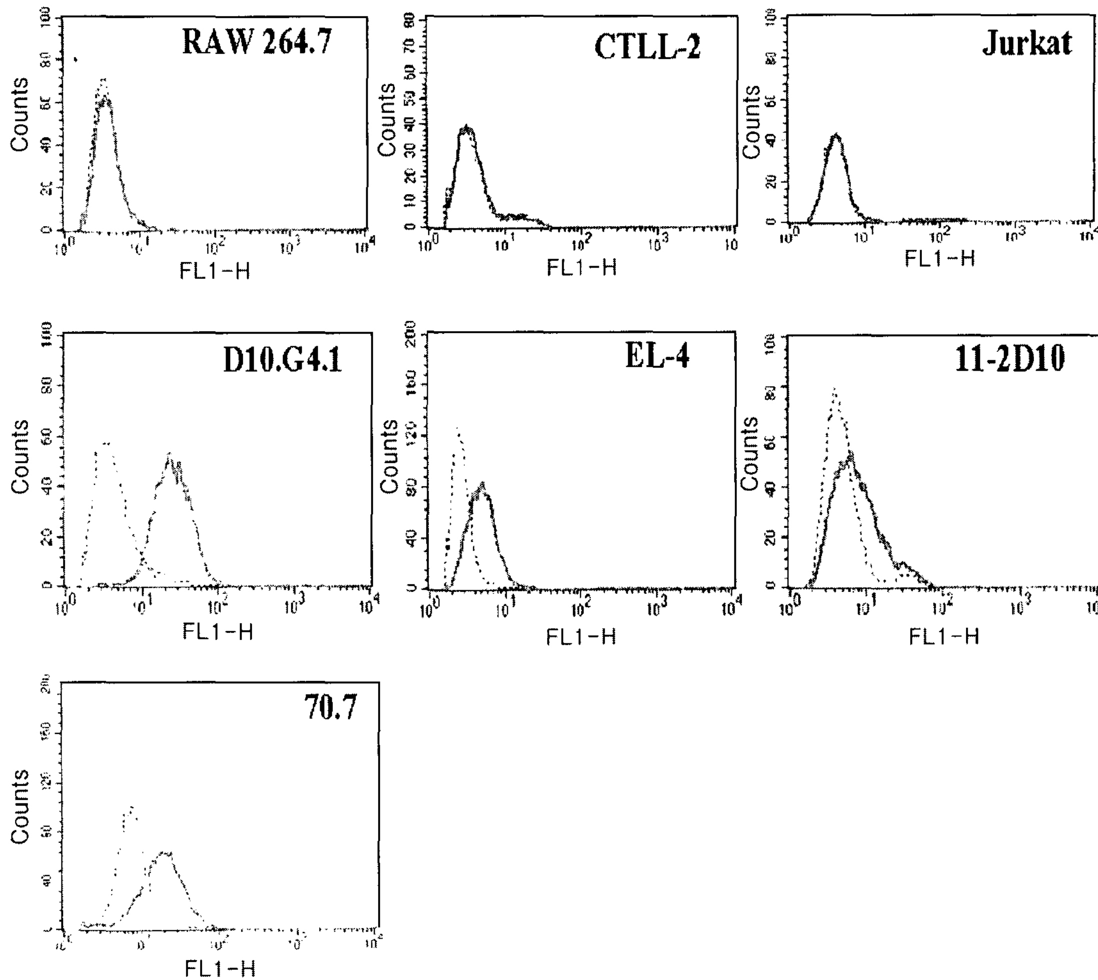

Fig. 1. Expression of CD3 on the various cell lines. Various cell lines were incubated with PBS (dotted line) or purified anti-CD3 antibody (solid line) and followed by FITC-conjugated goat anti-hamster IgG antibody. The stained cells were analyzed by flow cytometry.

on T lymphocytes. Indirect surface staining of CD3 expression was performed on various cell lines and analyzed by flow cytometry (Fig. 1). Compared with other cell lines of different origins, the mouse T cell lines D10.G4.1, 11-2D10, 70.7 and EL-4 all expressed CD3 molecules on cell surface, but RAW264.7 (macrophages), CTLL-2 (an IL-2 dependent human cytotoxic T cell line) and Jurkat (human T cell lymphoma line) did not. This result confirms that the purified anti-CD3 antibody has maintained the reactivity to mouse CD3 molecules. In a model system for AICD, T cell hybridoma cell line 11-2D10 was adopted. It is often assumed that AICD occurs in T cell hybridoma and in thymocytes via the same or similar mechanisms.

SNP-induced apoptotic cell death in T hybridoma

Nitric oxide itself is known to inhibit proliferation of T lymphocytes (Fehsel et al., 1995; Tai et al., 1997). NO production was measured by determining nitrite accumulation from the culture medium of cells using Griess reaction (Ding et al., 1988). To study the effect of exogenous nitric oxide on T cells, proliferation and cell death of T cells treated with SNP were examined (Fig. 2). At concentrations of 1 μM to 1 mM, SNP generated 10-150 μM of nitric oxide. T cell proliferation was not affected by the SNP at concentrations of 1-100 μM, but was significantly reduced at 1 mM. Similar effect was consistently observed for up to 3 days. Cell death was also prominent at 1 mM SNP, but was not obvious at lower concentrations (Fig. 2C).

Purified anti-CD3 antibody induced the AICD in T cells

The purified anti-CD3 antibody was tested for its inducibility of AICD. When 11-2D10 cells were incubated top of the immobilized anti-CD3 antibody, AICD was induced (Fig. 3A). In the plate coated with 100 μg/ml anti- CD3 antibody, proliferation of 11-2D10 cells was reduced by approximately 50%. The reduced cell growth

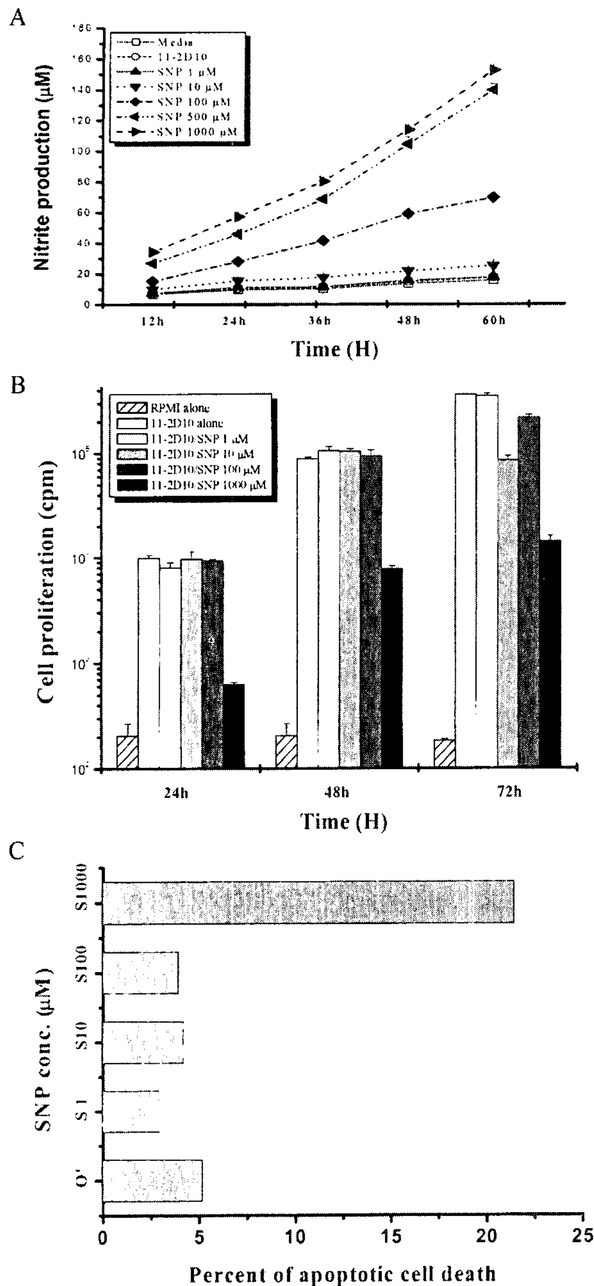

Fig. 2. Effects of SNP on the proliferation and the DNA fragmentation in T lymphocytes. Various concentrations of SNP were treated on 11-2D10 cells. After incubation for the indicated time periods, NO release was measured by the method of Griess (A). The effect of SNP on the proliferation of T cells was evaluated by ^{3}H-thymidine pulse labeling and measuring its incorporation into cells (B). After propidium iodide staining after incubation for 24 h with SNP, the effect of SNP on the cell death was evaluated by measuring the subgenomic contents(C).

resulted from cell death, which was confirmed by tryphan blue exclusion experiment (data not shown). The NO generation by the cells incubated with anti-CD3 antibody in the presence of SNP was equivalent to that from the cells incubated with SNP alone (Fig 3B). However, the cell death evaluated by subgenomic DNA fragmentation was significantly reduced by SNP (Fig 3C). This

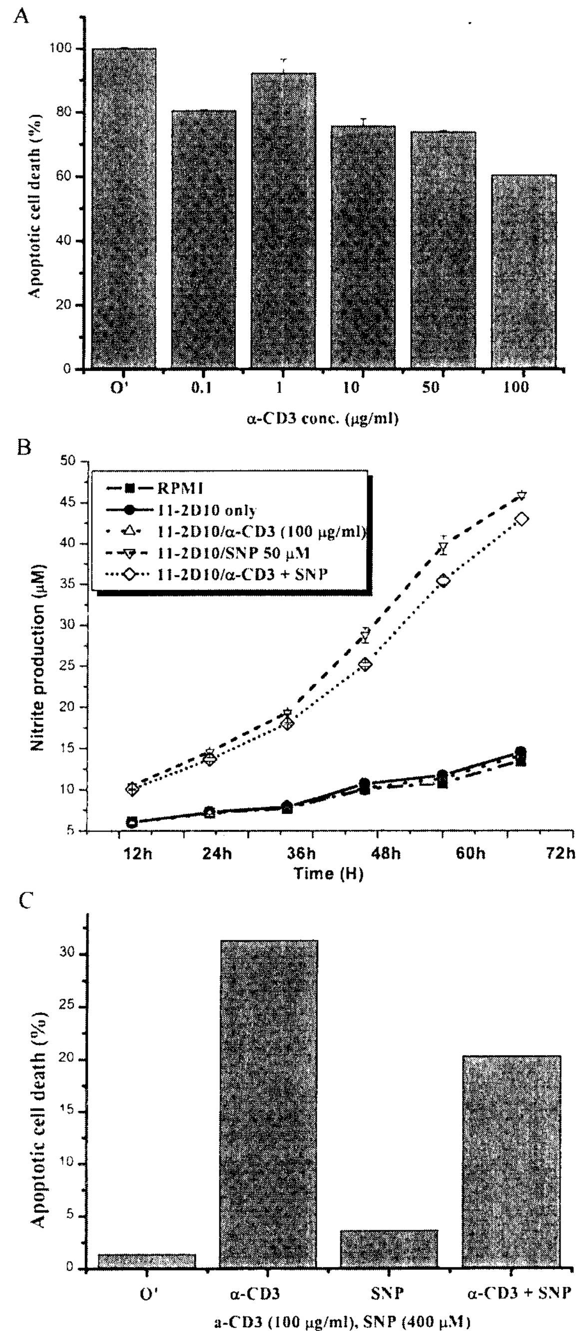

Fig. 3. Anti-CD3 antibody induced apoptosis in T cell hybridoma. 11-2D10 cells were stimulated with indicated concentration of anti-CD3 antibody immobilized on plastic wells. After three days, cell death was confirmed by trypan blue staining and the cell death was quantified by measuring the incorporation of ^{3}H-thymidine (A). After 11-2D10 cells were incubated onto immobilized anti-CD3 antibody in the presence of SNP, the production of nitrite was determined by Griess method (B). After the cells were incubated onto immobilized anti-CD3 antibody in the presence/absence of SNP, the subgenomic contents (apoptotic cells) were evaluated (C).

result suggests that endogenous NO generation during T cell AICD was negligible and that exogenous NO inhibits apoptotic cell death induced by immobilized anti-CD3 antibody.

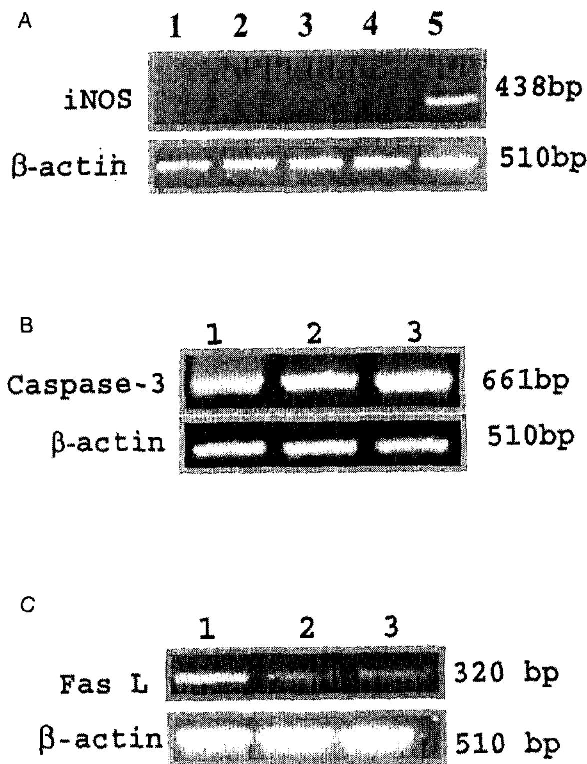

Fig. 4. Expression of iNOS and caspase-3 during the anti-CD3 antibody induced cell death. RT-PCR was conducted with mRNA prepared from 11-2D10 cells incubated with immobilized anti-CD3 antibody (A). Amplified PCR products using specific primers (Table 1) were separated on 1.2% agarose gel. Lane 1, untreated cells; lane 2, cells incubated with immobilized anti-CD3 antibody (50 μg/ml) ; lane 3, cells incubated with immobilized anti-CD3 antibody (100 μg/ml), lane 4, normal RAW264.7 cells; lane 5; RAW264.7 cells treated with IFN-γ (10 U/ml) and LPS (1 μg/ml). The expressions of mRNAs of both caspase-3 (B) and Fas ligand (C) in 11-2D10 cells were detected by RT-PCR. Lane 1, untreated; lane 2, anti-CD3 antibody (50 μg/ml) treated; lane 3, anti-CD3 antibody (100 μg/ml) treated.

Expression of caspase-3, iNOS, and FasL mRNA in the cells undergoing AICD

To analyze the expression pattern and enzymatic activity of iNOS, caspase-3, and FasL, we performed RT-PCR and Western blot analysis. Fig. 4 illustrates the mRNA expressions of iNOS (Fig. 4A), caspase-3 (Fig. 4B), and FasL (Fig. 4C). Induction of iNOS mRNA was not detected in the cells of AICD, but the expressions of mRNA of caspase-3 and FasL were detected. These results indicate that cross-linking of CD3 molecules on T cells does not induce iNOS expression, but the expressions of caspase-3 and FasL are maintained. Similar results were obtained from the apoptotic cells induced by dexamethasone (Fig. 5B right panel). When the cells were treated with dexamethasone, DNA fragmentation was clearly induced at 1 or 5 μM in 20-48 h (Fig. 5A). Similar to anti-CD3 antibody-induced cell death in 11-2D10 cells, there was no iNOS mRNA induction in the cells treated with dexamethasone (Fig. 5B left panel).

The cell death induced by immobilized anti-CD3 antibody does not involve caspase-3 enzyme activity.

Next, to verify caspase-3 precursor processing, the presence of the active form of caspase-3 was accessed in the cells treated with the immobilized anti-CD3 antibody (Fig. 6A). The 17 kDa caspase-3, an activated form of caspase-3, was significantly reduced in the cells undergoing anti-CD3 antibody-induced cell death. However, the cleavage of poly (ADP-ribose) polymerase (PARP), a common phenomenon in the cells undergoing apoptosis, was increased in the apoptotic cells (Fig. 6B). These results suggest that the AICD by anti-CD3 antibody does not involve caspase-3 activity directly. Furthermore, the increase in PARP cleavage indicates that another pathway not involving caspase-3 may function in the induction of cell death.

Taken together, exogenous NO inhibits the AICD of T cells induced by immobilized anti-CD3 antibody. In the AICD, the caspase-3 mRNA was increased, but the conversion from the inactive form of caspase-3 (32 kDa) to the active form (17 kDa) was significantly reduced. With the result of increased PARP cleavage in the AICD, we propose that another PARP cleavage pathway not involving caspase-3 may function in the anti-CD3 antibody induced AICD.

Discussion

NO appears to be involved in the T cell responses

including activation, differentiation, and proliferation (Huang et al., 1998; Brito et al., 1999). NO also modulates cytokine production by lymphoid cells (Eigler et al., 1995; Marcinkiewicz et al., 1995) and down-regulates IFNγ-induced MHC class II expression on astrocytes (Colasanti et al., 1993). However, it is generally not known what the critical role of NO in the immune responses is and in what phase NO plays a pivotal role in the immune response. Since naive, activated, and apoptotic T cells express different repertoire of genes, the effect of NO would be different depending on the specific stages of differentiation. In this study, we focused on the NO effect in the AICD induced by immobilized anti-CD3 antibody, which may occur in late stage of the T cell immune response.

In our study, exogenous NO at low concentrations significantly reduced the apoptosis of T cells in the AICD induction condition. T cells at day 3 undergoing apoptosis by immobilized anti-CD3 antibody were also induced to express caspase-3 mRNA, but there was little conversion to the activated form of caspase-3. However, we could not detect iNOS messages by RT-PCR, which may generate endogenous NO. These results suggest that exogenous NO, which may be provided by macrophages *in vivo*, prolongs the survival of T cells in the late stage of immune response. NO was reported to inhibit the proliferation of T cells in a mixed T lymphocytes culture (Hoffman et al., 1990; Albina et al., 1991; Mills, 1991; Huang et al., 1998) and CD8 T cells (Stefani et al., 1994). However, recent reports suggest that activity of CPP32 family in non-apoptotic cells is higher than in apoptotic cells

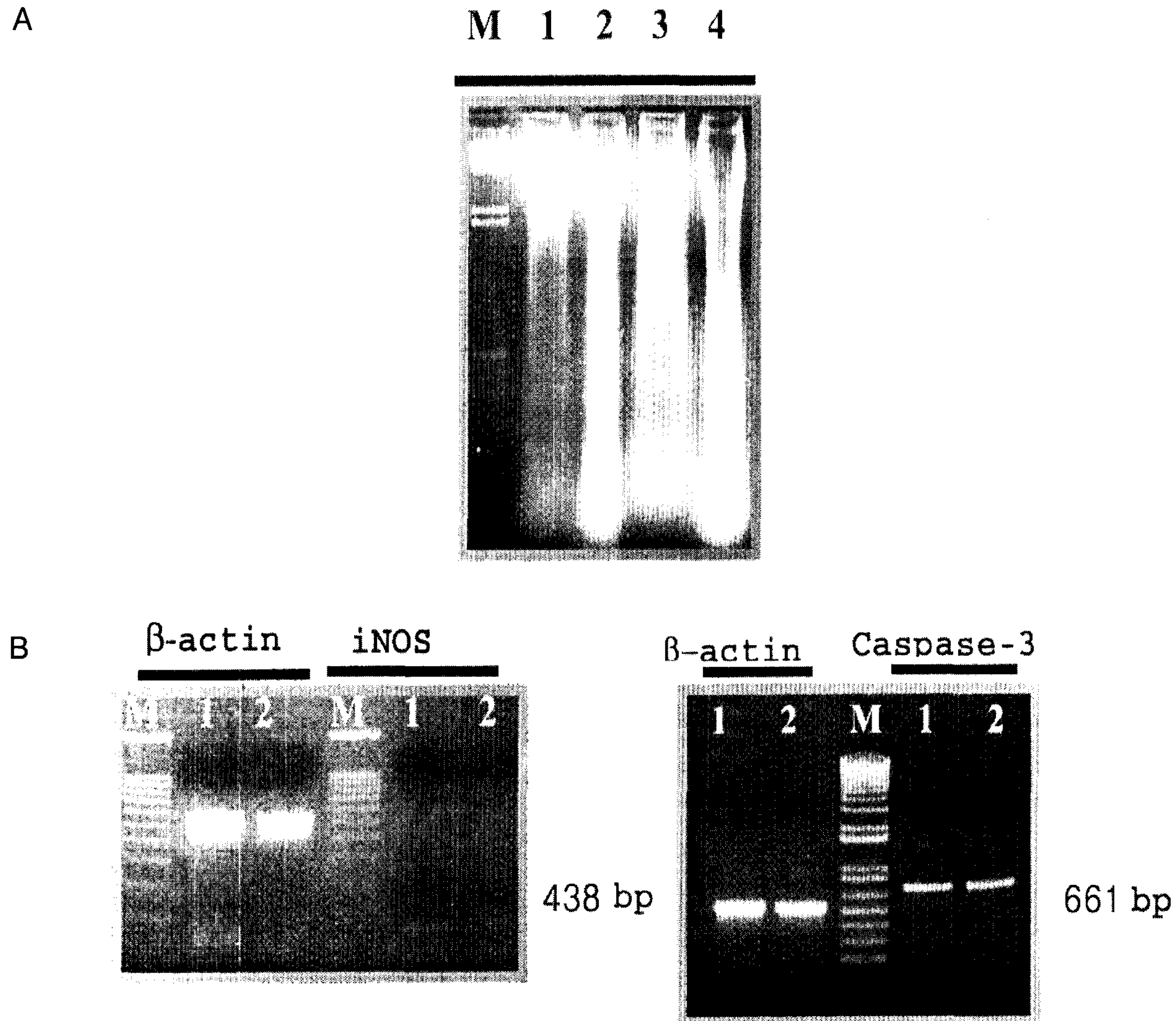

Fig. 5. *Dexamethasone-induced apoptosis in 11-2D10 cells. Cells* (2×10^6 *cells per group) were incubated with* 1 μM (lane 4) or 5 μM (lane 2) dexamethasone for 24 h (lane 1 and 2) or 48 h (lane 3 and 4). Cells were then harvested and DNA fragmentation was visualized in 2% agarose gel (A). The expressions of iNOS (B, left) and caspase-3 (B, right) were assessed by RT-PCR in the T cells treated with dexamethasone (5 μM) for 24 h.

(Miossec et al., 1997; Wilhelm et al., 1998; Woo et al., 1998; Alam et al., 1999; Kennedy et al., 1999). During apoptosis induced by Fas, NO donor suppressed Fas-induced caspase-3 activation (Mannick et al., 1999). A significant proportion of caspase-3 is S-nitrosylated intracellularly on the active site cysteine residues. Generally, the function of caspase-3 in cell death and cell proliferation is not clear. Thymocytes from caspase-3 null and wild-type mice were equally sensitive to induction of apoptosis by anti-CD95, dexamethasone, ceramide, staurosporin, and gamma-irradiation (Kuida et al., 1996), but peripheral T cells from caspase-$3^{-/-}$ mice were less susceptible to activation-induced cell death triggered by anti-CD3 (Woo et al., 1998). All these results with ours indicate that the caspase-3 may function differentially depending on in what stage the cells are, what the origins of cells are, and what the stimuli are. Further systemic analysis would clarify the function of caspase *in vivo*.

There was PARP cleavage, which is an indicative event in apoptosis, in the cells of AICD by anti-CD3 antibody. Also, redundancy in caspases function and its substrates was reported (Los et al., 1999). With the result of increased PARP cleavage in the cells of AICD, we propose that another PARP cleavage pathway not involving caspase-3 may function for the anti-CD3 antibody-induced AICD in the T cell hybridoma.

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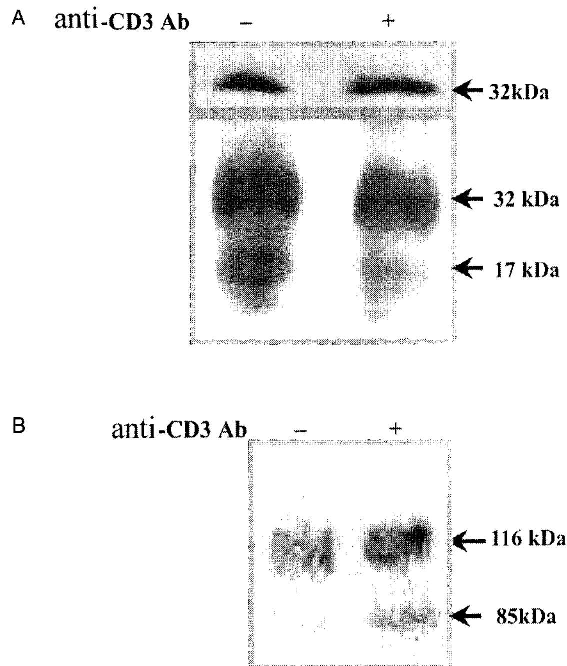

Fig. 6. Caspase-3 processing and PARP cleavage in immobilized anti-CD3 antibody and dexamethasone-induced cell death. 11-2D10 cells were incubated with immobilized anti-CD3 antibody and harvested after 3 d later in culture. Cell pellets were lyzed in ice-cold NP-40 lysis buffer for 1 h. Cell extracts (50 μg of proteins) were run on SDS-PAGE and proteins were transferred onto PVDF membrane. The membranes were probed with either anti-caspase-3 antibody (A) or anti-PARP antibody (B) and were followed by HRP-conjugated goat anti-rabbit IgG antibody. Bands were visualized using the ECL detection system.

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