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Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids

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Abstract

Rutinoside (rhamnoglucoside; rhamnose + glucose) addition has been examined extensively in the metabolism of flavonoids, however the effect of rutinoside on apoptosis-inducing activity of flavonoids is still unknown. In the present study, the two pairs of flavonoids of hesperetin (HT) and hesperidin (HD; HT-7-rutinose), and naringenin (NE) and naringin (NE-7-rutinose), were used to study their apoptosis-inducing activities in HL-60 cells. Both HD and NI are flavonoids which contain a rutinoside at the C7 of HT and NE, respectively. Results of the MTT assay showed that HT and NE, but not HD and NI, exhibited significant cytotoxic effect in HL-60 cells, accompanied by the dose- and time-dependent appearance of characteristics of apoptosis including an increase in DNA ladder intensity, morphological changes, appearance of apoptotic bodies, and an increase in hypodiploid cells by flow cytometry analysis. HT and NE, but not HD and NI, caused rapid and transient induction of caspase-3/CPP32 activity, but not caspase-1 activity, according to the cleavage of caspase-3 substrates poly(ADP-ribose) polymerase and D4-GDI proteins, the appearance of cleaved caspase-3 fragments detected in HT- or NE-, but not in HD- or NI-treated HL-60 cells. A decrease in the anti-apoptotic protein, Mcl-1, was detected in HT- and NE-treated HL-60 cells, whereas other Bcl-2 family proteins including Bax, Bcl-2, Bcl-XL, and Bag remained unchanged. The caspase-3 inhibitor, Ac-DEVD-FMK, but not the caspase-1 inhibitor, Ac-YVAD-FMK, attenuated HT- and NE-induced cell death. Interestingly, neither HT nor NE induced apoptosis in the mature monocytic cell line THP-1 and primary human polymorphonuclear cells, as characterized by a lack of DNA ladders, caspase-3 activation, poly(ADP-ribose) polymerase cleavage, and Mcl-1 decrease, compared with those in HL-60 cells. In addition, the rutinoside group in HD and NI was removed by hesperidinase and naringinase, accompanied by the production of HT and NE, respectively, according to HPLC analysis. Accordingly, hesperidinase and naringinase digestion recovered the apoptosis-inducing activity of HD and NI in HL-60 cells. Our experiments provide the first evidence to suggest that rutinoside in flavonoids prevents the induction of apoptosis, and that activation of the traditional caspase-3 cascade participates in HT- and NE-induced apoptosis.

Keywords: Rutinoside; Hesperetin; Hesperidin; Naringenin; Naringin; Apoptosis; Caspase-3

1. Introduction

Flavonoids exist extensively in all parts of plants including fruits, vegetables, nuts, leaves, flowers, and bark, and their biological functions such as apoptosis-inducing activity, free radical scavenging activity, and antitumorigenic activity have been identified [1–4]. Flavonoids are a large group of low molecular weight polyphenolic compounds, and have been shown to be one of the most important classes in the free state and as glycosides [5,6]. Both glycone and aglycone occurred in the metabolic process of flavonoids, however there is a lack of evidence to suggest the effect of glycoside addition on the biological activities of flavonoids. In the present study, flavonoids in the aglycone form including hesperetin (HT; 3,5,7-trihydroxy-4-methoxyflavanone) and naringenin (NE; 4′,5,7-trihydroxyflavanone), and in the glycone form including

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Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribosyl) polymerase; Bcl-2, B-cell lymphoma 2; ROS, reactive oxygen species; NE, naringenin; NI, naringin; HT, hesperetin; HD, hesperidin; NIase, naringinase; HDase, hesperidinase; HPLC, high performance liquid chromatography; DEVD, Asp-Glu-Val-Asp; YVAD, Tyr-Val-Ala-Asp.

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hesperidin (HD; HT-7-rhamnoglucoside) and naringin (NI; NE-7-rhamnoglucoside) were used to evaluate the effect of glycoside on the induction of apoptosis in leukemia cell line HL-60. HT, HD, NE, and NI are flavonoids present extensively in the plant kingdom especially in many citrus fruits, and commonly used in traditional medicines [7]. NI and HD, but not NE or HT, possess a rhamnoglucoside (rutinoside) moiety at the 7-position of the flavone and flavanone skeleton, and both NE and HT are aglycone metabolites of NI and HD, respectively. NI was reported to possess anti-inflammatory, anti-ulcer, and antioxidant activities. Evidence was provided that orally administrated NI was hydrolyzed by enterobacteria to aglycones such as NE before absorption [8,9]. NE has been demonstrated to inhibit CYP3A4 activity, and exhibit aorta dilatory, antioxidant, anitproliferative effects [10,11]. Result of *in vitro* anticarcinogenesis assay showed that NE, but not NI, inhibited aflatoxin B1-induced carcinogenesis [12]. Kawaguchi *et al.* indicated that NI suppressed the lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF) release, followed by the blocking of LPS-induced liver injury [13]. In addition to NE and NI, HT and HD showed the similar glycoside substitution as NI and NE, and some biological activities of HD were identified. Previous study demonstrated that HD inhibits tumor promotion in 12-*O*-tetradecaboylphorbol-13-acetate-treated CD-1 mice [14], and Tanaka *et al.* indicated that HD reduced *N*-methyl-*N*-amylnitrosamine-induced rat esophageal tumorigenesis by decreasing cell proliferation [15]. HD also showed antihypertensive and diuretic effects on normotensive and spontaneously hypertensive rats [16]. In contrast to HD, HT is able to inhibit the hydroxylation of benzo(*a*)pyrene and aflatoxin B1 to mutagenic products [17], and Melzig and Loose reported that HT attenuated LPS-induced cytotoxicity via inhibition of specific tyrosine kinases [18]. These data suggest that NE, NI, HT, and HD possess several different biological functions; however their apoptosis-inducing activities and the importance of rutinoside at C7 are still unresolved.

Apoptosis, a cell death process which has been extensively studied, is characterized by cellular morphological changes, chromatin condensation, and apoptotic bodies associated with DNA cleavage into ladders. Several pathways have been described to regulate apoptosis during development, tumorigenesis, and chemical treatments [19,20]. Several gene expressions have been demonstrated to be critical in the regulation of apoptosis such as caspase cascades and Bcl-2 family proteins. Members of the Bcl-2 family proteins can be divided into two subfamilies, one is anti-apoptotic including Bcl-2, Mcl-1, and Bcl-XL proteins and the other is pro-apoptotic including Bax, Bcl-Xs, and Bad. Induction of pro-apoptotic Bcl-2 family proteins and inhibition of anti-apoptotic family proteins have been detected in apoptosis induced by chemicals [21,22]. Human caspases-1 to -10 have been described, and activation of the caspase cascade is involved in chemical- and agent-induced apoptosis. Caspase-9 is an apoptosis initiator and activated caspase-9 then cleaves and activates executioner caspase-3, which exists as an inactive pro-caspase-3 in the cytoplasm and is proteolytically activated by multiple cleavages of pro-caspase-3 to generate the cleaved fragments in cells undergoing apoptosis [23]. Some specific substrates for caspase-3 such as poly(ADP-ribose) polymerase (PARP) and D4-GDI proteins are cleaved which is important for the occurrence of apoptosis. PARP is required for DNA repair and activated caspase-3 cleaves PARP at Asp 216 to generate the 85- and 31-kDa apoptotic fragments in coordination with DNA fragmentation during apoptosis [24]. D4-GDI is a negative regulator of the ras-related Rho family of GTPases, and activation of Rho GTPases promoted cytoskeletal and membrane changes associated with apoptotic cells. Activated caspase-3 cleaves D4-GDI to 23- and 5-kDa fragments, and activates Rho GTPases to produce apoptotic morphological changes [25].

Glycosylation commonly occurs in the metabolism of flavonoids, and increase their hydrophilic properties by addition of sugar moieties into the structure. The relation of the effect of glycosylation on the biological function of flavonoids is still undefined, and our previous studies suggested that glycosylation attenuated the anti-inflammatory activity of flavonoids in macrophages [26,27]. In order to demonstrate the effect of glycosylation on apoptosis-inducing activity of flavonoids, NE, NI, HT, and HD were used to study their apoptosis-inducing activities in the human promyeloleukemia cell line HL-60. Results of the present study suggest that adding a rutinoside group attenuates the apoptosis-inducing activity of flavonoids, and that the activation of caspase-3 cascade is involved in the apoptotic mechanism.

2. Materials and methods

2.1. Cell culture and chemicals

HL-60 and THP-1 are leukemia cell lines and obtained from ATCC (American Type Culture Collection). Previous study and comments of ATCC indicated that HL-60 is a poorly differentiated promyelocytic cell line, and THP-1 is a mature monocytic cell line [28]. Human polymorphonuclear (PMN) cells were isolated from human heparinized venous blood of healthy volunteers, followed by centrifugation of the granulocyte-rich supernatant on a cushion of a mixture of Ficoll and Hypaque, as previous described [29]. HL-60, THP-1, and PMN cells were maintained at 37° in RPMI 1640 containing 10% heat-inactivated fetal bovine serum in an atmosphere containing 5% CO_2. The plates for HL-60, THP-1, and PMN cells were purchased from Gibco (GIBCO/BRL). The colorigenic synthetic peptide substrates for caspase-3-like proteases (Ac-DEVD-pNA) and for caspase-1 (Ac-YVAD-pNA) were purchased from Calbiochem. The inhibitors for caspase-3-like proteases

(Ac-DEVD-FMK) and for caspase-1 (Ac-YVAD-FMK) were purchased from Calbiochem. Propidium iodide was obtained from Sigma. Naringenin (NE), naringin (NI), hesperetin (HT), and hesperdin (HD) were obtained from Sigma. Antibodies for PARP, caspase-3, and D4-GDI detection in Western blotting were obtained from IMGENEX. And, antibodies for detecting Bcl-2 family proteins were purchased from Santa Cruz. DCHF-DA was obtained from Molecular Probe. Hesperindinase from *Aspergillus niger* and naringinase from *Pencillium* species were purchased from Sigma. All chemical solvent was purchased from Merck.

2.2. Cell viability

Cell viability was assessed by MTT staining as described by Mosmann [30]. Briefly, HL-60, THP-1, or PMN cells were plated at a density of 10^6 cells/mL into 24-well plates and treated with different concentrations of indicated compounds for 8 hr. At the end of treatment, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (10 mg/mL) was added, and cells were incubated for further 4 hr. Cell viability was obtained by scanning with an ELISA reader (Molecular Devices) with a 600-nm filter.

2.3. Acridine orange staining

A 6 mL of cell suspension was mixed on a slide with an equal volume of an acridine orange solution (10 mg/mL in phosphate-buffered saline). Green fluorescence was detected between 500 and 525 nm using an Olympus microscope. Cells with brightly staining condensed chromatin were recognized as apoptotic cells.

2.4. Western blots

Total cellular extracts (30 μg) were prepared and separated on 8% SDS-polyacrylamide minigels for PARP detection and 12% for SDS-polyacrylamide minigels for caspase-3, cleaved D4-GDI, Bcl-2 family, and α-tubulin protein detection, and then transferred to Immobilon polyvinylidenedifluoride membranes (Millipore). The membrane was incubated overnight at 4° with 1% bovine serum albumin at room temperature for 1 hr and then incubated with indicated antibodies for a further 3 hr at room temperature followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for an hour. Protein was visualized by incubating with the colorimetric substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as described in our previous paper [26].

2.5. DNA gel electrophoresis

Cells (10^6 mL^{-1}) under different treatments were collected, washed with PBS twice, and then lysed in 100 μL of lysis buffer (50 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/mL proteinase K) for 3 hr at 56° and treated with 0.5 mg/mL RNase A for another hour at 56°. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting point agarose, 0.025% (w/w) bromophenol blue) and loaded onto a pre-solidified 2% agarose gel containing 0.1 μg/mL ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer. The gels were observed and photographed under UV light.

2.6. Activities of caspase-3 and caspase-1 (ICE) proteases

After different treatments, cells (10^6 mL^{-1}) were collected and washed three times with PBS and resuspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid. Cell lysates were clarified by centrifugation at 25,000 *g* for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific colorimetric substrates including Ac-DEVD-pNA for caspase-3/CPP32 and Ac-YVAD-pNA for caspase-1 at 37° for 1 hr. The alternative activity of caspase-3 or caspase-1 was described as the cleavage of the colorimetric substrate by measuring the absorbance at 405 nm.

2.7. Flow cytometry analysis

Trypsinized cells (10^6 mL^{-1}) were washed with ice-cold PBS and fixed in 70% ethanol at −20° for at least 1 hr. After fixation, cells were washed twice, incubated in 0.5 mL of 0.5% Triton X-100/PBS at 37° for 30 min with 1 mg/mL of RNase A, and stained with 0.5 mL of 50 mg/mL propidium iodide for 10 min. Fluorescence emitted from the propidium–DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickenson).

2.8. Deglycosylation by hesperindinase and naringinase

Hesperindinase and naringinase were dissolved in 50 mM Tris–HCl buffer, pH 4.0. The reaction buffer contained each compound and hesperindinase and naringinase were added, respectively. Adjust the reaction buffer to 5 mM, pH 4 and 5 and incubated at 40° for 30 min. The HPLC (Jasco, PU1580) analysis used RP-C18 column (5 μm, Merck), with acetonitrile:water (32:68, v/v) at a flow of 0.8 mL/min. Samples were detected by their absorbance at 280 nm (Jasco, UV1575). By cell culture assay, the reaction buffer pH was adjusted to 7.0–7.4 by NaOH and sterilized by 0.22 μm filter and added the 0.5 mL reaction buffer and 0.5 mL culture medium to cell culture assay [31].

2.9. Statistics

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's *t*-test for each paired experiment. A P value of <0.01 or <0.05 was regarded as indicating a significant difference.

3. Results

3.1. Induction of apoptosis by hesperetin and naringenin, but not hesperidin and naringin

Four structurally related flavonoids with or without rutinoside at C7 including aglycone HT, glycone HD, aglycone NE, and glycone naringin (NI) were applied to investigate their apoptosis-inducing effects. The chemical structures of HT, HD, NE, and NI are shown in Fig. 1. An arrow indicates the rutinoside (rhamnoglucoside) group in HD and NI. When HL-60 cells were treated with various concentrations of each indicated compound (40 and 80 μM) for 24 hr, the viability of HL-60 cells was significantly reduced in the presence of NE and HT, but not NI or HD (Fig. 2B). DMSO, even at the highest dose of 0.5%, showed no effect on cellular viability of HL-60 cells. To characterize cell death induced by NE and HT, the integrity of genomic DNA, the occurrence of apoptotic bodies, and the ratio of hypodiploid cells were examined. With analysis of DNA integrity by agarose electrophoresis, NE and HT treatment caused the digestion of genomic DNA into ladders in a concentration- and time-dependent way, associated with a decrease in intact DNA (Fig. 2A). However, with NI and HD, even at 80 μM, no significant DNA ladders were found. In the same part of the experiment, morphological changes and the ratio of hypodiploid cells were examined under microscopic observation and flow cytometric analysis, respectively. NE and HT, but not NI or HD, induced the occurrence of apoptotic bodies under microscopic observation, accompanied by an increased ratio of hypodiploid cells under flow cytometric analysis (Fig. 3A). In the acridine orange staining assay, chromosome condensation was observed in NE- and HT-treated cells by fluorescence microscopy (Fig. 3B). No obvious DNA ladders, apoptotic bodies, or hypodiploid cells were detected in DMSO-treated HL-60 cells. These data demonstrate that the aglycones NE and HT, but not the glycones NI and HD, are effective apoptosis inducers in HL-60 cells.

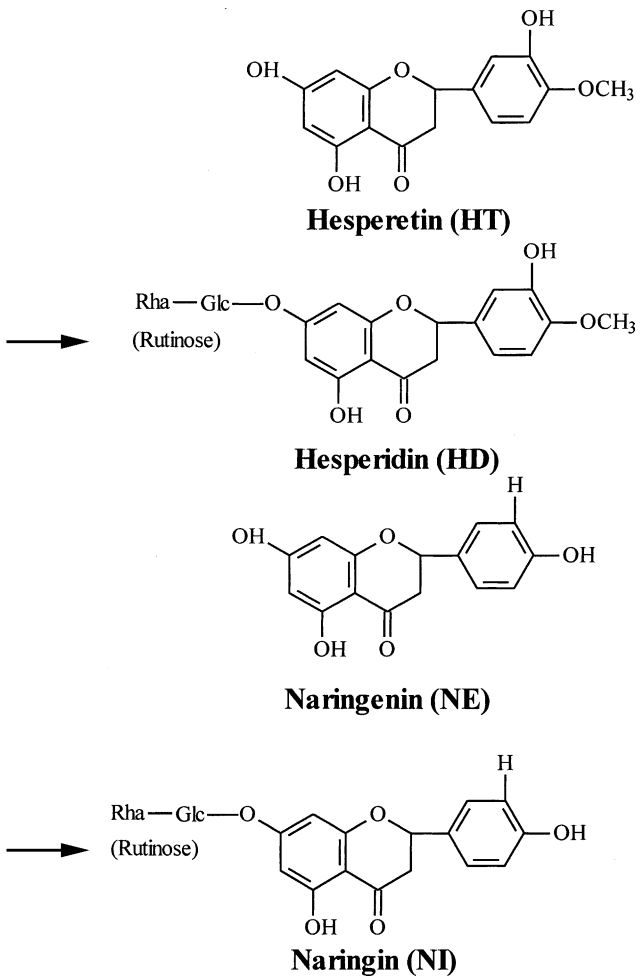

Fig. 1. Structurally related flavonoids including HT, HD, NE, and NI. HD and NI contain a rutinoside at the C7 of HT and NE, respectively. Glc, glucose; Rha, rhamnose; rutinose, rhamnose + glucose.

3.2. Stimulation of caspase-3-like activities, not caspase-1-like activities, in NE- and HT-induced apoptosis

Previous data indicated that induction of caspases activities is an essential event in apoptosis. Caspases-1 and -3 have been shown to be extensively involved in the apoptotic process. To detect the enzymatic activity of caspases-1 and -3 in NE-, NI-, HT-, and HD-treated HL-60 cells, two colorimetric substrates, Ac-DEVD-pNA for caspase-3-related activities and Ac-YVAD-pNA for caspase-1-related activities, were used in this study. As illustrated in Fig. 4, NE and HT induced a dramatic increase in DEVD-specific, but not YVAD-specific, caspase activity in HL-60 cells. In contrast, none of the DEVD-specific and YVAD-specific enzyme activities was stimulated in NI- or HD-treated cells. This suggests that activation of caspase-3, but not caspase-1, activity participated in NE- and HT-induced apoptosis. To determine if the activation of caspase-3-like protease is necessary for apoptosis induced by NE and HT, caspase-inhibitors including the caspase-3-like protease inhibitor, Ac-DEVD-FMK, and the caspase-1-like protease inhibitor, Ac-YVAD-FMK, were used to block intracellular protease, and NE- and HT-induced DNA ladders were analyzed by agarose electrophoresis. Results in Fig. 4D show that the caspase-3-like inhibitor, Ac-DEVD-FMK (20 and 40 μM), inhibited the occurrence of DNA ladders induced by NE or HT (80 μM). However, Ac-YVAD-FMK, an inhibitor of caspase-1-like activity, showed no

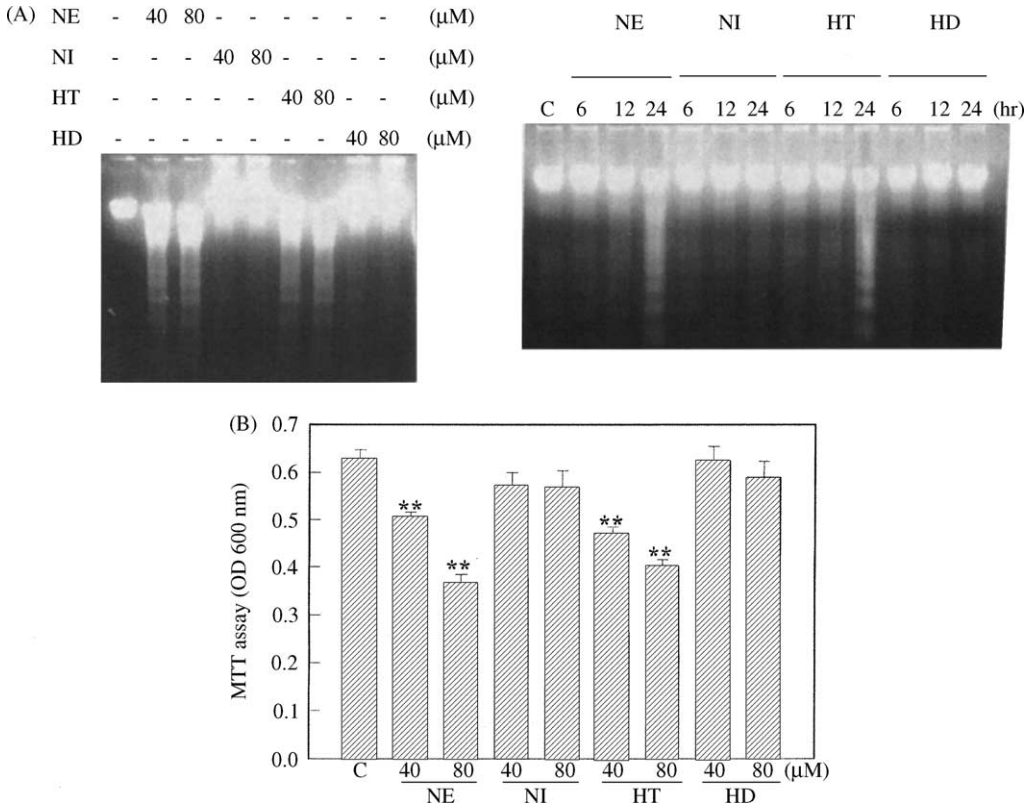

Fig. 2. Analysis of cell viability and DNA integrity in HT-, HD-, NE-, and NI-treated HL-60 cells by MTT assay and agarose electrophoresis. (A) HL-60 cells were treated with different concentrations (40 and 80 μM) of the indicated compound for 24 hr (left panel). HL-60 cells were treated with NE, NI, HT, and HD (80 μM) for 6, 12, and 24 hr (right panel). Integrity of DNA in cells was extracted and electrophoresed through a 1.8% agarose gel and visualized by staining with ethidium bromide. (B) HL-60 cells were plated into 24-well plates for 24 hr and then treated with different concentrations (40 and 80 μM) of indicated compound for a further 24 hr. MTT was added into medium for an additional 4 hr. The viability of cells was detected by measuring the absorbance at a wavelength of 600 nm. Each value is presented as the mean ± SE of three independent experiments. $^{**}P < 0.01$ significantly different from the control, as analyzed by Student's *t*-test.

obvious effect at the same concentrations. Similarly, Ac-DEVD-FMK, but not Ac-YVAD-FMK, attenuated NE- or HT-induced cytotoxicity according to the MTT assay (Fig. 4E). These data demonstrate that activation of caspase-3-like activity is involved in NE- and HT-induced apoptosis.

3.3. Involvement of PARP and D4-GDI cleavage, caspase-3 protein procession, and a decrease in Mcl-1 protein in NE- and HT-, but not in NI- and HD-treated HL-60 cells

Activation of caspase-3 leads to the cleavage of a number of proteins, two of which are PARP and D4-GDI, another hallmark of apoptosis. Results in Fig. 5A show that exposure of HL-60 cells to NE or HT (80 μM) caused the degradation of 116-kDa PARP into 85-kDa fragments and the production of cleaved D4-GDI protein (23 kDa) in a concentration-dependent manner, associated with the protein procession of caspase-3 brought about by its cleavage, represented here as a decline in its pro-level and the appearance of cleaved fragments on the Western blot. However, NI and HD showed no obvious effects on PARP, D4-GDI, or caspase-3 cleavage in HL-60 cells. Bcl-2 family proteins act as important regulators of apoptosis and are located upstream of caspase activation. In NE- or HT-treated HL-60 cells, a decrease in the Mcl-1 protein was detected in a dose-dependent manner. In contrast to a decrease in Mcl-1 proteins, Bcl-2, Bcl-XL, Bag, and Bax proteins remained unchanged in NE- or HT-treated HL-60 cells (Fig. 5A). No obvious change was detected in the expression of any of the Bcl-2 family proteins in NI- or HD-treated HL-60 cells. Furthermore, a dose-dependent induction of caspase-3 cleavage, PARP, and D4-GDI cleavage, and a decrease in the Mcl-1 protein was examined in NE- and HT-treated HL-60 cells (Fig. 5B).

3.4. NE and HT showed no significant apoptosis-inducing activity in mature monocytic cells THP-1 and primary polymorphonuclear (PMN) cells

THP-1 is a mature monocytic cell line, and PMN cells were isolated from human venous blood of healthy volunteers. In order to identify if NE and HT exhibit differential apoptosis-inducing activities among poorly differentiated promyelocytic leukemia cells HL-60, mature monocytic

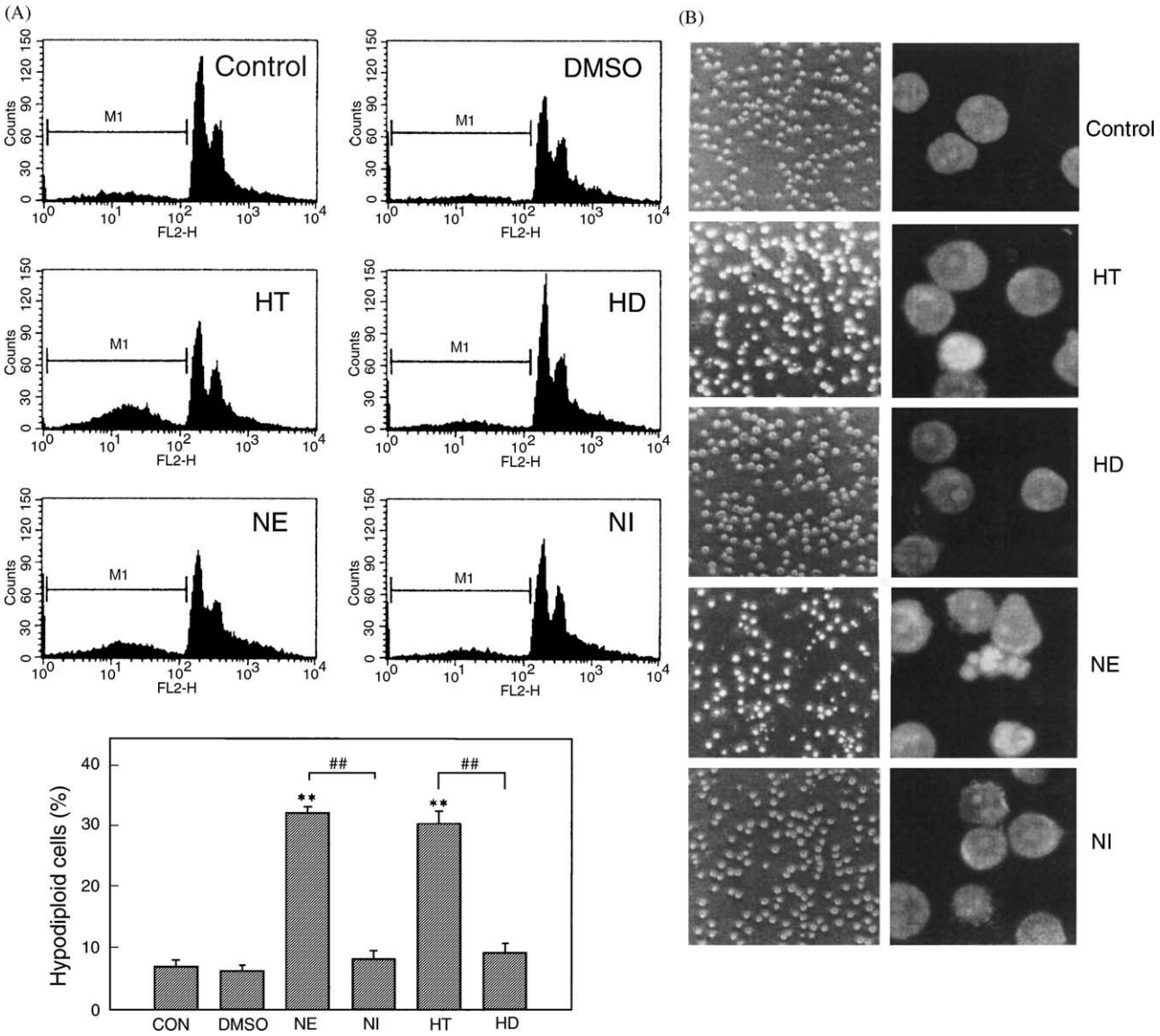

Fig. 3. Appearance of chromosomal condensation, hypodiploid cells and apoptotic bodies in HT- and NE-, not HD- and NI-treated HL-60 cells. (A) Induction of hypodiploid cells in HT- and NE-, but not HD- and NI-treated HL-60 cells. HL-60 cells were treated with or without indicated compound (80 μM) for 24 hr, and the appearance of hypodiploid cells was detected by flow cytometry using PI staining. Upper panel: A representative of the result of flow cytometry analysis was shown. Lower panel: The M1 apoptotic value expressed as the mean ± SE of three independent experiments by flow cytometry analysis was described. $^{**}P < 0.01$ significantly different from the control and $^{\#\#}P < 0.01$ significant difference between indicated groups, as analyzed by Student's *t*-test. (B) Occurrence of apoptotic bodies and chromosomal condensation in HL-60 cells was detected in 80 μM HT- and NE-, but not HD- and NI-treated HL-60 cells under light microscopic observation (left panel) and fluorescent microscopy using acridine orange staining (right panel), irrespectively.

cells THP-1 and primary normal PMN cells, a comparison of the effects of NE and HT in HL-60, THP-1, and PMN cells was performed in the present study. Results in Fig. 6A show that NE and HT induced DNA ladders in HL-60 cells, but not on THP-1 and PMN cells. Results of the MTT assay also supported that both NE and HT significantly exhibited cytotoxic effects in HL-60, but not in THP-1 and primary PMN cells (Fig. 6C). Induction of caspase-3 protein procession, PARP and D4-GDI cleavage by HT and NE in proleukemia cell HL-60, but not in the mature monocytic cells THP-1, were detected by Western blotting (Fig. 6B). This indicates that both NE and HT reserve differential apoptosis-inducing effects among promyelocytic leukemia cells HL-60, mature monocytic leukemia cells THP-1 and primary normal PMN cells.

3.5. Removing rutinoside by nariginase and hesperidinase recovers the apoptosis-inducing activity in NI- or HT-treated HL-60 cells

Previous data suggested that the rutinoside at C7 might be a determinant involved in the induction of apoptosis by flavonoids. Both naringinase (NIase) and hesperidinase (HDase) have been shown to digest rutinose from the glycones NI and HD to produce the aglycones NE and HT, respectively. In the absence of enzymes, glycones (NI

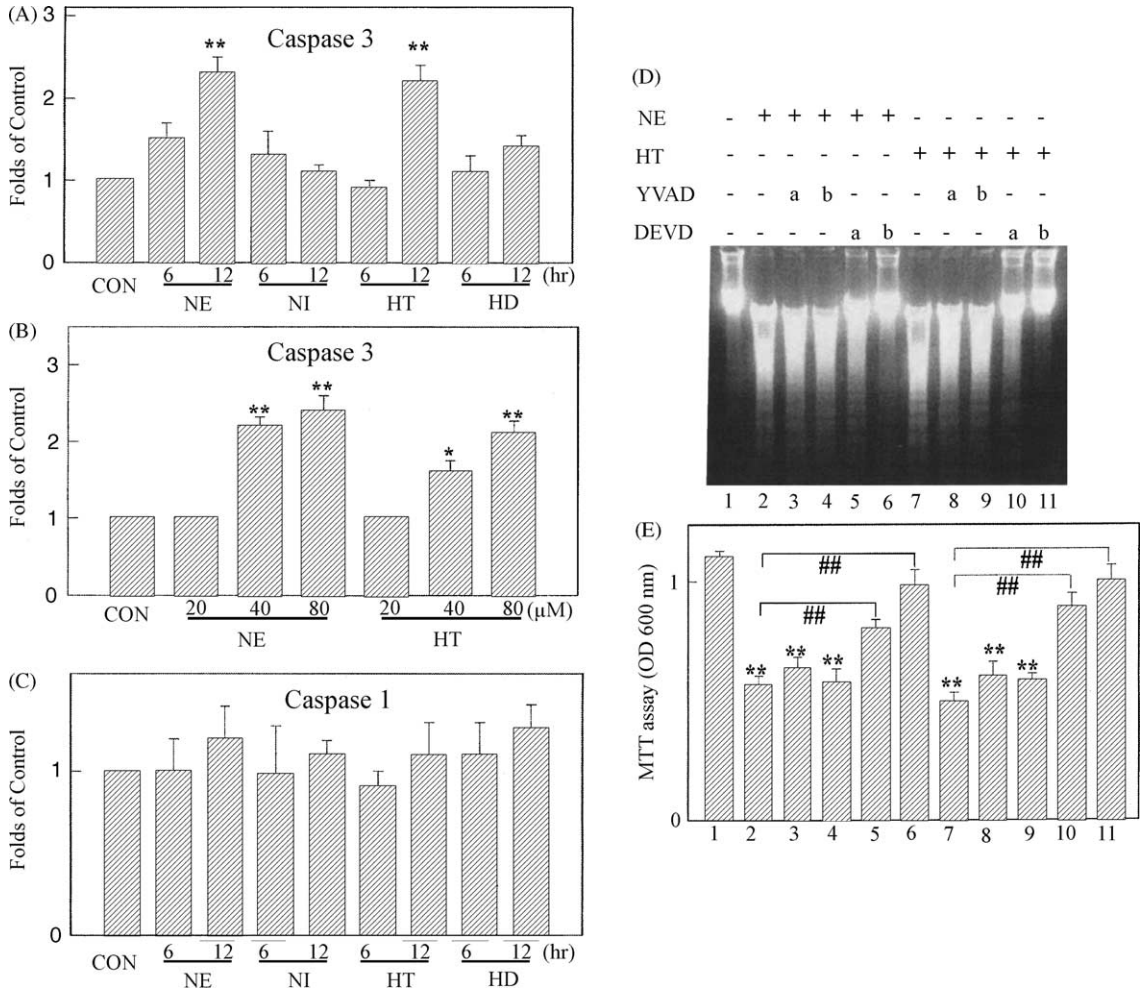

Fig. 4. Activation of caspase-3 but not caspase-1 activity in NE- and HT-treated (80 μM) HL-60 cells. (A) HL-60 cells were treated with NE, NI, HT, and HD (80 μM) for 6 and 12 hr, and cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3-like proteases were determined by incubation with specific colorigenic substrates, Ac-DEVD-pNA. (B) HL-60 cells were treated with different doses of NE and HT (20, 40, and 80 μM), and the caspase-3 activity was detected using Ac-DEVD-pNA as a substrate. (C) Cells were treated as described in (A) and caspase-1 activity in cells was measured by using Ac-YVAD-pNA as a substrate. (D) Caspase-3 peptidyl inhibitor Ac-DEVD-FMK (DEVD), but not caspase-1 peptidyl inhibitor Ac-YVAD-FMK (YVAD), inhibited NE- and HT-induced apoptosis. Cells were treated with or without different doses (a: 20 μM; b: 40 μM) of indicated inhibitors for 1 hr, followed by NE- or HT-treatment (80 μM) for a further 24 hr. The integrity of DNA in cells was examined as described in Fig. 2. (E) The viability of cells under different treatments as those in (D) was evaluated by MTT assay. $^{**}P < 0.01$ significantly different from the control and $^{\#\#}P < 0.01$ significant difference between indicated groups, as analyzed by Student's *t*-test.

and HD) and algycones (NE and HT) were detected by HPLC analysis with different retention time points. Result in Fig. 7A shows that the retention times of the glycone (NI and HD) and the algycone (NE and HT) are about 2.5 and 5.3 min after injection, respectively. In the presence of enzyme digestion including NI digested by NIase and HD digested by HDase, we detected a time-dependent decrease in the glycones NI and HD, accompanied by a time-dependent increase in aglycone NE and HT, by HPLC analysis. Interestingly, the addition of NIase-digested NI or HDase-digested HD significantly induced cytotoxicity in HL-60 cells, compared with NI-, HD-, NIase-, and HDase-treated cells (Fig. 7C). Furthermore, results of DNA integrity analysis showed that induction of DNA fragmentation was found in NIase-digested NI or HDase-digested HD but not in NI-, HD-, NIase-, or HDnase-treated HL-60 cells. Results in Fig. 7 provide direct evidence to suggest that the rutinoside moiety at C7 is an important determinant for the induction of apoptosis by flavonoids.

4. Discussion

Several biological functions of flavonoids have been identified, whereas there is a lack of evidence to support the relationship between rutinoside- and apoptosis-inducing activity. In the present study, we demonstrate that the aglycones NE and HT are able to induce apoptosis in the human leukemia cell line HL-60, however the glycones NI and HD, even at a dose of 80 μM, did not affect the viability of cells. The induction of apoptosis by NE and HT is consistent with the activation of the apoptotic machinery

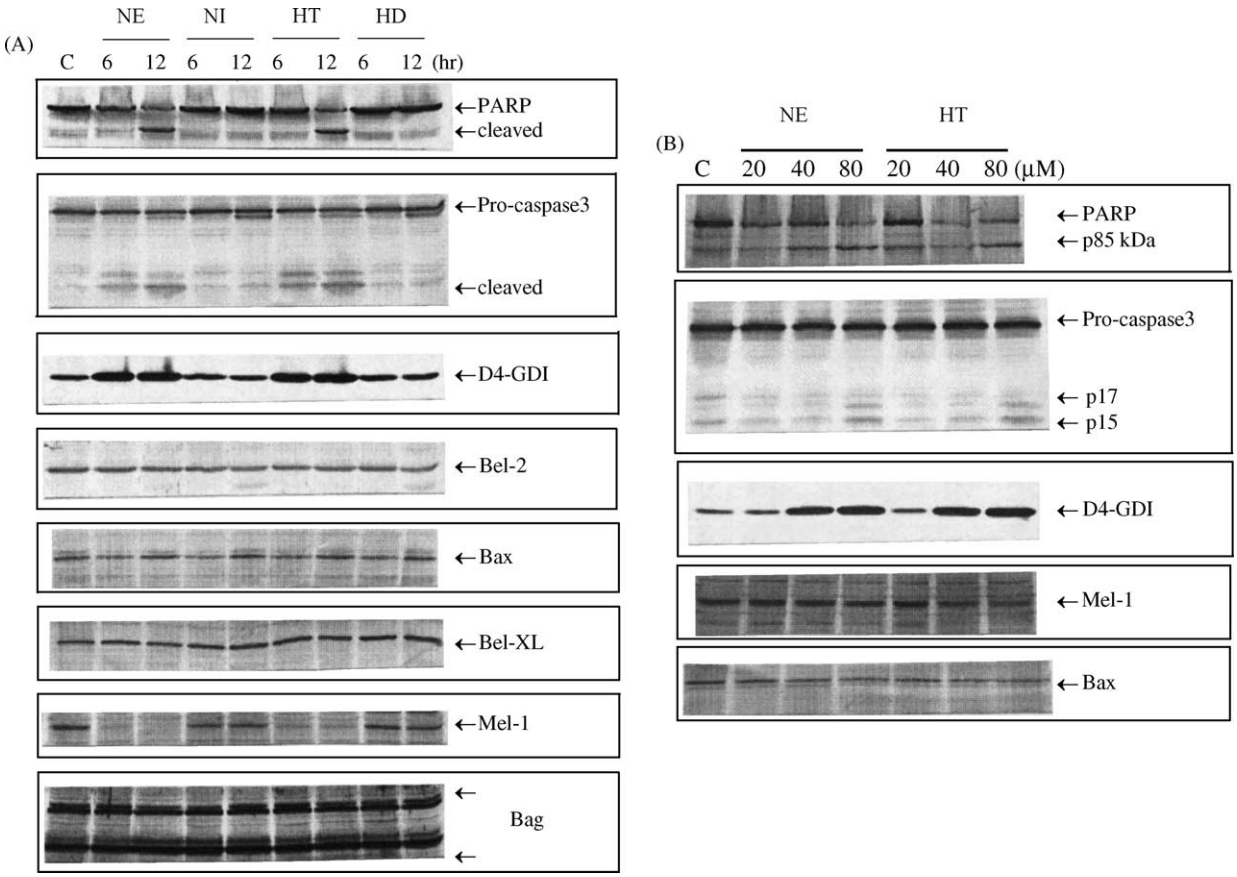

Fig. 5. Induction of caspase-3 protein processing, PARP and D4-GDI protein cleavage, and a decrease in Mcl-1 protein in NE- and HT-, but not NI- and HD-treated HL-60 cells. (A) HL-60 cells were treated with, NE, NI, HT, HD (80 μM) for 6 or 12 hr. Bcl-2 family proteins including Bcl-2, Bcl-XL, Bax and Mcl-1, PARP and D4-GDI cleavage and induction of caspase-3 protein procession were analyzed by Western blotting as described in Section 2. (B) Dose-dependent response of NE and HT in the caspase-3 protein procession, PARP and D4-GDI cleavage, and decreasing Mcl-1 protein. HL-60 cells were treated with different doses of NE and HT (20, 40, and 80 μM) for 12 hr, and expression of indicated genes was analyzed by Western blotting.

including activating the caspase-3 (not the caspase-1) cascade. Additionally, neither NE nor HT induced apoptosis in normal monocytic THP-1 cells, a preferential normal monocytic cell line. Removing the rutinoside moiety from NI and HD by NIase and HDase stimulated the occurrence of apoptosis in cells. Results of the present study suggest that (1) both aglycones NE and HT, but not the glycones NE and HD, possess apoptosis-inducing activity in HL-60 cells through activation of the caspase-3 cascade; (2) rutinoside at C7 acts as a negative moiety for the induction of apoptosis by flavonoids; and (3) both NE and HT show the apoptosis-inducing activity in the promyelocytic cells HL-60, but were less cytotoxic to the mature monocytic cells THP-1 and human PMN cells.

Flavonoids as either simple or complex glycosides exist in many plants, and humans are estimated to consume approximately 1 g flavonoids per day. Several biological functions of flavonoids have been identified, however their use in the pharmaceutical field is limited by their aqueous solubility [32]. Glycosylation occurred in the metabolism of flavonoids, and increased their hydrophilic properties, and oral ingestion of glycone flavonoids including HD or NI in rats produces several metabolic products in the urine such as the aglycones by the intestinal bacteria-producing rhamnosidase or glucosidase [33,34]. Kim *et al.* reported that the anti-platelet activity and cytotoxicity of the metabolites formed in the human intestine were more pronounced than that of the parent compound [35]. Ioku *et al.* suggested that dietary flavonoid glucosides were primarily hydrolyzed and liberated aglycones in the jejunum [36]. These data indicate a conversion of glycones to aglycones in the metabolism of flavonoids. In relation to the compounds used in the present study, several biological activities between aglycone (HT and NE) and glycone (HD and NI) were described in Section 1. However, the importance of the rutinoside moiety on the apoptosis-inducing activities of flavonoids is still undefined. Results of the present study show that the aglyones HT and NE, but not the glycones HD and NI, are apoptosis inducers, and digestion of the rutinoside group from NI and HD by NIase and HDase, respectively, stimulates their apoptotic effects. This suggests that rutinoside at C7 of flavonoids acts as a negative moiety in apoptosis induction.

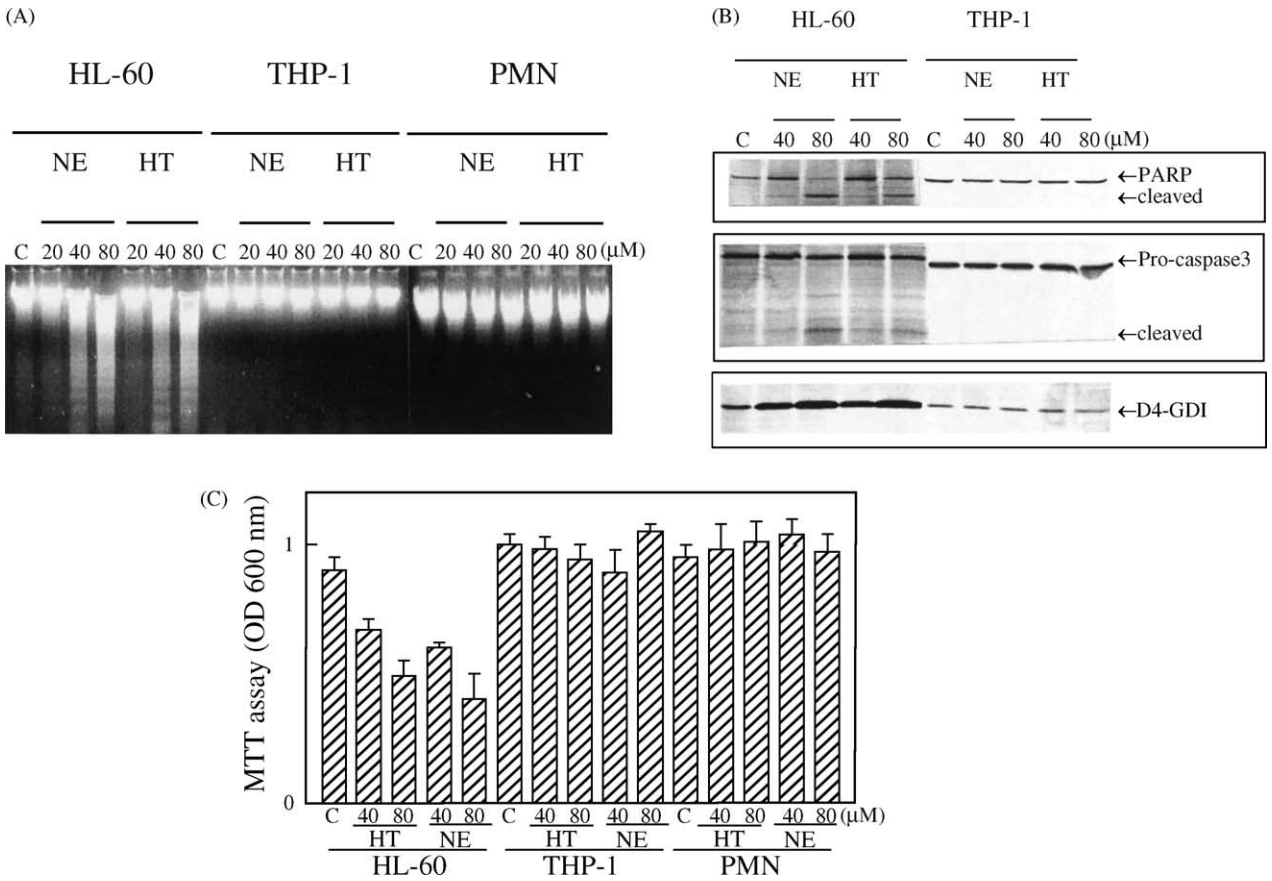

Fig. 6. Differential apoptosis-inducing effect of NE and HT in promyelocytic leukemia cells HL-60, mature monocytic cells THP-1, and PMN. (A) NE and HT showed the significant apoptosis-inducing activity in HL-60, but not in THP-1 and PMN cells. Cells were treated with NE and HT (20, 40, and 80 μM) for 24 hr, and the integrity of DNA in cells was analyzed. (B) NE and HT did not induce caspase-3 protein procession, PARP and D4-GDI cleavage in THP-1 cells, compared with those in HL-60 cells. Both cells were treated with different doses (40 and 80 μM) of NE and HT for 12 hr, and the expression of indicated genes was examined as described in Fig. 5. (C) MTT assay for cellular viability as described in Fig. 2B was performed to identify differential cytotoxicity of NE and HT in HL-60, THP-1 and PMN cells. $^{**}P < 0.01$ significantly different from the respective control, as analyzed by Student's *t*-test.

None of apoptotic mechanisms induced by HT, HD, NE or NI was identified previously. NE has been shown to scarcely cause cell death in B16 melanoma cells and was ineffective in inhibiting growth in human prostatic tumor cells (PC-3), however NE enhanced tumor necrosis factor-α (TNF-α) cytotoxicity [37,38]. In contrast to NE, NI suppressed LPS-induced TNF release and liver injury, and recent studies demonstrated that NI was able to prevent hepatocytes from alga toxins-induced apoptosis [39]. No previous papers reported the apoptotic effect of HT and HD. The caspase cascade has been shown to be involved in the action of apoptosis, and caspase-3 is an executioner caspase and exists in the cytoplasm as an inactive pro-caspase-3 that becomes proteolytically activated by multiple cleavages of its precursor 32 kDa to generate the 20/11- or 17/11-kDa active forms in apoptotic cells. Additionally, Bcl-2 family proteins have been demonstrated to be involved in the process of apoptosis, and pro-apoptotic and anti-apoptotic Bcl-2 family proteins including Bax, Bak, and Bcl-Xs for pro-apoptosis and Bcl-2, Bcl-XL, and Mcl-1 for anti-apoptosis have been identified. Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins participated in the process of apoptosis. In the present study, we found that apoptosis induced by the aglycones NE and HT was mediated by activation of caspase-3 and decreased the anti-apoptotic Bcl-2 family protein Mcl-1, whereas neither of them was found in the glycones NI- and HD-treated cells. This suggests that differential activation of the caspase-3 cascade is involved in the modulation of induction of apoptosis by flavonoids.

Both free radical scavenging and production activities of flavonoids have been previously described [40]. Our previous studies indicated that the production of reactive oxygen species (ROS) was not involved in the induction of apoptosis by flavonoids [19,41], however several previous studies supported ROS acting as an apoptotic initiator to turn on signal transduction for apoptosis [42,43]. Therefore, it is interesting to investigate if ROS are involved in the differential apoptosis-inducing activity of the aglycones NE and HT and the glycones NI and HD. As described in our previous paper, DCHF-DA was used to examine if alternative ROS production in aglycone- and glycone-treated HL-60 cells. Results of DCHF-DA assay showed that neither aglycones (NE and HT) nor glycones

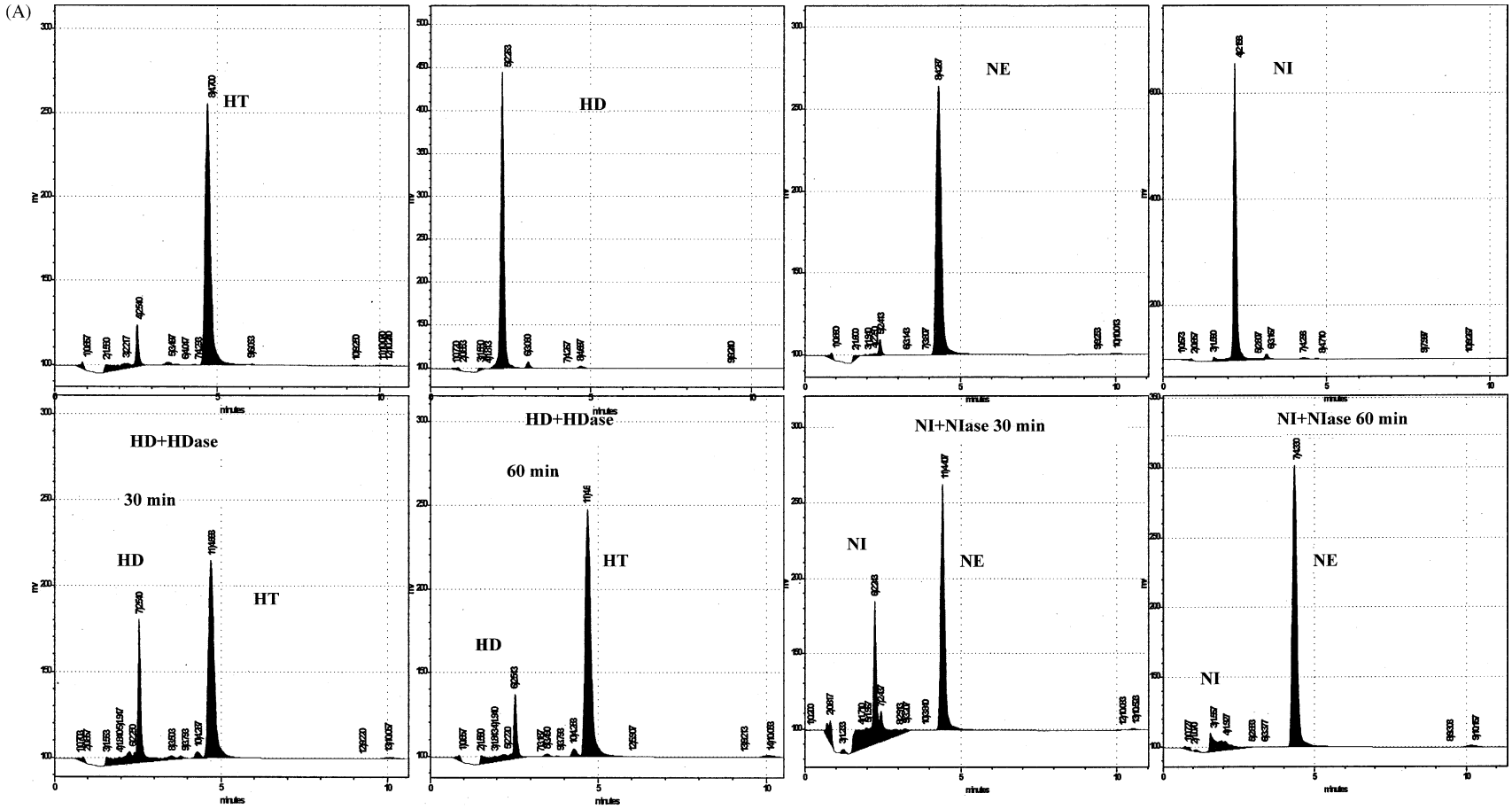

Fig. 7. Digestion of rutinoside from HD and NI by HDase and NIase, respectively, induces apoptosis in HL-60 cells. (A) *In vitro* analysis of HT, HD, NE, and NI by HPLC analysis as described in Section 2. HD + HDase and NI + NIase indicated that HD and NI incubated with HDase and NIase for indicated time points (30 and 60 min), and the conversion of glycones to aglycones was detected by HPLC analysis. (B) Effect of enzyme digestion on the DNA integrity of HL-60 cells. HL-60 cells were treated with HD (lane 1), HDase (lane 2), HD + HDase 30 min (lane 3), HD + HDase 60 min (lane 4), NI (lane 5), NIase (lane 6), NI + NIase 30 min (lane 7), and NI + NIase 60 min (lane 8) for 24 hr. The integrity of DNA was analyzed by electrophoresis as described in Fig. 2. (C) HL-60 cells were treated as those in (B) and the viability of cell was examined by MTT assay as described in Fig. 2. $^{**}P < 0.01$ significantly different from the control, as analyzed by Student's *t*-test.

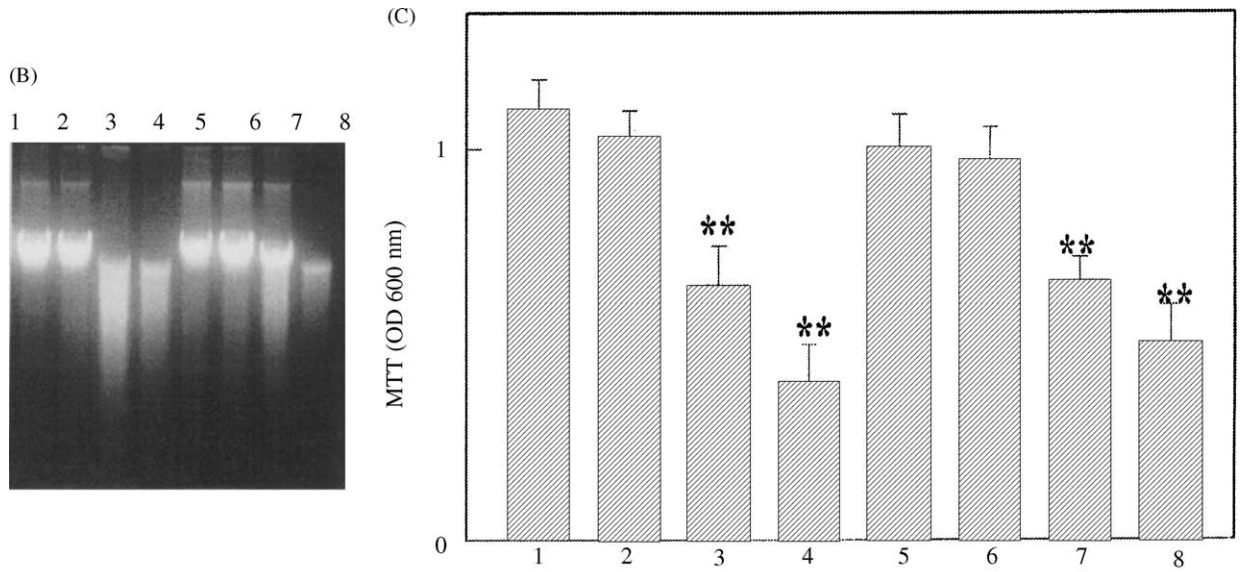

Fig. 7. (*Continued*).

(NI and HD) showed an effect on the intracellular ROS level by flow cytometry analysis. The antioxidants *N*-acetyl-cysteine and catalase inhibited H_2O_2-induced DNA fragmentation, but no prevention of aglycones NE- or HT-induced cell death was detected (data not shown). This suggests that ROS production is not involved in the differential apoptosis-inducing activities of aglycones (NE and HT) or glycones (NI and HD).

In conclusion, results of the present study provide scientific evidence to support the addition of rutinoside at C7 attenuating the apoptosis-inducing activity of flavonoids, and that the caspase-3 (not caspase-1) cascade is involved. More evidence to demonstrate the effect of the glycosylated position and other substituted glycosides such as glucoside or complex glucosides on the induction of apoptosis by flavonoids needs to be further evaluated.

Acknowledgments

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