Note

Apoptosis Induction by Wheat-flour Sphingoid Bases in DLD-1 Human Colon Cancer Cells

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Received March 4, 2002; Accepted May 7, 2002

The apoptotic effects of plant sphingoid bases prepared from wheat-flour cerebroside on human colorectal cancer DLD-1 cells were examined. The viability of DLD-1 cells treated with such plant sphingoid bases was reduced in a dose-dependent manner and was similar to that of cells treated with sphingosine. Morphological changes such as condensed chromatin fragments were found, so those sphingoid bases reduced cell viability through causing apoptosis in these cells.

Key words: apoptosis; colon cancer; sphingoid bases; wheat flour

Sphingolipids are ubiquitous in eukaryotic organisms. The most common sphingoid base of mammalian sphingolipids is trans-4-sphingenine (sphingosine), and smaller amounts of others such as sphinganine and 4-hydroxysphinganine are often found. Compared with animal-origin sphingolipids, plant-origin sphingolipids have more structural variation of their sphingoid bases, such as in 8-sphingenine, 4,8-sphingadienine, and 4-hydroxy-8-sphingenine.1) The mean daily intake of plant-origin sphingolipids has been estimated to be 50 mg for humans.2) However, there is no evidence that dietary sphingolipids from plant sources contribute to human health. Recent reports have indicated that the intake of sphingomyelin, a major animal-origin sphingophospholipid, reduces in the numbers of colonic aberrant crypt foci and adenocarcinoma in CF1 mice treated with 1,2-dimethylhydrazine.3–5) A possible mechanism for this suppression of dietary sphingolipids may be via hydrolysis to bioactive ceramide and sphingosine, because these breakdown products of sphingolipids are now recognized as being intracellular mediators of cell differentiation and apoptosis.5–9) Our hypothesis is that sphingoid bases from plant sources may induce apoptosis in cancer cells, and that dietary plant sphingolipids help to prevent colon cancer. The object of the present study was to evaluate the apoptotic effect on DLD-1 human colorectal cancer cells of sphingoid bases prepared from wheat-flour, which is a major dietary source of plant sphingolipids.11)

Cerebroside was isolated from wheat flour as described previously12) and degraded with aqueous methanolic 1 N HCl for 18 h at 70°C.12) The reaction mixture was chilled, and extracted three times with hexane to remove fatty acid methyl esters. After the methanolic phase was alkali-zed to pH 9 with 4 N NaOH, the sphingoid bases were extracted three times with diethyl ether. The composition of sphingoid bases was analyzed by oxidation with NaIO4 and GC of the resultant fatty aldehydes.13) The major constituents were cis- and trans-8-sphingenine (Table 1). Sphingoid bases were measured by the spectrophotometric method of Lauter and Trams14) with sphinganine as the standard, and stored in stock solution in ethanol. The stock solution was diluted into culture medium (final ethanol concentration, <0.1%).

DLD-1 cells were obtained from the Cancer Cell Repository at Tohoku University School of Medicine (Sendai, Japan) and cultured in RPMI1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2. DLD-1 cells were seeded in 96-well plates (100 μl/well at a density of 1×

Table 1. Composition of Wheat-flour Sphingoid Bases

<table>
<thead>
<tr>
<th>Sphingoid bases</th>
<th>wt%</th>
</tr>
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<tbody>
<tr>
<td>4-Hydroxysphinganine</td>
<td>1.4</td>
</tr>
<tr>
<td>4-Hydroxy-cis-8-sphinganine</td>
<td>1.4</td>
</tr>
<tr>
<td>Sphinganine</td>
<td>7.3</td>
</tr>
<tr>
<td>trans-8-Sphingenine</td>
<td>23.2</td>
</tr>
<tr>
<td>cis-8-Sphingenine</td>
<td>50.8</td>
</tr>
<tr>
<td>trans-4-Sphingenine (sphingosine)</td>
<td>1.3</td>
</tr>
<tr>
<td>trans-4, trans-8-Sphingadienine</td>
<td>5.4</td>
</tr>
<tr>
<td>trans-4, cis-8-Sphingadienine</td>
<td>9.2</td>
</tr>
</tbody>
</table>
Wheat-flour Sphingoid Bases Induce Apoptosis

10^7 cells/ml). After incubation for 24 h, medium was removed and the cells were cultured in the medium containing different doses of the wheat-flour sphingoid bases and sphingosine. Control experiments were done with 0.1% ethanol as the vehicle. The cell viability of the sphingoid bases was measured by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Both wheat-flour sphingoid bases and commercial sphingosine decreased the number of viable cells in a dose-dependent manner (Fig. 1). The reduction of cell viability by wheat sphingoid bases (IC50, 17 μM) was similar to that by sphingosine (IC50, 14 μM).

We examined apoptosis-associated DNA ladder formation. DLD-1 cells (1 × 10^5/ml) were seeded onto 10-cm dishes and treated with 20 μM sphingoid bases for 24 h. Cells were collected by centrifugation and washed with phosphate-buffered saline (pH 7.4). Pellets were suspended in 100 μl of lysis buffer (10 mM EDTA, 10 mM Tris-HCl, and 0.5% (v/v) Triton X-100, pH 8) and incubated at 4°C for 10 min. After centrifugation at 15,000 × g for 20 min to separate intact chromatin (in the pellet) from DNA fragments (in the supernatant), 2 μl of RNase A (1 mg/ml) was added to the supernatant, and the mixture was incubated at 37°C for 1 h. Two microliters of proteinase K (1 mg/ml) were then added and incubation was continued for an additional 1 h. DNA was precipitated with a mixture of 20 μl of 5 M NaCl and 120 μl of 2-propanol overnight at −20°C. Following centrifugation, pellets were air-dried and dissolved in 20 μl of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.4). Extracted DNA was electrophoresed in a 2.0% agarose gel in a mixture of 90 mM Tris, 90 mM boric acid, 2 mM EDTA buffer (pH 8.4) at 100 V. Each gel was stained with ethidium bromide and photographed under UV light. Figure 2 shows that 24 h of exposure of DLD-1 cells to 20 μM wheat-flour sphingoid bases or commercial sphingosine caused apoptosis-associated DNA fragmentation.

The apoptotic cells were evaluated by morphological changes; condensed chromatin fragments were seen under a fluorescence microscope after being stained with 4′,6-diamidino-2-phenylindole (Fig. 3). DLD-1 cells were incubated for 24 h in the presence or absence of wheat-flour sphingoid bases (20 μM), and then stained with 4′,6-diamidino-2-phenylindole. Nuclear morphology was assessed by fluorescence microscopy (magnification, ×400). A, cells without sphingoids; B, cells cultured with wheat-flour sphingoid bases.
The eluent was monitored with a UV-ODS 80Ts, 250 μm column (TSKgel, Tosoh, Tokyo, Japan) was eluted with methanol/water (70/30, v/v) containing 2% aqueous ammonia at the flow rate of 1 ml/min. The purity of sphingoid bases was higher than that in control wells (0.6 ± 0.4%, n = 5). This percentage of apoptotic cells evaluated by morphological changes under a microscope was lower than the cell viability estimated by the MTT assay (Fig. 1), because we ignored floating cells in the calculation.

The mixture of wheat-flour sphingoids was purified by reverse-phase HPLC to give both cis- and trans-isomers of 8-sphingenine. An ODS column (TSKgel-ODS 80Ts, 250 × 4.6 mm, Tosoh, Tokyo, Japan) was eluted with methanol/water (70/30, v/v) containing 2% aqueous ammonia at the flow rate of 1 ml/min. The purity of cis- and trans-8-sphingenine prepared by HPLC was >95% by GC as described above. These plant-origin sphingoids reduced the cell viability of DLD-1 cells in a dose-dependent manner (Fig. 4). This result suggested that the reduction of cell viability by sphingoid bases from wheat flour was mainly due to cis- and trans-8-sphingenine.

These results indicate that wheat-flour sphingoid bases induced apoptosis in DLD-1 human colon cancer cells. We report for the first time an apoptotic effect of plant-origin sphingoids on human cancer cells. It has been known that both mammalian ceramide and sphingosine induce apoptosis in various cultured cell lines, but the mechanism by which sphingosine induced apoptosis is different from that of ceramide. Previous studies showed that the apoptotic effect of sphingosine on cancer cells is related to the activation of caspases and the regulation of the bcl-2 gene family. Hung et al. reported that other long-chain bases (including sphinganine, dimethylsphingosine, and stearylamine) induce apoptosis in Hep3B hepatoma cells, but that octylamine (a short-chain analog of sphinganine) dose not. Therefore, a long-chain structure may be essential for this apoptotic effect of sphingoid bases on cancer cells. Plant sphingolipids in nature are composed of a variety of different sphingoid bases. For example, kidney beans have mostly 4,8-sphinigadienine (80%), and spinach leaves have mostly 4-hydroxy-8-sphingenine (60–70%).

The digestion of plant sphingolipids has not been reported. Several studies have indicated that dietary animal-origin sphingolipids can be hydrolyzed by intestinal enzymes. The nutritional contribution of plant-origin sphingolipids in vivo remains unknown.

Acknowledgment

This study was supported by grants from the Takano Life Science Research Foundation.

References


