GP7 induces internucleosomal DNA fragmentation independent of caspase activation and DNA fragmentation factor in NB4 cells

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Abstract. DNA fragmentation into internucleosomal fragments is the best recognized biochemical event of apoptosis. Two major caspase pathways have been identified in the signal transduction leading to DNA fragmentation: the receptor pathway and the mitochondrial pathway. DNA fragmentation factor (DFF) has been identified as a major apoptotic endonuclease in the internucleosomal DNA fragmentation process. However, the potential roles of caspases and DFF in internucleosomal DNA fragmentation induced by specific stimuli still need to be investigated since caspase-independent pathways and nuclease(s) other than DFF also play important roles during this process. In the present study, we investigated the activity of GP7 (4-[4"-(2",2",6",6"-tetramethyl-l"-piperidinyloxy) amino]-4'-demethyl epipodophyllotoxin), a new spin-labeled derivative of podophyllotoxin semi-synthesized by our university, to induce apoptosis of the human leukemia cell line NB4. GP7 induced the release of cytochrome-c from mitochondria, activations of caspase-3, -8, and -9, cleavage of DFF45/inhibitor of caspase-activated DNase, activation of DFF40/caspase-activated DNase, and apoptotic DNA fragmentation in NB4 cells. The broad-spectrum caspase inhibitor zVAD-fmk abrogated GP7-induced caspase-3, -8, and -9 activations but could not inhibit GP7-induced apoptotic DNA fragmentation in NB4 cells. Our findings suggest that GP7-induced apoptotic DNA fragmentation in NB4 cells is independent of caspase activation and DFF, although they are closely involved in this process.

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Introduction

Apoptosis is the mechanism of cell death activated in mammalian cells following exposure to a wide variety of stimuli including anticancer agents. DNA fragmentation into internucleosomal fragments is the best recognized biochemical event of apoptosis (1,2). Internucleosomal DNA fragmentation can minimize the risk of transferring genetic information from apoptotic cancer cells to the neighboring cells (3).

Two major pathways, the receptor pathway and the mitochondrial pathway, have been identified in the signal transduction of caspases leading to apoptosis. The receptor pathway involves the activation of caspase-8 by death-inducing signaling complexes formed by receptor trimerization and adapter proteins. The activation of caspase-8 leads to the subsequent cleavage and activation of caspase-3, which is responsible for the targeting of cellular substrates downstream. The mitochondrial pathway differs from the receptor pathway in that caspase-8 targets the Bcl-2 family member protein Bid. Bid is cleaved by caspase-8, whereupon its truncated form translocates to the mitochondrial membrane and initiates the mobilization of cytochrome-c (cyt-c) into the cytoplasm from the mitochondrial intermembrane space. Cytoplasmic cyt-c complexes with pro-caspase-9 and Apaf-1 to form the apoptosome. The apoptosome formation perpetuates pro-caspase-9 cleavage to the active caspase-9 form. Caspase-9 targets caspase-3 for cleavage in a manner similar to that of caspase-8 within the receptor pathway (4).

DNA fragmentation factor (DFF) has been identified as a major apoptotic endonuclease in the internucleosomal DNA fragmentation process (reviewed in ref. 5). DFF is composed of two subunits of 40 and 45 kDa termed DFF40/CAD (caspase-activated DNase) and DFF45/ICAD (inhibitor of caspase-activated DNase), respectively. DFF40/CAD is a Mg^{2+}-dependent endonuclease specific for double-stranded DNA that generates double-strand breaks with 3'-hydroxyl ends. DFF40/CAD is activated by caspase-3 that cuts the nuclease's inhibitor DFF45/ICAD (5).

GP7 (4-[4"-(2",2",6",6"-tetramethyl-l"-piperidinyloxy) amino]-4'-demethyl epipodophyllotoxin) is a new spin-labeled derivative of podophyllotoxin semi-synthesized by our university. GP7 has lower toxicity and higher total

chemical yield (based on podophyllotoxin) when compared with etoposide and thus is a promising anticancer drug of the podophyllotoxin class (6,7).

To study the anticancer mechanisms of GP7 in human leukemia cells, we investigated the activity of GP7 to induce apoptosis of the human leukemia NB4 cell line. GP7 induced the release of cyt-c from mitochondria, activations of caspase-3, -8, and -9, cleavage of DFF45/ICAD, activation of DFF40/CAD, and apoptotic DNA fragmentation in NB4 cells. The broad-spectrum caspase inhibitor zVAD-fmk abrogated GP7-induced caspase-3, -8, and -9 activations but could not inhibit GP7-induced apoptotic DNA fragmentation in NB4 cells. Our findings suggest that GP7-induced apoptotic DNA fragmentation in NB4 cells is independent of caspase activation and DFF, although they are closely involved in this process.

Materials and methods

Cell culture. The human myelogenous leukemia cell line NB4 was obtained from ATCC (Rockville, MD, USA). RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) was used for all cell cultures. Cell cultures were kept at 37˚C and in 5% atmospheric CO_2. Cells displayed exponential growth before all experiments were conducted.

Materials. GP7 (purity ≥98%) was kindly provided by Professor Tian Xuan (School of Chemistry and Chemical Engineering, Lanzhou University), dissolved in Me_2SO to achieve a stock solution of 10 mM and stored at -20˚C. Anti-cytochrome-c mAb (7H8.2C12) was provided by BD Biosciences Pharmingen (San Diego, CA, USA). ICAD (C-19) and anti-goat IgG-horseradish peroxidase (HRP) were provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-CAD/DFF40/CPAN mAb was provided by Imgenex Co. (San Diego, CA, USA). Sheep anti-mouse Ig-HRP-linked whole antibody was provided by Amersham Pharmacia Biotech (NJ, USA). Ac-Asp-Glu-Val-Asp-MCA (substrate for caspase-3), Ac-Ile-Glu-Thr-Asp-MCA (substrate for caspase-8), Ac-Leu-Glu-His-Asp-MCA (substrate for caspase-9) and zVAD-fmk were supplied by Peptide Institute (Osaka, Japan). During the experiment, all samples, including controls, contained 0.1% Me_2SO which had no observed effect on any of the assays performed.

DNA extraction and agarose gel electrophoresis. After GP7 treatment, the cells were lysed in a solution containing 500 mM Tris-HCl (pH 8.0), 20 mM EDTA, 10 mM NaCl, 1% (w/v) SDS and 100 μg/ml proteinase K at 37˚C for 48 h. DNA was extracted by standard phenol-chloroform-isoamyl alcohol extraction procedures (8), and treated with 100 μg/ml of RNase A at 37˚C for 30 min. DNA samples were electrophoretically separated on 1.2% agarose gels, and then gels were stained with ethidium bromide.

DNA fragmentation assay. DNA fragmentation was quantified by scanning photographic negatives with a densitograph gel documentation system (ATTO-densitograph, Osaka, Japan) and integrating the area under the curves. Integrated areas were divided at the 2000-bp molecular weight marker position into high- and low-molecular weight DNA, and the percentage of DNA fragmentation was determined by dividing the area of low-molecular weight DNA by the total area.

Preparation of cell lysates. Cell lysates were prepared by washing cells three times in ice-cold phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) and then incubating the cells for 15 min on ice at a density of $1x10^8$/ml in TKM buffer [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 50 mM KCl] containing 0.25% Triton X-100. The cell lysates were centrifuged (10,000 x g for 15 min at 4˚C), and the pellets were discarded. The supernatants were aliquoted and frozen at -80˚C. The lysates were used to detect caspase activation or the release of cyt-c from mitochondria by Western blot analysis.

Measurement of caspase-3, -8 and -9 activities. Caspase-3, -8 and -9 activities were respectively analyzed by the cleavage of their specific fluorometric substrates: Ac-Asp-Glu-Val-Asp-MCA (substrate for caspase-3), Ac-Ile-Glu-Thr-Asp-MCA (substrate for caspase-8), and Ac-Leu-Glu-His-Asp-MCA (substrate for caspase-9). Enzyme reactions were performed in 96-well plates (Dainippon Seiyaku, Osaka, Japan) with 100 μl of the cell lysates prepared as described above, and a specific fluorometric substrate (final concentration, 20 μM). Each sample was seeded in triplicate. After incubation at 37˚C for 90 min, AMC (7-amino-4-methyl coumarin) released from the substrates was measured at excitation and emission wavelengths of 355 and 460 nm using a fluorescence microplate reader (Fluoroscan Ascent, Dainippon Seiyaku).

Western blot analysis of DFF45/ICAD cleavage and DFF40/CAD activation. Cells ($4x10^7$) were washed and solubilized in Laemmli buffer. Equivalent amounts of proteins were mixed with an equal volume of 2X ME buffer, boiled, and resolved by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The proteins were transferred from the gels to nitrocellulose membranes using an electroblotting apparatus (Bio-Rad) (12 V, 60 min). Membranes were incubated in PBST (PBS with 0.1% Tween-20) containing 5% nonfat dried milk overnight to inhibit nonspecific binding. The membranes were then incubated with specific antibody for 60 min. After washing in PBST for 30 min, membranes were incubated for another 60 min with HRP-conjugated secondary antibody. The membranes were then washed and developed with enhanced chemiluminescence (ECL) (Pierce, Rockford, USA). Films were exposed for 1-15 min. ß-actin expression was used as the internal control.

Results

GP7 induces DNA fragmentation in NB4 cells. To examine the effect of GP7 on DNA integrity, NB4 cells were incubated with 18 μM GP7 for varying lengths of time or with different concentrations of GP7 for 24 h. Treatment of NB4 cells with 18 μM GP7 for 6, 12, or 24 h or with 4.5, 9, 18 μM of GP7 for 24 h induced internucleosomal DNA fragmentation, and a

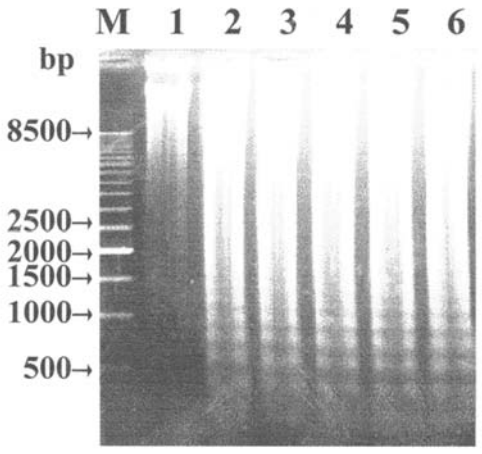
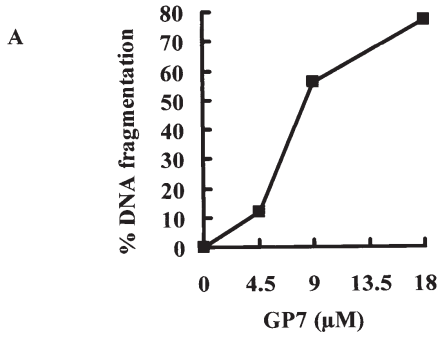


Figure 1. Agarose gel electrophoresis of DNA in NB4 cells treated with GP7. Cells were untreated (lane 1) or treated with 4.5, 9, or 18 μM GP7 for 24 h (lanes 2-4, respectively), or treated with 18 μM GP7 for 12 or 6 h (lanes 5 and 6). Left lane (M) shows the migration of 500-bp DNA size markers. Cellular DNA was extracted and subjected to agarose gel electrophoresis as described in Materials and methods.



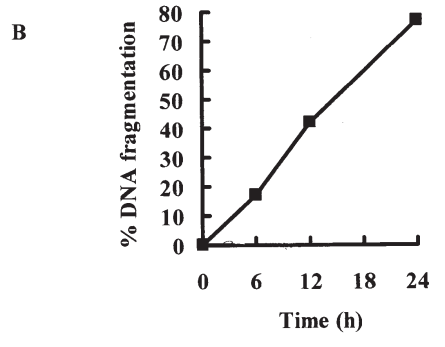
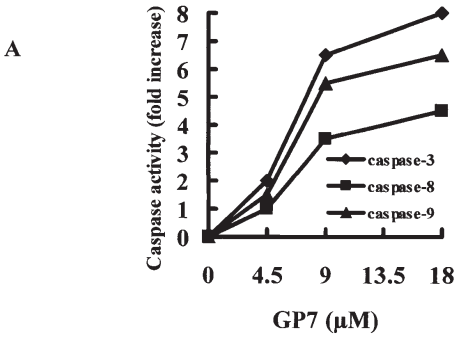


Figure 2. GP7 induces apoptotic DNA fragmentation in NB4 cells. (A) The concentration-response curve of GP7-induced DNA fragmentation in NB4 cells. NB4 cells were incubated with different concentrations of GP7 for 24 h. (B) The time course of GP7-induced DNA fragmentation in NB4 cells. NB4 cells were treated with 18 μM GP7 for the indicated times. Cellular DNA of NB4 was extracted and subjected to agarose gel electrophoresis. The percent of DNA fragmentation was determined from a densitometric scan as described in Materials and methods. Results are the mean values from three separate experiments.

'DNA ladder' was observed (Fig. 1). The amounts of DNA fragmentation induced by GP7 in NB4 cells were quantified by the densitograph gel documentation system. The results



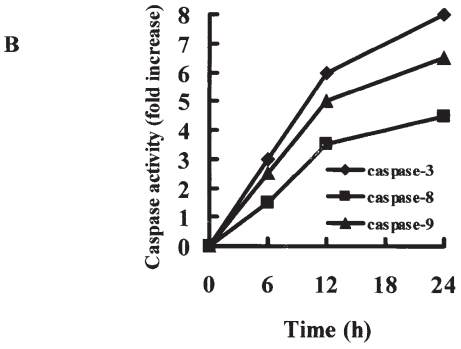


Figure 3. GP7 induces caspase-3, -8 and -9 activations in a concentration- and time-dependent manner in NB4 cells. (A) The concentration-response curve of GP7-induced caspase-3, -8 and -9 activations in NB4 cells. NB4 cells were incubated with different concentrations of GP7 for 24 h. (B) The time course of GP7-induced caspase-3, -8 and -9 activations in NB4 cells. NB4 cells were treated with 18 μM GP7 for the indicated times. Aliquots were collected and assayed for caspase-3, -8 and -9 activities with Ac-Asp-Glu-Val-Asp-MCA (substrate for caspase-3), Ac-Ile-Glu-Thr-Asp-MCA (substrate for caspase-8), or Ac-Leu-Glu-His-Asp-MCA (substrate for caspase-9), respectively. Assays were performed as described in Materials and methods. Results are the mean values from three separate experiments.

show that there was a time- or concentration-dependent relationship in GP7-induced DNA fragmentation (Fig. 2).

GP7 treatment activates caspase-3 in NB4 cells in a time- and concentration-dependent manner. Based on observations of DNA fragmentation noted in NB4 cells, we hypothesized that caspase-3 was activated during NB4 cell apoptotic DNA fragmentation. As expected, GP7 activated caspase-3 in NB4 cells in a time- and concentration-dependent manner (Fig. 3), and there was a correlation between the kinetics of caspase-3 activation and that of DNA fragmentation in NB4 cells (Fig. 2).

GP7 treatment activates caspase-8 and -9 in NB4 cells. Caspase-3 can be activated by caspase-8 or -9. To further characterize the pathway of apoptosis used by GP7 in NB4 cells, we checked caspase-8 and -9 activities in this cell line. As shown in Fig. 3, treatment with GP7 induced activations of caspase-8 and -9 in the NB4 cell line, although the activity of caspase-8 was not as high as that of caspase-9.

GP7 treatment induces cyt-c release, DFF45/ICAD cleavage and DFF40/CAD activation in NB4 cells. To clarify the

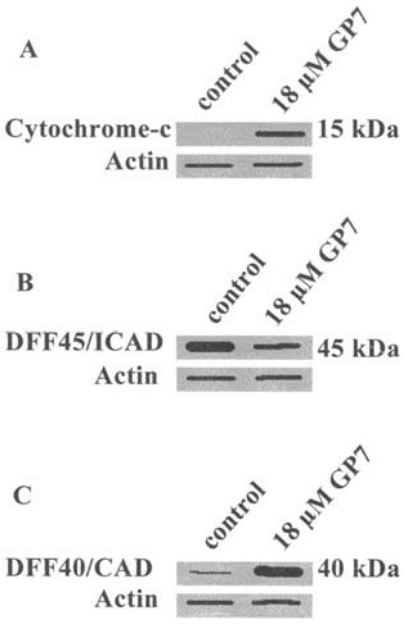


Figure 4. Western blot analysis of cytochrome-c release (A), DFF45/ICAD cleavage (B) and DFF40/CAD activation (C) in NB4 cells. Cells were untreated or treated with 18 μM GP7 for 24 h. Western blotting was performed as described in Materials and methods. ß-actin expression was used for the control of equal protein loading.

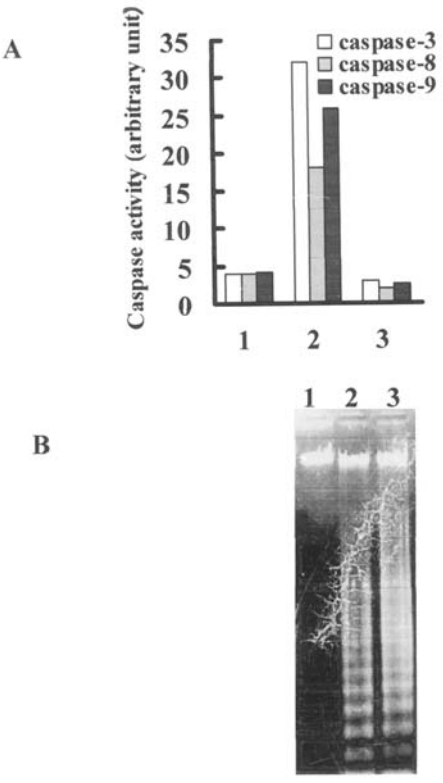


Figure 5. Effects of the broad-spectrum caspase inhibitor zVAD-fmk on GP7-induced caspase activation and DNA fragmentation in NB4 cells. (A) Effects of zVAD-fmk on GP7-induced caspase activation. After washing cells three times with PBS, cell lysates were prepared and assayed for caspase-3, -8, and -9 activities. Assays were performed as described in Materials and methods. Results are the mean values from three separate experiments. (B) Effects of zVAD-fmk on GP7-induced DNA fragmentation. Cellular DNA was extracted and subjected to agarose gel electrophoresis as described in Materials and methods. NB4 cells were treated with 18 μM GP7 in the absence or presence of zVAD-fmk (100 μM) for 24 h. zVAD-fmk was added 60 min prior to GP7 administration. 1, Untreated NB4 cells; 2, NB4 cells treated with 18 μM GP7; and 3, NB4 cells treated with 100 μM zVAD-fmk plus 18 μM GP7.

signaling cascade of caspase-3 activation and DNA fragmentation in GP7-treated NB4 cells, the involvement of cyt-c release from mitochondria and DFF activation was investigated in the GP7-treated NB4 cell line by Western blotting. Treatment with 18 μM GP7 for 24 h led to cyt-c release from mitochondria into the cytosol, DFF45/ICAD cleavage and DFF40/CAD activation in NB4 cells (Fig. 4A-C).

zVAD-fmk abrogated GP7-induced caspase-3, -8, and -9 activations but did not inhibit GP7-induced apoptotic DNA fragmentation in NB4 cells. To further clarify the roles of caspase activation and DFF in GP7-induced apoptotic DNA fragmentation in NB4 cells, NB4 cells were pretreated with the broad-spectrum caspase inhibitor zVAD-fmk. Pretreatment of NB4 cells with 100 μM of zVAD-fmk for 60 min abrogated GP7-induced caspase-3, -8, and -9 activations in NB4 cells (Fig. 5A). However, pretreatment of NB4 cells with 100 μM of zVAD-fmk for 60 min did not inhibit GP7-induced apoptotic DNA fragmentation in NB4 cells (Fig. 5B).

Discussion

Internucleosomal DNA fragmentation is an important biochemical hallmark of apoptosis. GP7 induced internucleosomal DNA fragmentation of human leukemia HL-60 and Jurkat as well as Burkitt's lymphoma Raji cells (6,7). In this study, GP7 induced DNA fragmentation of NB4 cells in a time- and concentration-dependent manner (Figs. 1 and 2), confirming that induction of apoptosis is one of the anticancer mechanisms of GP7.

The role of caspase-3 in GP7-induced DNA fragmentation is controversial (6,7). This incited us to further investigate the role of caspase-3 in GP7-induced DNA fragmentation in NB4 cells. In the present study, GP7 treatment activated caspase-3 in NB4 cells in a time- and concentration-dependent manner (Fig. 3), and the kinetics of caspase-3 activation was well correlated with that of DNA fragmentation, suggesting that caspase-3 is closely involved in GP7-induced DNA fragmentation in NB4 cells.

Caspase-3 can be activated through the receptor pathway and/or the mitochondrial pathway. In this experiment, we investigated the upstream events of caspase-3 in GP7-induced DNA fragmentation of NB4 cells. Caspase-8 and -9 were activated (Fig. 3) and cyt-c was released (Fig. 4A) by GP7 treatment of NB4 cells. These results indicate that caspase-8 and -9 are involved in the activation of caspase-3 in GP7-treated NB4 cells.

Apoptotic endonuclease is a key enzyme that mediates internucleosomal DNA fragmentation and chromatin condensation in response to apoptotic signals such as Fas ligand, radiation, and anticancer agents. A number of candidate apoptotic nucleases have been reported to be associated with apoptotic DNA cleavage, including deoxyribonuclease I (DNase I) (9), Nuc 18 (cyclophilin) (10), DNase II (11,12) as well as several Ca^{2+}/Mg^{2+}-dependent endonucleases (13-16). Recently, DFF has been identified as a major apoptotic endo-

nuclease in the internucleosomal DNA fragmentation process (5). In this study, GP7 treatment induced DFF45/ICAD cleavage and DFF40/CAD activation in NB4 cells (Fig. 4B and C), suggesting that DFF is involved in GP7-induced DNA fragmentation of NB4 cells.

Studies have found that CAD is not expressed ubiquitously in human tissues (17) and mice lacking DFF45/ICAD are viable and still show residual DNA fragmentation (18). This motivated us to further clarify the specific role of DFF in GP7-induced DNA fragmentation of NB4 cells. DFF is a substrate of caspase-3 (19), thus abrogation of caspase-3 activity should eliminate DFF activity. In our study, the broad-spectrum caspase inhibitor zVAD-fmk was employed to identify the roles of caspase and DFF in GP7-induced DNA fragmentation of NB4 cells. zVAD-fmk (100 μM) abrogated GP7-induced caspase-3, -8 and -9 activations (Fig. 5A) but did not inhibit GP7-induced apoptotic DNA fragmentation (Fig. 5B) in NB4 cells. These results suggest that caspase activation and DFF are dispensable for GP7-induced apoptotic DNA fragmentation in NB4 cells although they are involved in this process. These results also suggest that other endonucleases may play an important role during GP7-induced apoptotic DNA fragmentation in NB4 cells.

Endonuclease-G has been previously reported as an apoptotic endonuclease released from mitochondria during apoptosis and induces caspase-independent DNA fragmentation (20). It is conceivable to postulate that endonuclease-G or other caspase-independent endonucleases are involved in GP7-induced DNA fragmentation of NB4 cells.

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