Induction of granulocytic differentiation in acute promyelocytic leukemia cells (HL-60) by water-soluble chitosan oligomer

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Abstract

Water-soluble chitosan oligomer (WSCO) has been reported to have anticancer activity, immuno-enhancing effect and antimicrobial activity. However, other biological activities are unknown. Herein, we have shown that WSCO is able to inhibit proliferation of human leukemia HL-60 cells and induce these cells to differentiate. Treatment with WSCO for 4 days resulted in a concentration-dependent reduction in HL-60 cell growth as measured by cell counting and MTT assay. This effect was accompanied by a marked increase in the proportion of G0/G1 cells as measured by flow cytometry. WSCO also induced differentiation of the cells as measured by phorbol ester-dependent reduction of NBT, morphological changes as examined by Wright-Giemsa staining and expression of CD11b but not of CD14 as analysed by flow cytometry, indicating differentiation of HL-60 cells toward granulocyte-like cells. A combination of low dose of WSCO with all-trans retinoic acid, a differentiating agent toward granulocyte-like cells, exhibited a synergistic effect on the differentiation. In addition, treatment of HL-60 cells with WSCO for 6 or 8 days resulted in the induction of apoptosis as assayed qualitatively by agarose gel electrophoresis and quantitatively by Annexin V technique using flow cytometry. Collectively, there is a potential for WSCO in the treatment of myeloid leukemia. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Water-soluble chitosan oligomer; Growth inhibition; Differentiation; HL-60; Leukemia; Apoptosis

1. Introduction

The conventional approach to treatment of acute myeloid leukemia (AML) has been the use of chemotherapy, which is cytotoxic to malignant clones, is also cytodestructive to normal cells. In addition, some leukemic cells develop resistance to chemotherapy and are therefore difficult to eradicate. Differentiation therapy, which is associated with less adverse effects, has been tested as a leukemia treatment modality. Differentiation therapy has been successful as a treatment for acute promyelocytic leukemia [1]. Several compounds including dimethyl sulfoxide, retinoic acid, phorbol ester and 1,25-dihydroxy vitamin D3 induce AML cells to differentiate toward mature cells. Among them, retinoic acid induces AML cells to differentiate toward granulocytes [2], whereas 1,25-dihydroxy vitamin D3 induces AML cells to differentiate toward monocytes [2].

Chitin (Fig. 1(a)), a polymer of N-acetylglucosamine, is cellulose-like biopolymer present in the exoskeleton of crustaceans and in cell walls of fungi, insects and yeast. Chitosan (Fig. 1(b)) is derived from chitin by deacetylation in the presence of alkali. Chitin and chitosan oligomers can be obtained by either chemical or enzymatic hydrolysis of chitin and chitosan [3]. They have lower viscosity, low molecular weights and short chains and are soluble in neutral aqueous solutions. Subsequently, they seem to be readily absorbed in vivo. Chitin and chitosan oligomers are known to have various biological activities including antitumor activities [4,5], immuno-enhancing effects [6], increased protective effects against infection with some pathogens [7], anti-
fungal activities [8], and antimicrobial activities [8]. To our knowledge, there is no report on the effect of water-soluble chitosan oligomer (WSCO) on leukemia cell proliferation and differentiation. Thus, the aim of the present investigation was to examine the differentiation-inducing effect of WSCO on the human myeloid leukemia HL-60 cells. To our knowledge, this is the first time it has been shown that WSCO is able to inhibit proliferation of HL-60 cells and induce these cells to differentiate.

2. Materials and methods

2.1. Cells and culture

Human myeloid leukemia HL-60 cells were cultured in suspension in RPMI-1640 medium (GIBCO) containing 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were split every 2–3 days to maintain in an exponential growth. Cell number was assessed by the standard procedure of leukocyte counting using a hemocytometer and cell viability was checked by the ability of cells to exclude trypan blue. The morphology of HL-60 cells was determined by Wright-Giemsa method [9].

2.2. Chemicals and treatment

Water-soluble chitosan oligomer (WSCO) which have greater than three but less than 10 saccharide residues was kindly provided by Intermedpharm Company. WSCO was dissolved in Hank’s balanced salt solution (Sigma, St. Louis, MO) to make the concentration of 1 g/ml and stored at 20°C. When used to treat the HL-60 cells, WSCO was diluted in a series of different concentrations with culture media. HL-60 cells were plated in 6-, 12-, or 96-well plate (Falcon) at 2 × 10^5 cells/ml with different concentrations of WSCO or all-trans retinoic acid (Sigma). Cells were kept in culture for 2–8 days before various assays were carried out.

2.3. MTT proliferation assay

Cellular proliferation was determined by the 3-(4,5-dimethylthiazol-2-diphenyl-tetrazolium bromide (MTT) (Sigma). Briefly, 10 µl of MTT (5 µg/ml) was added to each well of 96-well plate containing 2 × 10^5 cells after treated with different concentrations of WSCO for 4–8 days. The reaction was stopped after 4 h incubation by adding 100 µl of 0.04 N HCl in isopropanol and OD570nm was determined by a ELISA reader. Each concentration treatment was done in triplicate wells.

2.4. NBT differentiation assay

Differentiation was determined by NBT (Sigma) reduction with some modification as previously reported [10]. Briefly, cells were incubated in 96-well flat bottom plate (2 × 10^5 cells/well) for 4 days. Then cells were resuspended in 200 µl of RPMI-1640 media containing 2 mg/ml of NBT and 1 mg/ml of 12-O-tetradecanoylphorbol 13-acetate (Sigma). After incubation at 37°C for 30 min, the cells were pelleted and resolved in 200 µl of DMSO, and their absorbance at 570 nm was determined.

2.5. Cell surface makers

CD11b and CD14 antigens were detected by two-colour immunofluorescence, using commercially available reagents. In brief, 106 cells were incubated simultaneously with fluorescein isothiocyanate (FITC)-conjugated CD11b and phycocerythrin (PE)-conjugated CD14 monoclonal antibodies (Immunotech, France) in the phosphate buffered saline (PBS) with 1% FBS and 0.1% sodium azide for 30 min at 4°C. After washing, at least 10^4 cells were analysed by FACS Vantage flow cytometer and CellQuest software (Becton Dickinson).

2.6. Flow cytometry with FITC-Annexin V and propidium iodide (PI) double staining

Double staining with FITC-Annexin V and PI for flow cytometry analysis was performed. After washing twice with PBS, 10^6 cells treated and untreated were resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2). FITC-Annexin V (Boehringer Mannheim, Germany) was added resulting in a final concentration of 1 µg/ml Annexin V. Then 10 µg/ml PI (Sigma) was added. The mixture was incubated for 10 min in the dark at room temperature and then measured by FACS Vantage flow cytometer and CellQuest software (Becton Dickinson).

2.7. Analysis of DNA fragmentation by agarose gel electrophoresis

DNA isolation was performed as described previously [9]. Briefly, HL-60 cells (2 × 10^5 cells) treated with

![Fig. 1. Idealized structures of chitin (a) and chitosan (b).](image)
WSCO were harvested and washed with ice-cold PBS. Cells were suspended in PBS (10^6 cells/ml) containing 0.1 mg/ml proteinase K (Sigma), 0.1 mg/ml RNase A (Sigma), and 1% SDS and were incubated at 37°C for 30 min. After the addition of a NaI solution (6 M NaI, 13 mM EDTA, 0.5% sodium N-lauryl sarcosine, 10 μg glycogen, pH 8.0), cells were incubated at 60°C for 15 min and diluted with an equal volume of isopropanol. Cells were mixed vigorously and placed for 15 min at room temperature. The mixtures were centrifuged at 10 000 × g for 5 min, and the supernatants were discarded. Precipitated DNA was dissolved in sample solution for analysis by agarose gel electrophoresis. The electrophoresis was performed in 2% agarose gel at 100 V. DNAs in the gels was visualized by ethidium bromide. The presence of DNAs in the gels was visualized by ethidium bromide (Sigma).

3. Results

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, provides a unique in vitro model for studying the cellular and molecular events involved in the differentiation of normal and leukemic cells [11]. Moreover, recent approaches in the treatment of leukemia include the use of differentiating agents such as all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA) [12]. Thus, the strategy of HL-60 cell differentiation has been accepted as a valid model in detecting or screening for potent differentiating agents in preclinical evaluation. Therefore, proliferation and differentiation were here studied after treatment of HL-60 cells with WSCO or WSCO plus ATRA.

3.1. The effect of WSCO on proliferation of HL-60 cells

It was found that after 4-day treatment with 100 μg/ml of WSCO, the growth of HL-60 was inhibited by 6% (Table 1). The inhibition rate for HL-60 cells at dose of 1000 μg/ml of WSCO was 56%. The MTT assay also showed the inhibition of cell growth from the doses of 50–1000 μg/ml (Table 1). The percentage of viable cells was >98% under all conditions through 4 days (data not shown), indicating that the inhibition of cell growth by WSCO could not be attributed to the reduction in viable cells. The time course of the effect of WSCO (500 μg/ml) on HL-60 cell growth is shown in Fig. 2. WSCO-treated cells did not achieve a growth plateau. Instead, after 6 days the cell number steadily declined.

Table 1

<table>
<thead>
<tr>
<th>WSCO (μg/ml)</th>
<th>Cell count a</th>
<th>MTT assay b</th>
<th>NBT reduction c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86 ± 5</td>
<td>0.65 ± 0.02</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>50</td>
<td>84 ± 6</td>
<td>0.64 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>100</td>
<td>81 ± 3</td>
<td>0.62 ± 0.02</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>250</td>
<td>72 ± 5 d</td>
<td>0.55 ± 0.02 d</td>
<td>0.44 ± 0.04 d</td>
</tr>
<tr>
<td>500</td>
<td>46 ± 7 d</td>
<td>0.35 ± 0.04 d</td>
<td>0.75 ± 0.04 d</td>
</tr>
<tr>
<td>1000</td>
<td>38 ± 4 d</td>
<td>0.29 ± 0.03 d</td>
<td>0.82 ± 0.03 d</td>
</tr>
</tbody>
</table>

a Cells × 10^6/ml after culture for 4 days with WSCO. The beginning inoculation concentration was 1 × 10^5 cells/ml.
b OD_{570nm}/1 × 10^6 HL-60 cells after treatment with WSCO for 4 days.
c OD_{570nm}/1 × 10^6 HL-60 cells after treatment with WSCO for 4 days.
d P < 0.01 compared with the control. Data were presented as means ± SD from three separate experiments.

3.2. In vitro induction of differentiation of HL-60 cells by WSCO

To explore the ability of WSCO to induce differentiation of HL-60 cells, HL-60 cells were treated for 4 days with various doses of WSCO (50–1000 μg/ml). As indicated in Table 1, WSCO induced differentiation of HL-60 cells dose-dependently which was determined by NBT reduction. When morphological changes were observed by Wright-Giemsa staining, untreated HL-60 cells were predominantly promyelocytes containing a large nucleus and granules in cytoplasm (Fig. 3(a)). On the other hand, differentiated myeloid cells including myelocyte, metamyelocyte, banded and segmented neutrophils were observed in WSCO-treated HL-60 cells for 4 days (Fig. 3(c)). Similar morphological changes were found in ATRA-treated HL-60 cells (Fig. 3(b)).
3.4. Effect of WSCO on CD11b and CD14 expressions of HL-60 cells differentiated with WSCO

Flow cytometric diagrams of CD11b and CD14 expressions after treatment with ATRA or WSCO from the representative experiment are shown in Fig. 5. CD11b (FITC-labelled) expression was used as a maker of granulocytic and monocytic differentiation, while CD14 (PE-labelled) expression was used to monitor monocytic differentiation. In comparison with the untreated cells (Fig. 5(a)), the amount of CD11b positive cells were increased significantly after 4-day treatment with 500 g/ml of WSCO (Fig. 5(c)). However, the expression of monocytic CD14 antigen was not significantly increased. Similar results were found in ATRA-treated HL-60 cells (Fig. 5(b)). These results further supported that WSCO induced HL-60 cell differentiation toward granulocyte-like cells.

3.6. Induction of apoptosis of HL-60 cells differentiated with WSCO

It has been suggested that apoptosis is a major physiologic mechanism of cell death in terminally differentiated hematopoietic cells [14]. For example, apoptosis plays an important role in the elimination of activated granulocytes [15] and monocytes [16]. Furthermore, apoptosis occurs in HL-60 cells induced to differentiate with ATRA [17]. On days 6 and 8, but not on day 4, it was found that cells treated with 500 μg/ml WSCO underwent apoptosis as assayed qualitatively by agarose gel electrophoresis (Fig. 6(a)) and quantitatively by Annexin V technique (Fig. 6(b)), suggesting that HL-60 cells terminally differentiated with WSCO undergo apoptosis.
Fig. 5. Effects of ATRA or WSCO on CD11b and CD14 expression. Representative results of flow cytometric analysis of CD11b (FITC labelled) or CD14 (PE labelled) expression after 4-day treatment with 1 μM of ATRA or 500 μg/ml of WSCO. (a) Untreated HL-60 cells (control). (b) ATRA-treated HL-60 cells (positive control). (c) WSCO-treated HL-60 cells; CD11b expression (marked as UL) is significantly increased.

Among the activities of WSCO shown here are the inhibition of growth of HL-60 cells and the differentiation of HL-60 cells to granulocytic cells. In addition, the combination of WSCO and all-trans retinoic acid (ATRA) synergically increased the differentiation of HL-60 cells.

Several leukemic diseases are characterized by a break-down in myeloid cell maturation. To restore the normal differentiation, such patients are treated with differentiation therapy using differentiating agents like ATRA. However, remission can not be reached in all cases and the therapy has side effects, such as drug resistance and hypercalcemia. Therefore, it seems worthwhile to search for other substances which induce differentiation alone or in combination with established inducers of differentiation. In the present study, WSCO was found to inhibit the proliferation of a human promyelocytic leukemia cell line HL-60 and induce this cell line to differentiate toward granulocytes from the following evidence. (1) WSCO inhibited HL-60 cell proliferation and increased NBT reduction activity. (2) WSCO caused morphological changes toward granulocyte-like cells after culture for 4 days; indented nuclei and banded neutrophils were observed in WSCO-treated cells. (3) WSCO-treated cells were arrested mainly at G1/G0 phase. (4) WSCO induced the expression of cell surface antigen CD11b but not CD14. (5) Long-term culture of HL-60 cell with WSCO induced apoptosis. However, the mechanism for the induction of differentiation by WSCO is unknown, and it remains unclear whether WSCO effectively induces the elimination of malignant cells via the differentiation in vivo.

When chitosan is dissolved in solution, the amino groups in the N-deacetylated subunits are conferring a high positive charge density [18]. These amino groups have intrinsic pKa values of 6.5 and thus it behaves as a polycation at acidic and neutral pH [19]. Chitosan is known to be mucoadhesive, and ionically interacts with the negative charged molecules [20]. It has been shown to bind mammalian cells by interacting with surface glycoproteins [21]. Such properties of chitosan led us to hypothesize that a possible interaction of the positive charge of chitosan with cell surface would lead a change in the ionic environment of the cell membrane which is of importance in maintaining cell integrity and the numerous functions necessary for cell growth and differentiation [22].

Many natural products or synthetic compounds induce the differentiation of HL-60 cells. The potential activity of these agents can be evaluated by the concentration at which these agents induce maximum differentiation. The most well-known agents induce 80% differentiation in HL-60 cells after 5–7 days (23,24). Among them, the polar-planar drugs (acetamine, pipe-

Table 2
The effects of WSCO on ATRA-induced differentiation of HL-60 cells

<table>
<thead>
<tr>
<th>ATRA (μM)</th>
<th>WSCO (100 μg/ml)</th>
<th>NBT reductiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>0.05</td>
<td>−</td>
<td>0.36 ± 0.04b</td>
</tr>
<tr>
<td>0.10</td>
<td>−</td>
<td>0.48 ± 0.03b</td>
</tr>
<tr>
<td>0.50</td>
<td>−</td>
<td>0.72 ± 0.03b</td>
</tr>
<tr>
<td>1.00</td>
<td>−</td>
<td>0.88 ± 0.04b</td>
</tr>
<tr>
<td>0.05</td>
<td>+</td>
<td>0.60 ± 0.03c</td>
</tr>
<tr>
<td>0.10</td>
<td>+</td>
<td>0.78 ± 0.03c</td>
</tr>
<tr>
<td>0.50</td>
<td>+</td>
<td>0.86 ± 0.02c</td>
</tr>
<tr>
<td>1.00</td>
<td>+</td>
<td>0.89 ± 0.03c</td>
</tr>
</tbody>
</table>

a OD570nm/1 × 106 HL-60 cells after 4-day treatment with WSCO alone or WSCO plus ATRA.
b P < 0.01 compared with untreated cells.
c P < 0.05 compared with ATRA-treated cells. Data were presented as means ± SD from three separate experiments.
Fig. 6. Induction of apoptosis of HL-60 cells by WSCO. Cells (2 × 10^5 cells/ml) were grown for indicated days with 500 μg/ml of WSCO. (a) Agarose gel electrophoresis of DNA extracted from WSCO-treated HL-60 cells; on days 6 and 8, DNA ladders are shown. (b) FITC-Annexin V/PI flow cytometry of WSCO-treated HL-60 cells; apoptotic population with PI-negative and Annexin V-positive cells (marked as LR) is increased on days 6 and 8.
ridine and triethylene glycol) induce a higher proportion (up to 80%) of cells to differentiate at higher concentrations (0.1–0.01 mM) [24]. Several cancer chemotherapeutic agents have a potent effect on granulocytic differentiation at lower concentration (1 μM–1 nM). Actinomycin-D and methotrexate (1–10 nM) induce 80 and 30% differentiation in the cells, respectively [25]. ATRA can also trigger differentiation of HL-60 cells after 1 week exposure at 1 nM [26]. The maximal differentiation of HL-60 cells (up to 90%) is induced at 1 M of ATRA. Our present study has demonstrated that ~ 50% of HL-60 cells treated with 500 μg/ml of WSCO for 4 days were differentiated. With respect to therapeutic agents, Suh et al. [23] investigated 398 natural extracts to test their potential activity in inducing differentiation in HL-60 cells. According to their criteria, active compounds for differentiation were judged as having a response of > 30% in one or more differentiation makers. In the aspect of the antileukemic activity of WSCO, our present results have indicated that this compound could be judged as an active antileukemic agent via the induction of differentiation of HL-60 cells.

One of beneficial effects of chitosan and its oligomer is the reduction of plasma cholesterol and triglycerides due to their ability to binding dietary lipids, thereby reducing intestinal lipid absorption [27]. The hypolipidemic influence of chitosan may also be due to interruption of the enterohepatic bile acid circulation. Plasma cholesterol in animals on cholesterol-free diet, however, is not affected by chitosan or its oligomer, indicating that endogenous biosynthesis of cholesterol remains intact in the presence of chitosan or its oligomer [28]. Plasma cholesterol concentrations are related to the degree of maturation in acute myeloid leukemia [29]. The lowest cholesterol levels are observed in acute myeloid leukemia patients. Although there is no evidence that hypo-cholesterolemia may directly induce acute myeloid leukemia, certain medical precaution should be observed with long-term ingestion of high doses of chitosan to avoid potential adverse metabolic consequences.

Our results showing synergism in the induction of differentiation of HL-60 cells by combinations of WSCO and ATRA may have clinical utility. While cytodifferentiation therapy by retinoic acid (RA), such as ATRA or 9-cis RA, for acute promyelocytic leukemia patients is encouraging, there are several limitations preventing better clinical outcomes. A high percentage of patients in complete remission induced by RA alone relapse within a few months [17]. Most relapsed patients are resistant to further treatment with RA even though their leukemia cells may respond to RA in vitro [30]. One explanation for the resistance of patients relapsing to RA treatment is an increased systemic cytochrome P-450-mediated metabolism of RA.

The rapid catabolism of retinoic acid makes it very difficult to maintain effective plasma levels of RA [31]. Another explanation for RA resistance is at the cellular level. Leukemia cells from patients treated with RA have increased levels of cellular RA-binding protein [32]. High levels of this protein are associated with alterations in RA metabolism [33] and reduced cell differentiation [34]. Whatever the reasons for relapse, treatment with combinations of RA and WSCO may be one approach to decrease the plasma levels of RA needed for clinical effects. It is likely that decreasing the therapeutic concentrations of RA would also reduce the induction of cytochrom P-450-mediated catabolism of RA and of cellular RA-binding protein. Studies are presently underway to determine the toxicity of WSCO in animals and to measure the effectiveness of WSCO alone or in combination with RA on tumor growing in animals.

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References


