J. Biochem. 131, 469–475 (2002)

Activation of Caspase-3, Proteolytic Cleavage of DFF and No Oligonucleosomal DNA Fragmentation in Apoptotic Molt-4 Cells

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Received September 25, 2001; accepted January 13, 2002

A variety of endonucleases has been implicated in apoptotic DNA fragmentation. DNA fragmentation factor (DFF) is one of the endonucleases responsible for DNA fragmentation. Since an oligonucleosomal DNA ladder is not induced in apoptotic Molt-4 cells, we investigated whether or not the absence of ladder formation is related to an inability of DFF endonuclease in the cells. Semiquantitative RT-PCR analysis showed that the mRNA level of DFF-40 and DFF-45 in Molt-4 cells was approximately the same, compared with in other cells, which exhibit different levels of the fragmentation in apoptosis. When Molt-4 cells were induced to undergo apoptosis by neocarzinostatin (NCS) treatment, both caspase-3 activation and DFF-45 cleavage were observed. Furthermore, DFF immunoprecipitated from Molt-4 cells exhibited DNA degradation activity. These results suggest that functional expression of DFF is not sufficient for the induction of DNA fragmentation in Molt-4 cells.

Key words: DFF, Molt-4, neocarzinostatin, oligonucleosomal DNA fragmentation.

Apoptosis is the programmed cell death for maintaining normal development, tissue homeostasis, and regulation of the immune system (*1*). Although many stimuli, including cytokines, hormones, growth factor withdrawal, and DNA-damaging agents, can trigger a variety of pathways to apoptosis, all the pathways converge to a common process involving the activation of cysteine aspartate proteases (caspases) (*2, 3*). Caspases usually exist in all living cells as inactive precursors that become activated when cells receive a signal to undergo apoptosis (*2, 3*). The activated caspases cleave multiple substrates, such as poly (ADP-ribose) polymerase (PARP), lamin, gelsolin, focal adhesion kinase, and DNA fragmentation factor-45 (DFF-45) (*2–7*). The activation of these caspases is linked to many of the well-characterized morphological changes associated with apoptosis, including cell shrinkage, apoptotic body formation, cytoskeletal changes, chromatin condensation, and DNA fragmentation (*2, 3*).

Oligonucleosomal DNA fragmentation is one of the hallmarks of apoptosis (*8, 9*). The DNA fragmentation results from activation of a variety of endogenous nucleases. Several nucleases have been proposed to be candidate apoptotic endonucleases, including caspase-activated endonucleases (*4–7*), Ca^{2+}/Mg^{2+}-dependent endonucleases (*10, 11*), acidic endonuclease (DNase II) (*12, 13*), and DNase γ (*14*). Among them, it has been well demonstrated that a caspase-activated endonuclease, DNA fragmentation factor-40 (DFF-40/CAD/CPAN), is associated with the internucleosomal cleavage of DNA during apoptosis (*4–7*).

DFF is a heterodimeric protein composed of DFF-40 and DFF-45 (ICAD) (*4–7*). DFF-45 is a dual function protein, serving as both an inhibitor of DFF-40 and as a specific molecular chaperone to mediate the correct folding of DFF-40 (*4–7, 15, 16*). Upon induction of apoptosis, DFF-45 is cleaved by caspase-3 at two sites and dissociates from DFF-40, resulting in the activation of DFF-40 endonuclease and leading to oligonucleosomal DNA fragmentation (*4–7, 17–19*).

In the present study, we have examined whether or not DFF is responsible for induction of oligonucleosomal DNA fragmentation in human leukemia Molt-4 cells, which are often used as a cell line exhibiting defective oligonucleosomal DNA fragmentation in apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Human promyelocytic leukemia HL-60, acute T-cell leukemia Jurkat, lymphoblastic leukemia Molt-4, non-B, non-T acute lymphoblastic leukemia NALL-1, and prostatic carcinoma LNCaP cells were cultured in RPMI-1640 medium containing 10% fetal calf serum under a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation—Cell proliferation was evaluated by measuring the fluorescence intensity in the presence of Alamar Blue (Wako Pure Chemical Industries) (*20*). Cells were seeded in 96-well multidishes (Costar) at a density of 1.8×10^4 cells per well in culture medium, incubated overnight, and then treated with neocarzinostatin (NCS) for 24 h. To each well, 20 μl of Alamar Blue was added, and then the plate was preincubated for 4 h. The fluorescence intensity was measured using a Cytofluor 2350 with excitation

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Abbreviations: Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide); DEPC, diethylpyrocarbonate; DFF, DNA fragmentation factor; FITC, fluorescein isothiocyanate; G3PDH; glyceraldehyde-3-phosphate dehydrogenase; NCS, neocarzinostatin; PBS, phosphate-buffered saline.

at 530 nm and emission at 590 nm.

Assaying of Apoptosis by Nuclear Staining—Cells were treated with NCS for the indicated times, collected, and then washed twice with ice-cold phosphate-buffered saline (PBS). The cells were fixed with 1% glutaraldehyde in PBS for 30 min and then washed twice with PBS. After washing, the cells were stained with 0.2 mM Hoechst 33258 in PBS, and then photographed by fluorescence microscopy under a Zwiss Axioplan microscope.

Assaying of Apoptosis by Annexin Staining—Annexin staining was performed as described previously (*20*). Briefly, after NCS treatment, Molt-4 cells were collected, and washed twice with PBS and then once with a binding buffer (Hepes-buffered saline solution supplemented with 2.5 mM calcium chloride). The cells were resuspended in the binding buffer at the concentration of 1×10^5 cells/ml. Propidium iodide and fluorescein isothiocyanate (FITC)-labeled annexin V were added to the solution, and then samples were incubated for 15 min before being analyzed within 1 h with a FACScan (Becton-Dickinson) using LYSYS-2.

DNA Fragmentation Assay—5×10^5 cells were collected at 600 ×*g* for 5 min, and then washed twice with ice-cold PBS. The cell pellets were lysed in 400 μl buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X100), and treated with 100 μg/ml proteinase K for 5 h at 37°C and then with 50 μg/ml RNase overnight at 37°C. Proteins were removed, and the DNA was precipitated with ethanol. The DNA was separated by electrophoresis on 2.0% gels and visualized by staining with ethidium bromide.

Semiquantitative RT-PCR—Total cellular RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and then quantified by measuring the absorbance at 260 nm. One microgram of total RNA was used to synthesize the first strand cDNA using a SuperScript II (Life Technologies), and PCR amplification was performed in a thermal cycler. The numbers of PCR cycles and conditions for denaturation, annealing and extension were 28 cycles, 1 min at 94°C, 1 min at 52°C, 1 min at 72°C for DFF-45; 28 cycles, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C for DFF-40; and 26 cycles, 45 s at 94°C, 45 s at 60°C, 2 min at 72°C for G3PDH. The primers used in this study were 5′-GATGCAGTAGACACGGGTATC-3′ and 5′-AAAATTGGTGGAACGGCGTA-3′ for DFF-45; 5′-CTCTGGGGTACTCGTTGGAT-3′ and 5′-ACTGCTGTTCAGATCCGCGT-3′ for DFF-40; and 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Amplification was performed under the described conditions, and followed a linear relationship (data not shown). The control reaction for RT-PCR was performed by replacing the RNA sample with DEPC-treated water. G3PDH served as an internal RNA control. The amplified DNA was then electrophoresed on 2.0% agarose gels and visualized with ethidium bromide. Band intensity analysis was performed with a Macintosh computer using the public domain NIH Image program.

Immunoprecipitation—HL-60 and Molt-4 cells were collected and lysed with buffer A (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin) for 15 min on ice and then homogenized. The cell extracts were centrifuged at 15,000 ×*g* for 10 min. To abolish the non-specific binding to protein A/G-agarose (Santa Cruz), the cell extracts (2 mg protein) were incubated with a 20 μl aliquot of protein A/G-agarose at 4°C overnight, and then supernatants were collected by centrifugation. The protein A/G-treated cell extracts were used for immunoprecipitation.

A 20 μl aliquot protein A/G-agarose was incubated with 5 μl of preimmune or immune serum for DFF-45 at 4°C for 2 h. The antibody-protein A/G-agarose beads were pelleted by centrifugation. After washing four times with buffer A containing 1 mg/ml BSA, the antibody-protein A/G agarose beads were incubated with the protein A/G-agarose-treated cell extracts at 4°C for 8 h, and then washed six times with buffer A. For the DNase assay, the beads were resuspended in 25 μl of buffer B (10 mM Hepes-NaOH, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml BSA), and then incubated with or without 370 ng recombinant caspase-3 (MBL) and 1 μg plasmid DNA. After 2 h, plasmid digestion was analyzed by 1.75% agarose gel electrophoresis.

Caspase-3–Like Protease Assay—The caspase activity in cell lysates was determined as described previously (*20*). Briefly, cells were lysed in extraction buffer (25 mM Hepes, pH 7.4, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin) for 20 min on ice and then sonicated. The lysates were centrifuged at 15,000 ×*g* for 15 min and supernatants were collected. The resulting cell extracts (100 μg protein) were incubated with 50 μM acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA; Peptide Institute) at 37°C for 60 min, the formation of amino-4-methylcoumarin being monitored fluorometrically with excitation at 380 nm and emission at 460 nm. Protein concentrations were determined by the Bradford assay (*21*) using BSA as a standard.

Western Blot Analysis—Cells (5×10^5) were collected by centrifugation, washed twice with ice-cold PBS, resuspended in 20 μl of SDS-sample buffer, and then boiled for 5 min. Samples were subjected to SDS-PAGE with 12% polyacrylamide. The proteins were then transferred to a nitrocellulose membrane, and probed with an anti–DFF-45 rabbit polyclonal antibody (MBL) or anti–caspase-3 rabbit polyclonal antibody (Pharmingen). Detection was accomplished with a horseradish peroxidase–conjugated goat anti–rabbit IgG and an ECL detection system (Amersham Life Science).

RESULTS

Effects of Antitumor Drugs on the Cell Growth of Various Human Cell Lines—The effects of antitumor drugs on cell viability were examined by means of the Alamar Blue assay. Antitumor drugs etoposide and NCS are inhibitors of DNA topoisomerase II and a DNA damaging agent, respectively (*22, 23*). As shown in Fig. 1A, NCS and etoposide reduced the cell viability in a dose-dependent manner. Molt-4 cells were more sensitive to the two antitumor drugs than the other cells, LNCaP cells exhibiting the lowest sensitivity. HL-60, Jurkat and NALL-1 cells exhibited intermediate sensitivity.

To determine whether or not the antitumor drug-induced cell death is apoptosis, the nuclear morphology of NCS-treated cells was investigated by fluorescent staining of cellular DNA with Hoechst 33258. As shown in Fig. 1B, when

the cells were treated with NCS, nuclear chromosome condensation, a typical morphological characteristic of apoptosis, was observed. To further confirm the cell death is apoptosis, the phosphatidylserine externalization of NCS-treated Molt-4 cells was examined. The externalization of phosphatidylserine could be detected on FITC-labeled annexin V staining since annexin binds phosphatidylserine exposed to the outer membrane in apoptotic cells (*24*). As shown in Fig. 1C, annexin-positive cells increased among NCS-treated cells. These results indicate that NCS induces apoptosis in these human cancer cell lines.

Lack of Oligonucleosomal DNA Fragmentation in NCS-Treated Molt-4 Cells—Nucleosomal DNA fragmentation is one of the major biochemical events for apoptosis. However, the fragmentation does not always occur in apoptotic cells (*25, 26*). To determine whether or not DNA fragmentation occurs in NCS-induced apoptotic cells, cellular DNA was extracted and electrophoresed in agarose gels. NCS was examined because it induces cell death more effectively than etoposide (Fig. 1A). The IC_{50} and IC_{80} values were used for the DNA fragmentation experiment, being approximately 0.3 and 1 μg/ml for HL-60, 0.5 and 1 μg/ml for Jurkat, 0.2 and 0.3 μg/ml for Molt-4, 0.3 and 0.5 μg/ml for NALL-1, and 5.0 and 10.0 μg/ml for LNCaP cells (Fig. 1A). Oligonucleosomal DNA fragmentation was observed in NCS-treated HL-60, Jurkat, NALL-1, and LNCaP cells (Fig. 2). On the other hand, in apoptotic Molt-4 cells, a high molecular weight DNA smear but no oligonucleosomal DNA fragmentation was detected (Fig. 2).

Activation of DFF in NCS-Treated Molt-4 Cells—It is known that an endonuclease (DFF-40/CAD) is activated on cleavage the inhibitor of DFF-45/ICAD, resulting in DNA fragmentation during apoptosis (*4–7*). Therefore, it is possible that induction of DNA fragmentation may be related to the expression level of DFF-40 or DFF-45. To determine whether the different levels of DNA fragmentation were correlated with different levels of the DNase or its inhibitor expression, we examined the expression level of DFF-40 and DFF-45 mRNA in various human cell lines.

After amplifying DFF-40 and DFF-45 mRNA by RT-PCR within a linear reaction rate range, mRNA expression was examined by agarose gel electrophoresis (Fig. 3A), and the mRNA levels were determined with NIH-image (Fig. 3B). The levels of DFF-40 mRNA in Jurkat and Molt-4 cells,

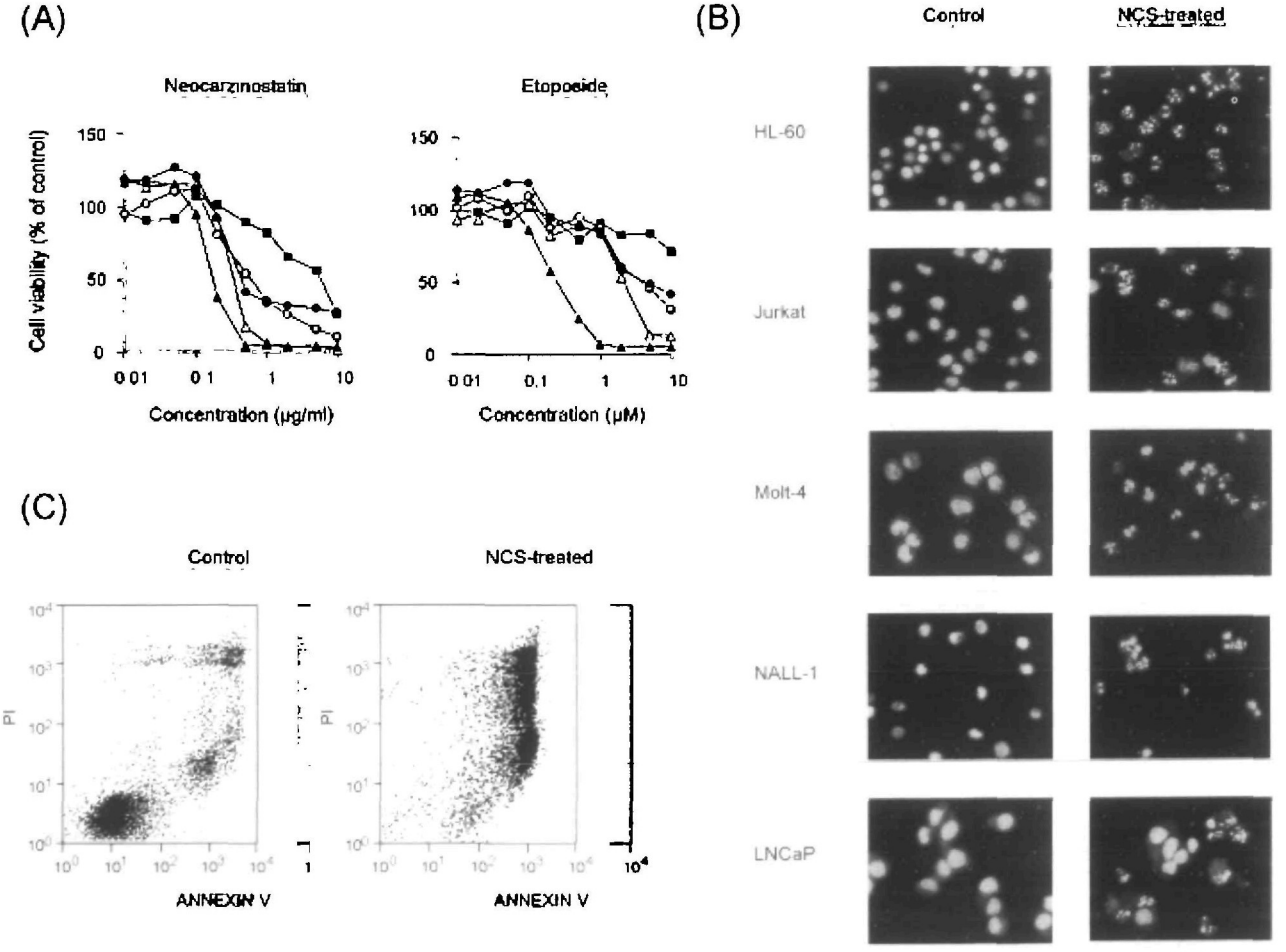

Fig. 1. **Induction of apoptosis by antitumor drugs in various human cell lines.** (A) Cytotoxic effects of antitumor drugs. HL-60 (●), Jurkat (○), Molt-4 (▲), NALL-1 (△), and LNCaP (■) cells were treated with the indicated concentrations of NCS or etoposide for 24 h, and then cell growth was examined by means of the Alamar Blue assay. Each value represents the mean for five cultures. (B) Nuclear morphological analysis. The cells were treated with NCS for 24 h and stained with Hoechst 33258, and then examined under a fluorescence microscope. (C) Phosphatidylserine externalization. Molt-4 cells were treated with NCS for 24 h. The treated cells were stained with FITC-annexin V and propidium iodide, and then examined by flow cytometry.

and DFF-45 mRNA in HL-60, Jurkat, and Molt-4 cells were approximately the same, but in LNCaP cells, the levels of both DFF-40 and DFF-45 were higher than in the other cells (Fig. 3, A and B). Similar results were obtained on Western blot analysis of DFF-45 (data not shown). The results show that the expression levels of DFF-40 and DFF-45 in Molt-4 cells were not significantly different from those in other cell lines.

To determine whether or not DFF-40 in Molt-4 cells functions as a DNase, the DFF complex was immunoprecipitated using antiserum against DFF-45 and nuclease activity of the immunoprecipitated protein was examined as to the ability to degrade plasmid DNA substrate in the presence and absence of caspase-3. As shown in Fig. 3C, the DFF complex immnoprecipitated from control HL-60 and Molt-4 cells exhibited endonucleolytic activity in the presence of recombinant caspase-3.

It is well known that the inhibitor (DFF-45) is cleaved by active caspase-3 (*4–7, 17–19*). Therefore, we assessed whether or not caspase-3 was activated in NCS-treated Molt-4 cells (Fig. 4, A and B). The activation of caspase-3 was examined using DEVD-MCA as a substrate and by means of proteolysis of procaspase-3. As shown in Fig. 4A, the caspase-3–like protease activities in the extract of Molt-4 cells increased in a time-dependent manner after NCS treatment. In addition, procaspase-3 was completely cleaved and converted to an active form in the cells (Fig. 4B).

Next, we examined whether or not DFF-45 is cleaved in NCS-treated cells, and found that it is (Fig. 4C). Although the inhibitor of DNase (DFF-45) was cleaved in apoptotic Molt-4 cells and DFF-40 in Molt-4 cells functions as a DNase, ladder formation was not detected (Fig. 2). These

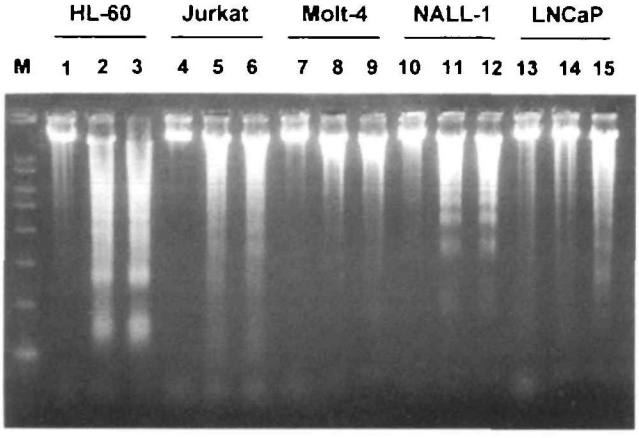

Fig. 2. **DNA fragmentation analysis of NCS-treated cells.** Cells were treated with NCS for 24 h and then cellular DNA was electrophoresed on a 2.0% agarose gel. Lanes 1, 4, 7, 10, and 13, control untreated-cells; lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, and 15, treated with concentrations 0.3, 1.0, 0 5, 1.0, 0.2, 0.3, 0.3, 0.5, 5.0, and 10 μg/ml NCS, respectively. Lane M, DNA marker.

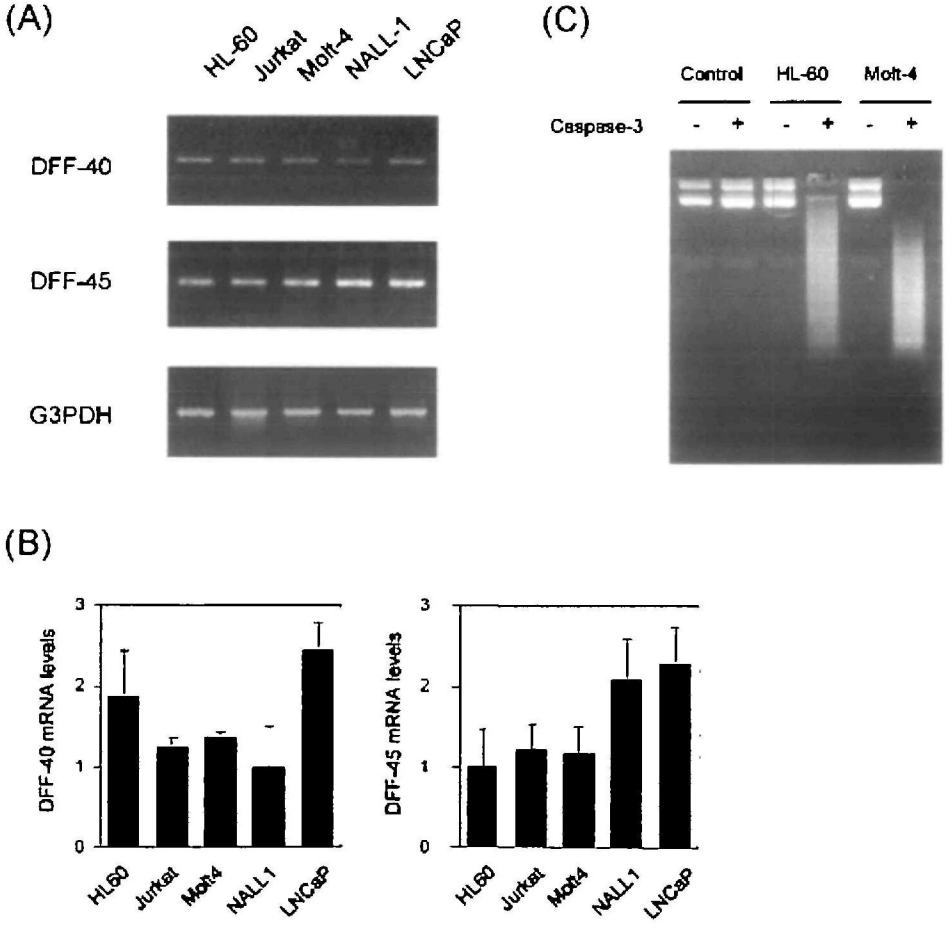

Fig. 3. **DFF expression in various human cell lines.** (A and B) Relative abundance of DFF-45 and DFF-40 mRNA transcripts. (A) Constitutive expression of DFF-45, DFF-40, and G3PDH mRNA was analyzed by RT-PCR with specific primers, as described under "EXPERIMENTAL PROCEDURES." (B) Band intensity analysis was performed using the NIH Image program. Columns indicate band intensities relative to the minimum intensity. The intensities were normalized as to the G3PDH standards. Each value represents the mean ± SD value for three analyses. (C) DNase activities of DFF immunoprecipitated with DFF-45. HL-60 and Molt-4 cytosol was immunoprecipitated with anti–DFF-45 IgG. The DNase activities were assayed in the presence or absence of 370 ng caspase-3.

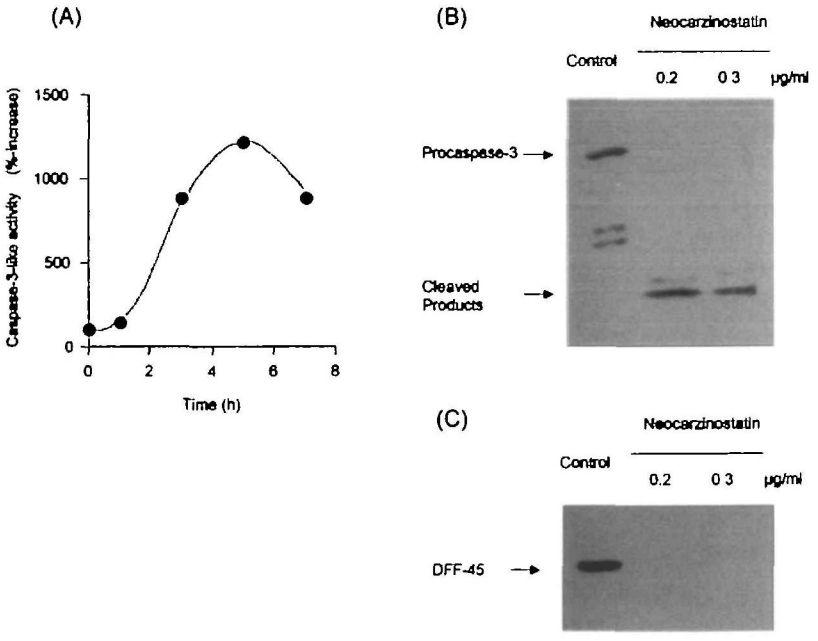

Fig. 4. **Activation of caspase-3 and cleavage of DFF-45 in NCS-treated Molt-4 cells.** (A) Molt-4 cells were treated with 0.5 μg/ml NCS for the indicated times and then cell extracts were incubated at 37°C for 60 min with 50 μM Ac-DEVD-MCA as a substrate. (B) Molt-4 cells were treated with NCS for 24 h, and then cell extracts were subjected to SDS-PAGE, transferred to a membrane, and then probed with anti–caspase-3 antibodies. (C) Molt-4 cells were treated with NCS for 24 h and then analyzed by Western blotting with anti–DFF-45 antibodies.

results suggest that the cleavage of DFF-45 by caspase-3 and the following activation of DFF-40 are not sufficient for the induction of oligonucleosomal DNA fragmentation in NCS-treated Molt-4 cells.

DISCUSSION

Oligonucleosomal DNA fragmentation has been implicated as a in biological characteristic of apoptosis (*8, 9*). There are many candidates for enzymes responsible for the induction of oligonucleosomal DNA fragmentation. These include Ca^{2+}/Mg^{2+}-dependent endonucleases (*10, 11*), a caspase-activated endonuclease (DFF-40/CAD/CPAN) (*4–7*), a cation-independent acidic endonuclease (DNase II) (*12, 13*), and so on. Among these endonucleases, DFF-40 is a more likely candidate apoptotic DNase. Here, we showed that the functional expression of DFF is not sufficient for the induction of oligonucleosomal DNA fragmentation in NCS-mediated apoptotic Molt-4 cells.

It has been reported that equal expression of DFF-40 and DFF-45 is indispensable for DFF-40 to function as a DNase (*5, 6, 15, 16*). Not only a defect of DFF-40 but also low or overexpression of DFF-45 does not result in DNA fragmentation, because DFF-45 acts as both a specific chaperone and an inhibitor of DFF-40 for regulating endonucleotyic activity. We therefore examined whether or not abnormal expression of DFF-40 and/or DFF-45 corresponds to no DNA ladder formation in Molt-4 cells. Unexpectedly, the results obtained on RT-PCR analysis revealed that DFF-40 and DFF-45 were expressed in all cell lines, the ratio of DFF-40 and DFF-45 not differing among the cell lines (Fig. 3, A and B). Moreover, the sequence of DFF-40 cDNA in Molt-4 cells was found to be identical to that in HL-60 cells (data not shown), and the DFF immunoprecipitated from Molt-4 cells retained DNase activity, which is activated by caspase-3 (Fig. 3C). These results indicated that the resistance to induction of oligonucleosomal DNA fragmentation in Molt-4 cells is not due to the lack of functional expression of DFF. To our knowledge, this is the first report that oligonucleosomal DNA fragmentation was not detected despite the expression of functional DFF.

It has been reported that caspase-3 activates the endonuclease (DFF-40), responsible for DNA fragmentation by specifically cleaving and inactivating DFF-45, the inhibitor of DFF-40 (*17–19*). MCF-7 cells, which do not express detectable level of caspase-3, exhibit no DNA fragmentation (*17*). TUR cells are also resistant to DNA fragmentation because of defective caspase-3 activation (*18*). We therefore examined whether or not the caspase-3 activation and DFF-45 cleavage occur in NCS-treated Molt-4 cells. The results we obtained on Western blot analysis revealed that caspase-3 and DFF-45 were normally expressed and cleaved into the active or inactive form upon NCS-treatment (Fig. 4, B and C). These observations suggest that neither caspase-3 activation nor cleavage of DFF-45 was sufficient to cause DNA fragmentation upon NCS-treatment in Molt-4 cells. Similar observations have been reported fort tetra-butylhydroperoxide-treated Raji cells (*27*) and Cl^- efflux–prevented Jurkat cells (*28*).

Why is oligonucleosomal DNA fragmentation not induced in apoptotic Molt-4 cells although the possible apoptotic DNase, DFF, is activated? Four possibilities can be envisaged to explain this observation. First, another co-factor for DFF-40 is necessary for nucleosomal DNA fragmentation and the factor is absent in Molt-4 cells. Specific chromosomal proteins, such as histone H1 or HMG-1/2, and topoisomerase II are known to further activate DFF nuclease on naked DNA substrates (*29–31*). CIDE-B (cell death–inducing DFF-45–like effector) protein regulates DFF enzymatic activity *in vitro* (*32*). Cl^- efflux was also reported to be a necessary co-factor for the activation of DFF nuclease (*28*). Second, the nuclear DNA of Molt-4 cells could be resistant to endonuclease. In fact, the chromatin structure in Molt-4 cells was reported to be more resistant to exogenously added endonucleases compared with that in other cell lines (*33*). Third, other nucleases could be in-

volved in NCS-induced apoptosis. For example, activation of acidic nucleases, such as DNase II, has been reported to be associated with apoptotic DNA fragmentation (*12, 13*). Since intracellular acidification is known to occur concomitantly with apoptosis in some types of cells (*34–36*), it is convenient for the activation of acidic nuclease in apoptosis. In preliminary studies, we found that intracellular acidification occurs in NCS-treated HL-60 cells but not in NCS-treated Molt-4 cells (data not shown). Moreover, induction of cellular DNA digestion in Nonidet P-40-treated Molt-4 cells occurred at acidic pH, but not at neutral pH (data not shown, *37*). Fourth, DFF degrades DNA into 50 kbp DNA, and subsequently other DNases such as Ca^{2+}/Mg^{2+}-dependent endonucleases or acid DNases cleave the latter into oligonucleosomal DNA fragments. In apoptotic Molt-4 cells, the intracellular DNA is known to be degraded into a 50 kbp DNA fragment (*38, 39*), which is possibly mediated by DFF (*40*). DFF may be related to the 50 kbp fragmentation, and not to the following oligonucleosomal DNA fragmentation in NCS-treated cells. Molt-4 cells may lack the endonuclease for inducing oligonucleosomal DNA fragmentation.

In this report, we have first demonstrated that Molt-4 cells, which are resistant to DNA fragmentation, exhibit a normal level of DFF, cleavage of DFF-45 and functional DFF-40, suggesting that suitable expression of DFF is not sufficient to induce oligonucleosomal DNA fragmentation in Molt-4 cells. Recently, a paper appeared that showed the absence of a DNA ladder, but cleavage of DFF-45 in staurosporine-treated neuroblastoma IMR-5 cells (*41*).

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