Hot Water Extract of Bark of Nikko Maple (Acer nikoense) Induces Apoptosis in Leukemia Cells

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In screening for antitumor constituents in traditional crude drugs, we used three cultured cell lines: mouse leukemia P388 cells, doxorubicin-resistant P388 cells and lecyzme (catalytic lectin)-resistant P388 cells. The hot water extract (HWE) of the bark of Nikko maple (Acer nikoense) showed concentration-dependent inhibitory effects on the growth of these three cell lines. DNA fragmentation and morphological changes, accompanied by condensed and fragmented nuclei, were observed in the leukemia cell lines cultured with HWE of the bark of Nikko maple. Treatment with this HWE increased the expression of sialylated glycoconjugates on the apoptotic cells. These results suggest that HWE induces cell death via apoptosis in vitro.

Key words  Bark; Nikko maple; Acer nikoense; antitumor activity; apoptosis; sialylated glycoconjugate

Some plant phenols, curcumin (diferuloyl methane) from Curcuma longa and baicalein from Scutellaria baicalensis, exhibit antiproliferative effects in tumor cell lines1-3 and cultured vascular smooth muscle cells.4,5 Curcumin, classified into a group of diarylethanol, is a major phenolic antioxidant and anti-inflammatory agent and an effective inhibitor of tumor production.6,7 In addition, the induction of apoptosis in tumor cells in vitro by plant (poly)phenolic substances with antioxidant activity such as tannins and gallic acid, has been reported.8,9 It has been reported that tannins are present in the natural kingdom in large amounts and show anti-tumor activity by modulating host immunity.9-10 and that gallic acid shows selective cytotoxicity to tumor cells compared with normal cells.11 In contrast, antioxidants such as retinol and α-tocopherol have been shown to be effective in protecting cells from cell death caused by oxygen toxicity.12,13 As mentioned above, antitumor-constituent substances have been obtained from many traditional crude drugs. The hot water extract (HWE) of the bark of Nikko maple (Acer nikoense, Aceraceae) has traditionally been used to treat hepatic diseases or as an eyewash.14 The bark of Nikko maple comprises several diarylethanol and their glycosides, termed acerogelin and aceroside, respectively.14,15 In this study, we sought to determine whether the HWE of Nikko maple suppresses cell growth in vitro, and whether HWE-treated cells display characteristics of apoptosis.

MATERIALS AND METHODS

Chemicals  HWE of the bark of Nikko maple was prepared by the method of Kosuge et al.16 The dried stem bark (6g) was cut into small pieces and extracted twice with distilled water (60 ml) under reflux for 1h. The extract was lyophilized (HWE). Alamar Blue™ and aphidicolin were purchased from Wako Pure Chemicals Industries (Osaka, Japan). H33258 was purchased from Calbiochem-Behring Corp. (San Diego, CA). Fluorescein-isothiocyanate (FITC)-labeled Limax fulvus agglutinin (FITC-LFA), and FITC-labeled Sambucus sieboldiana agglutinin (FITC-SSA) and FITC-labeled Maackia amurensis agglutinin (FITC-MAM) were from E-Y Laboratories, Inc. (San Mateo, CA) and Seikagaku Kogyo (Tokyo, Japan), respectively. Other general reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Cell Culture  Murine leukemia P388 cell lines of varying status were used in this study. P388 cells (wild-type, P388/S) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Doxorubicin-resistant P388 cells (P388/Dox) and lecyzme-resistant P388 cells (RC150) were developed from parent P388/S cells by growth in progressively increasing concentrations of doxorubicin17 and lecyzme obtained from the eggs of Rana catesbeiana,18 respectively. These cells were cultured synchronously with aphidicolin, a tetacyclic diphenidol obtained from Cephalospolium aphidicola Petch,19 and then maintained in medium R (RPMI 1640 supplemented with 10% fetal calf serum) at 37°C in a humidified 5% CO₂ incubator.

Effects of HWE of Nikko Maple on Cell Growth  HWE of Nikko maple was dissolved in medium R. To study the cytotoxic effect of HWE, cells (1.8×10⁵/100 μl) were exposed to different concentrations of HWE at 37°C for 48 h. HWE-treated cells in 96-well plates were incubated with alamar Blue™ for 4h. Optical density was determined using a microplate reader MPR-A4i (Tosoh, Tokyo, Japan) at 570 nm/600 nm. All testing was done in triplicate and the percent cytotoxicity was calculated.

Determination of DNA Fragmentation in Agarose Gel  At the end of each treatment described above, HWE-treated cells were centrifuged at 400×g. After being washed with phosphate-buffered saline (PBS), cells were lysed in Tris-HCl buffer (50 mm, pH 7.8) containing 10 mm EDTA and 0.5% w/v sodium-N-lauroylsarcosinate (20 μl). RNase A (1 μl, 10 mg/ml) was added and the mixture was then incubated at 50°C for 30 min. Then, proteinase K (1 μl, 10 mg/ml) was added and the mixture was treated at 50°C for 1h.20 DNA samples were run on 1.6% agarose gel and visualized by ethidium bromide staining under UV light.

Analysis of Morphological Changes  Cells were exposed to HWE in medium R for 48 h. At the end of the experiment, cells were washed with PBS, fixed in 1% glutaraldehyde/PBS and then stained with 1% osmium tetroxide/PBS.21 Alterations in cell nuclei were photographed under a fluorescence microscope.

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Flow Cytometry Analysis of Cell Surface Sialylated Glycoconjugate Expression After HWE treatment, cells were washed with ice-cold PBS. Cells were incubated with FITC-LFA, FITC-SSA or FITC-MAM at 4 °C for 30 min. Samples were analyzed using FACScan (Becton Dickinson, San Jose, CA) at a laser setting of 36 mM and an excitation wavelength of 488 nm.

Flow Cytometry Analysis of Production of Intracellular Peroxides After HWE treatment, cells were washed with ice-cold PBS. Cells were recultured with 5 mM 2,7’-dichlorofluorescein diacetate (DCFH-DA)/dimethylsulfoxide (10 µl) at 37 °C for 30 min. Samples were analyzed using FACScan.

RESULTS AND DISCUSSION

HWE of Nikko Maple Inhibited Tumor Cell Growth Mouse tumor cell lines were exposed to 2.0—250 µg/ml of HWE for 48 h. At the end of the experiment, the number of viable cells was determined by an alamar Blue assay method. Figure 1 shows the concentration-dependent growth inhibitory effect caused by HWE on the different cell lines. The IC_50s were as follows: 25 µg/ml for P388/S and P388/Dox cells, and 42 µg/ml for RC150 cells. At a concentration of 31 µg/ml of HWE, P388/S and P388/Dox cell lines showed about 70% growth inhibition, while RC150 cells showed 20% inhibition. Since microvilli and microspikes are rarely seen on RC150 cells, it may be hard for RC150 cells to uptake the cytotoxic substance(s) of HWE. Since P-gp was expressed on P388/Dox cells (data not shown), P388/Dox cells have the properties of multiple drug resistance. HWE inhibits not only chemotherapeutic drug-sensitive but also multiple drug-resistant tumor cell growth. Because HWE is composed of many substances, such as tannins and acerossides, it is necessary to identify its apoptosis-inducible heat-stable substance(s) in further studies.

HWE of Nikko Maple Induced Apoptosis in P388 Cell Lines The significant influence of HWE on the growth of these cells led us to investigate whether the effect of this extract was a result of triggering programmed cell death. After treatment of these cells with HWE for 48 h, the genomic DNA from the cells was subjected to gel electrophoresis. Apoptosis due to this treatment was recognized by the appearance of a typical DNA fragmentation ladder detected by gel electrophoresis in all three cell lines (Fig. 2). DNA fragmentation was concentration-dependent; after 48 h, about 90% of the DNA was fragmented in each cell line treated with 120 µg/ml of HWE evaluated by densitometric assay.

Apoptosis was further observed under a fluorescence microscope. The H33258 staining shows apoptotic nuclei, either condensed or fragmented. The typical morphological changes of P388/S cell nuclei after 48 h incubation with 60 µg/ml of HWE is shown in Fig. 3. The percentage of apoptotic cells (the number of apoptotic cells out of 100 total cells counted) in P388/S cells was 80%.

Expression of Sialylated Glycoconjugates Upregulated on the Cell Surface of Nikko Maple-Treated Cells Lectin-like molecules mediate macrophage recognition of changes in the surface carbohydrates on apoptotic cells. Apoptotic rat liver cells displaying surface carbohydrate deavd of sialic acid residues are ingested by neighboring liver cells which express an asialoglycoprotein receptor. To study whether sialic acid residues of surface carbohydrates on apoptotic cells are reduced in three P388 cell lines by the treatment of HWE, we studied the expression of sialylated glycoconjugates by flow cytometry analysis using FITC-labeled sialic acid-recognizing lectins.

Treatment with HWE increased the expression of LFA- and SSA-reactive sialylated glycoconjugates on the three cell lines (Fig. 4). LFA recognizes sialic acid residues of carbohydrate chains, regardless of its linkage. SSA and MAM also recognize sialic acid residues: SSA and MAM have a strong affinity against Siaα2-6Gal/GalNAc and tri- or tetra-antenary complex carbohydrate chains containing Siaα2-3Gal sequence, respectively. These results suggested that carbohydrate structures containing a Siaα2-6Gal/GalNAc sequence were increased.
Fig. 3. Morphological Changes in P388/S Cells Treated with HWE of Nikko Maple
A and C: phase-contrast microscopic view of B and D. B and D: fluorescence microscopic view of H33258 staining. ×400. A and B: untreated control. C and D: P388/S cells treated with HWE of Nikko maple for 48 h.

Fig. 4. FACSscan Analysis of HWE of Nikko Maple-Induced Sialylated Glycoconjugate Expression
Cells were treated for 48 h with HWE of Nikko maple (----) or with medium R (-----). Then cells were stained with FITC-LFA (A, B and C), FITC-SSA (D, E and F) or FITC-MAM (G, H and I). A, D and G: P388/S cells. B, E and H: P388/Dox cells. C, F and I: RC150 cells.
quence increase in the HWE-treated apoptotic cells, and that a novel sialic acid binding lectin-like molecule may be involved in the phagocytosis of the HWE-treated apoptotic cells.

**Intracellular Peroxides Were Not Produced in HWE of Nikko Maple-Treated P388/S Cells** To elucidate whether intracellular peroxides were produced in P388/S cells undergoing apoptosis by HWE, P388/S cells were incubated with DCFH-DA and analyzed by FACScan. After treatment with HWE for 3, 6, and 21 h, no change in the histogram pattern was observed in comparison with that of untreated cells (data not shown). The induction of apoptosis by HWE is not through a reactive oxygen species production pathway, although the mechanism needs to be further addressed.

Kakeya *et al.* reported that cytorterin A isolated from *Streptomyces* sp. induced apoptosis in HL60 cells. Although inhibitors of caspase-3 or -1-like proteases did not block the cytorterin A-induced apoptosis, Z-Asp-CH$_2$-DCB strongly inhibited both the apoptosis of HL60 cells and the activation of p36 myelin basic protein kinase (MAPK superfamily molecule) induced by cytorterin A. Myricanone from the bark of *Myrica rubra* is a cyclic diarylheptanoid, and induces apoptosis in P388 cells. Myricanone-induced P388 cell death was inhibited by Z-Asp-CH$_2$-DCB but not by inhibitors of caspase-3 or -1 (unpublished observation). The experiments reported here open a new area of research concerning the usage of dietary growth inhibitors of tumor cells.

**REFERENCES**