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Induction of apoptosis in human acute leukemia HL-60 cells by oligochitosan through extrinsic and intrinsic pathway

Jiangli Dou[a,b], Pan Ma[a,b], Chuannan Xiong[a], Chengyu Tan[c], Yuguang Du[a,*]

[a] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Graduate University of Chinese Academy Sciences, China
[c] College of Oceanic Environment Engineering, Dalian Ocean University, Dalian 116023, China

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ABSTRACT

The underlying cellular and molecular mechanism about oligochitosan induced HL-60 cell apoptosis remained unknown. In the present study, we found oligochitosan treated HL-60 cells showed enhanced cleavage of poly (ADP-Ribose) polymerase PARP and p21 expression, accompanied with G0/G1cell cycle arrest. After treatment with oligochitosan, the pro-apoptotic molecules Fas, FADD and Bax were up-regulated whereas anti-apoptotic Bcl-2 expression in HL-60 cells was significantly decreased. Furthermore, galectin-9 mRNA expression was increased in the presence of oligochitosan. We thus over-expressed galectin-9S or galectin-9L and found galectin-9 could significantly increase the oligochitosan induced apoptotic rate of HL-60 cells. Collectively, our data demonstrated that oligochitosan might regulate cell cycle progression and induce HL-60 cell apoptosis through both extrinsic and intrinsic pathways.

1. Introduction

Chitosan has been widely used in pharmaceutics, foods and oligochitosanmetics because of its renewable, nontoxic, biocompatible and biodegradable properties. Oligochitosan are obtained by chemical and enzymatic hydrolysis of chitosan. In addition, oligochitosan have been reported to have antimicrobial, antibacterial, antitumour and antiangiogenesis functions (Lee, Park, Choi, Yi, & Shin, 2003; Lee, Park, Jung, & Shin, 2002; Muzzarelli, 2010; Yan, Wanshun, Baoqin, Bing, & Chenwei, 2006; Yang, Chung, Kim, Choi, & Moon, 2007). Moreover, oligochitosan affect the mitogenic response and the chemotactic activities of animal cells (Kosaka, Kaneko, Nakada, Matsuura, & Tanaka, 1996). They have the ability of inducing human acute myeloid leukemia cells (HL-60) differentiation into granulocytic cells and the following apoptosis (Pae et al., 2001), however, the mechanism of apoptosis is unclear.

Recent studies have documented that two major pathways are involved in the occurrence of apoptosis (Fulda & Debatin, 2006). The extrinsic apoptotic pathway involves cell surface death receptors, such as Fas/CD95 and TNFR1, which up-regulate down stream signaling cascade leading to the activation of caspase-8. The intrinsic pathway is dependent on various cell stress stimuli leading to altering ratio of Bcl-2 family members that causes activation of caspase-9. The active forms of caspase-8 and caspase-9 activate downstream effectors such as caspase-3, -6, and -7 then lead to DNA fragmentation and cleavage of PARP (Cartee et al., 2002).

Galectins is one kind of the animal lectins possessing one or two carbohydrate recognition domains (CRD) of 14 kDa with a high affinity to β-galactosides of glycoconjugates. At least 13 members of galectins (galectin-1 through galectin-13) and 5 galectin-like proteins (GRIFIN, HSPC159, PP13, PPL13, and OvGal11) have been identified in mammals. Galectins modulate a variety of biological processes, such as cell activation, proliferation, adhesion, and apoptosis (Hernandez & Baum, 2002). Like other galectins, galectin-9 exhibits various biological functions, like promoting cell aggregation and chemoattraction of eosinophils, as well as inducing apoptosis of murine thymocytes and T cells (Chabot et al., 2002), and human melanoma cells (Irie et al., 2005).

In this article, we uncover the molecular mechanisms involved in the apoptosis induced by oligochitosan in human leukemia HL-60 cells. Our studies provide a deeper insight into the events leading to oligochitosan induced apoptosis in HL-60 cells. The apoptotic activity of oligochitosan is associated with the activation of extrinsic and intrinsic pathways, the cell cycle arrest and participation of galectin-9.

2. Materials and methods

2.1. Chemicals and reagents

Oligochitosan (the degree of deacetylation was above 95%) were produced in our lab (Zhang, Du, Yu, Mitsutomi, & Aiba,

* Corresponding author. Tel.: +86 411 84379061; fax: +86 411 84379060.
E-mail address: articles1805@gmail.com (Y. Du).

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1999). The weight percentages of oligochitosan with degree of polymerization 2–6 in the oligomixture were 3.7%, 16.1%, 28.8%, 37.2% and 14.2%, respectively. Dimethylsulfoxide (DMSO) was purchased from Sigma. Trizol and RT-PCR kit was purchased from TAKARA. Bio-Rad protein assay kit was acquired from TIANGEN. Anti-human β-tublin rabbit polyclonal antibody was from Santcruiz, anti-human Bcl-2 rabbit polyclonal antibody, anti-human phosphor-Bcl-2 (ser70)rabbit polyclonal antibody, anti-human Bax rabbit polyclonal antibody, anti-human p21 mouse polyclonal antibody, anti-human FADD rabbit polyclonal antibody, anti-human PARP rabbit polyclonal antibody HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG were acquired from Cell Signal.

2.2. Cell culture

HL-60 cells were cultured in RPMI-1640 medium supplemented 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere and used for assays during exponential phase of growth. HL-60 cells were treated with 1.25% DMSO as positive control for the apoptosis and cell cycle assay.

2.3. Flow cytometric analysis of apoptosis and cell cycle

The assessment of cell apoptosis by flow cytometry was performed according to a previously described technique (Cendoroglo et al., 1999). The rates of apoptosis and DNA content were analyzed by Annexin-V- FITC labeling and PI staining respectively. For cell cycle analysis, cells were treated as the past report (Henry, Lynch, Eapen, & Quelle, 2001). Cell-cycle histograms were generated after analysis of PI-stained cells.

2.4. Reverse transcription (RT)-PCR

Total RNA (1 μg) extracted from intact and chitosan oligosaccharides-treated HL-60 cells were used as template for cDNA synthesis. Primers used for PCR are as follows (5′ to 3′):

p21 forward AGGAGGCCCGTGAGCGATGGAAC,
reverse ACAAGTGGGGAGGAGGAAGTAGC;

bcl-2 forward AGATGTCCAGCCAGCTGCACCTGAC,
reverse AGATAGGCACCCAGGGTGATGCAAGCT;

bax forward AAGCTGAGCGAGTGTCTCAAGCGC,
reverse TCCCGCCACAAAGATGGTCACG;

fas forward ATAAGCCCTGTCCTCCAGGT,
reverse TGATGCCAATTACGAAGCAG;

galectin-9 forward GAGATGGCCTTCAGCAGTTCC,
reverse CGCCTATGTCTGCACATGGGT,
length galectin-9(with Sal I and Xho I restriction sites):
forward TACTCGAGATGGCCTTCAGCGGTTCCC
reverse GAGTCGACTTTGTCTGCACATGGGTCAG
β-actin forward CTGTCTGGCGGCACCACCAT′
reverse GCAACTAAGTCATAGTCCGC.

The PCR cycles were as follows: one cycle at 94 °C for 3 min as an initial denaturation step, then denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 1 min (the number of cycles was 30–35), followed by further incubation for 5 min at 72 °C (one cycle). The annealing step was carried at 55 °C (bax, galectin-9), 57 °C (β-actin), 59 °C (bcl-2, fas, p21). After amplication, an aliquot of the PCR reaction product was electrophoresised onto a 1.5% agarose gel.

2.5. SDS–PAGE and Western blot analysis

About 10^7 cells were pelleted by centrifugation, washed with ice-cold PBS twice, and lysed with protein lysis buffer (1 × SDS, 2.5% β-mercaptoethanol, 1 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin). After measuring with a Bio-Rad protein assay kit, proteins were separated through 12% SDS-polyacrylamide gels, transferred to PVDF membranes, and subjected to immunological detection. The PVDF membranes were incubated for 2 h with respective specific antibodies after blocking for 2 h in 5% defatted milk in PBST buffer. After washing with PBST for three times, the PVDF membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h at room temperature. The PVDF membranes were then developed by using the enhanced chemiluinescent detection systems.

2.6. Plasmid construction

The full length of galectin-9 was amplified using human thymus cDNA as completes, the cDNA of galectin-9 without the stop code was cloned into pEGFP-N1 vector via Sal I and Xho I restriction sites to generate GFP tagged galectin-9S and -9L.

2.7. Transfection

The pEGFP-galectin-9S and -9L were transfected by electroporation into HL-60 cells. Harvest the cells in the exponential growth phase and centrifuge them, set the cell concentration to 2.5×10^6 cells/ml. Add and mix plasmid DNA (25 μg/ml final concentration, in bi-distilled H2O). Transfer 800 μl cell suspensions into electroporation cuvettes (4 mm gap width). Electroporation parameters: 270 V, 975 μF, 12–13 ms. After the pulse, allow the cell suspension to stand in the cuvette for 5–10 min at room temperature then carefully transfer the cell suspension to 4 ml RPMI 1640/10% FCS, and cultivate it in a 35 mm culture dish. The expression of the GFP- galectin-9s and -9L was detected after 24 h by flow cytometric analysis and fluorescence microscope.

3. Results and discussion

3.1. Indcuction of apoptosis in oligochitosan-treated HL-60 cells

The resluts revealed that oligochitosan induced the DNA into the sub-G0 phase which represents the apoptosis of cells. After incubation with oligochitosan for 3, 5, 7 days, the rates of apoptosis increased through a dose and time dependently manner, shown in Fig. 1A. Meanwhile, the cell apoptosis was further determined by Annexin V-FITC labeling at 7 days, the results demonstrated that most cells dead by the apoptosis way not the necrosis one, shown in Fig. 1B. In addition, the cleavage of PARP was up-regulated dominantly, especially by the treatment of 400 μg/ml oligochitosan (Fig. 1C).

3.2. Cell cycle arrest in HL-60 induced by oligochitosan

As shown in Fig. 2A, the cell cycle in HL-60 cells was arrested in G0/G1 phase by treatment with oligochitosan for 5 days. The percentage of HL-60 cells in S phase was decreased from 47.28% to 25.51% and 22.33% after being treated with 400 μg/ml and 800 μg/ml oligochitosan. However, the change of cell cycle was not prominent at 3 and 7 days. The gene expression of p21 was clearly up-regulated after the oligochitosan treatment, shown in Fig. 2B. Meanwhile, the protein expression of p21 was also increased at 5 and 7 days (Fig. 2C). The improvement of p21 expression was in agreement with the cell cycle arrest.

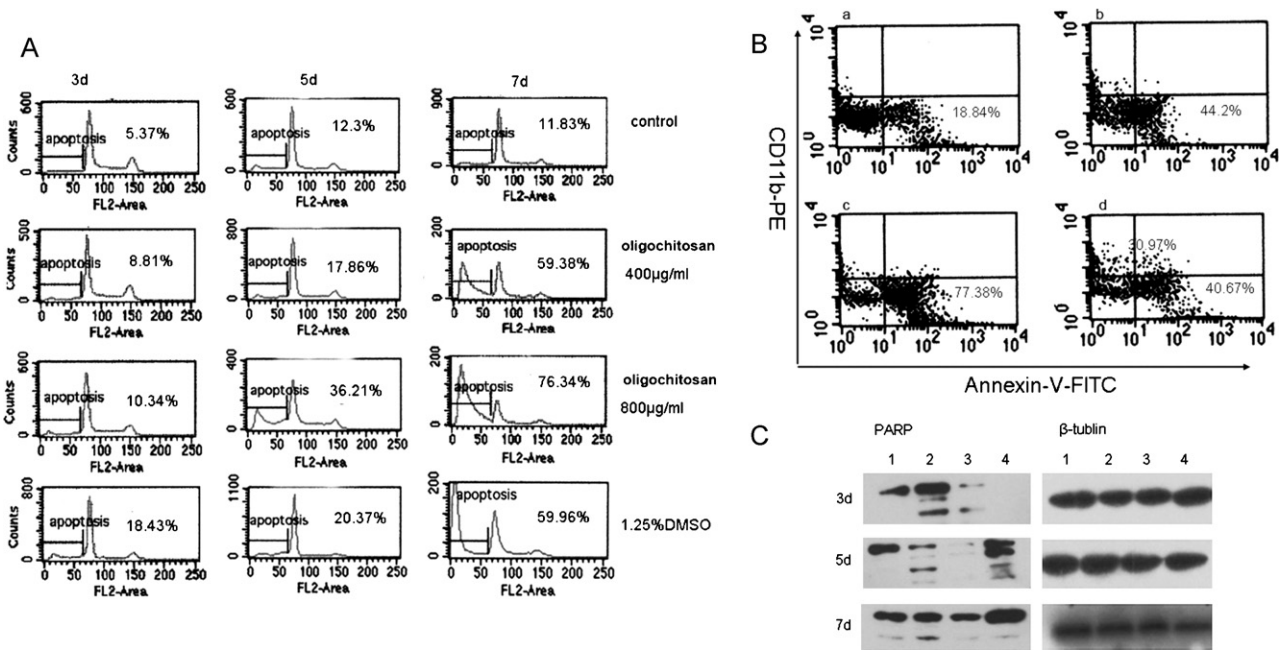

Fig. 1. The apoptosis of HL-60 cells induced by oligochitosan. (A) Flow cytometric analysis of oligochitosan induced apoptosis in HL-60 by PI-staining. (B) Flow cytometric analysis of apoptosis and differentiation of HL-60 by Annexin V-FITC/CD11b-PE. a: Control; b: oligochitosan 400 μg/ml; c: oligochitosan 800 μg/ml; d: 1.25%DMSO. (C) Cleavage of PARP detected by Western blotting. Lane 1: control; lane 2: oligochitosan 400 μg/ml; lane 3: oligochitosan 800 μg/ml; lane 4: 1.25%DMSO.

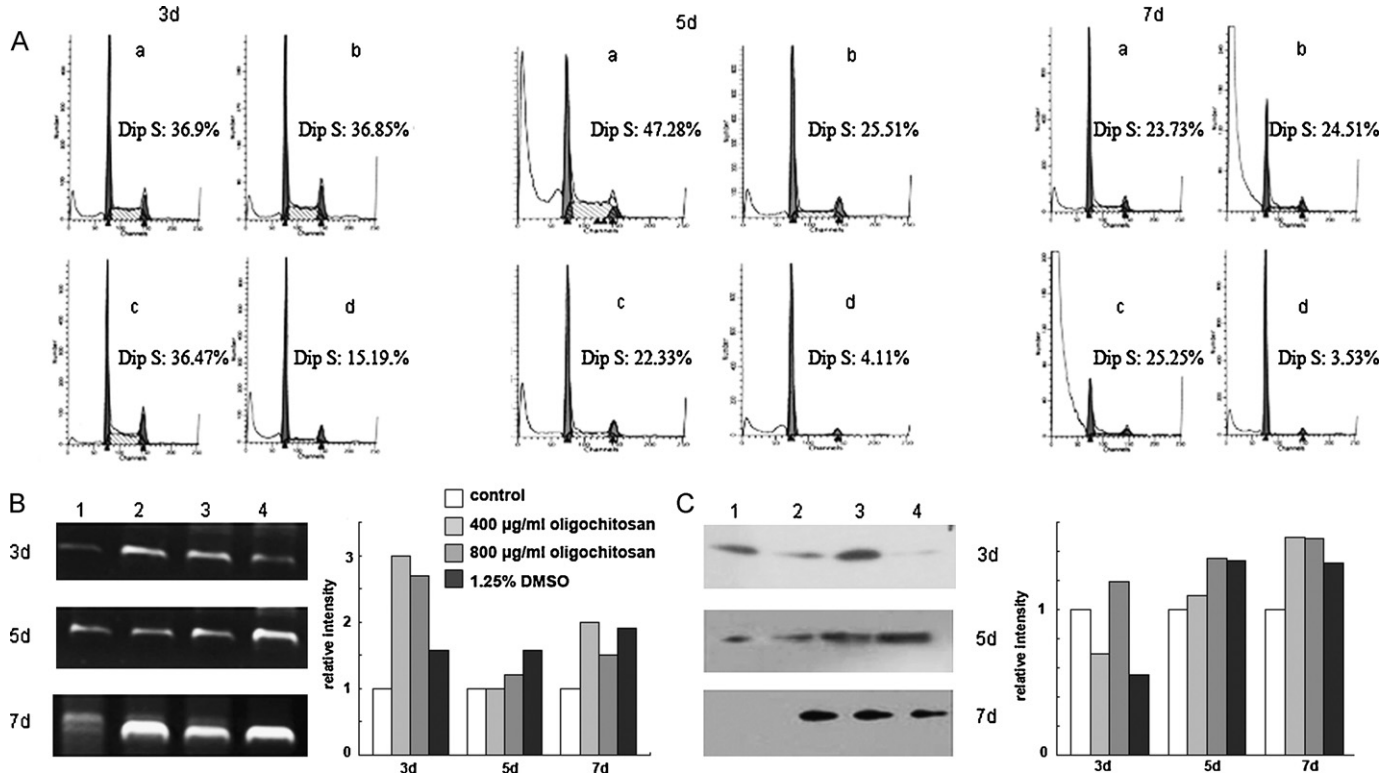

Fig. 2. Cell cycle arrest induced by oligochitosan. (A) Flow cytometric analysis of cell cycle by PI-staining. a: Control; b: oligochitosan 400 μg/ml; c: oligochitosan 800 μg/ml; d: 1.25%DMSO. (B) Gene expression of p21 by RT-PCR. Lane 1: control; lane 2: oligochitosan 400 μg/ml; lane 3: oligochitosan 800 μg/ml; lane 4: 1.25%DMSO. (C) Protein expression of p21 by Western blotting. Lane 1: control; lane 2: oligochitosan 400 μg/ml; lane 3: oligochitosan 800 μg/ml; lane 4: 1.25%DMSO. The right figures were the statistic analysis of the p21 expression.

It has been suggested that cell cycle is regulated by p21, p27 and other cyclin-dependent kinases (CDK) (Green, Freiberg, & Giaccia, 2001), p21 is a CDK inhibitor, which is best known for arresting the cell cycle at G0/G1 phase, besides its effects on apoptosis and differentiation (Halevy et al., 1995). The results demonstrated that oligochitosan supplementation increased the expression of p21 at gene and protein level clearly, so we supposed that oligochitosan regulated the cell cycle through the over expression of p21. It has been demonstrated that p21 is a transcriptional target of the tumour suppressor gene p53 (Kachnic et al., 1999), but the HL-60

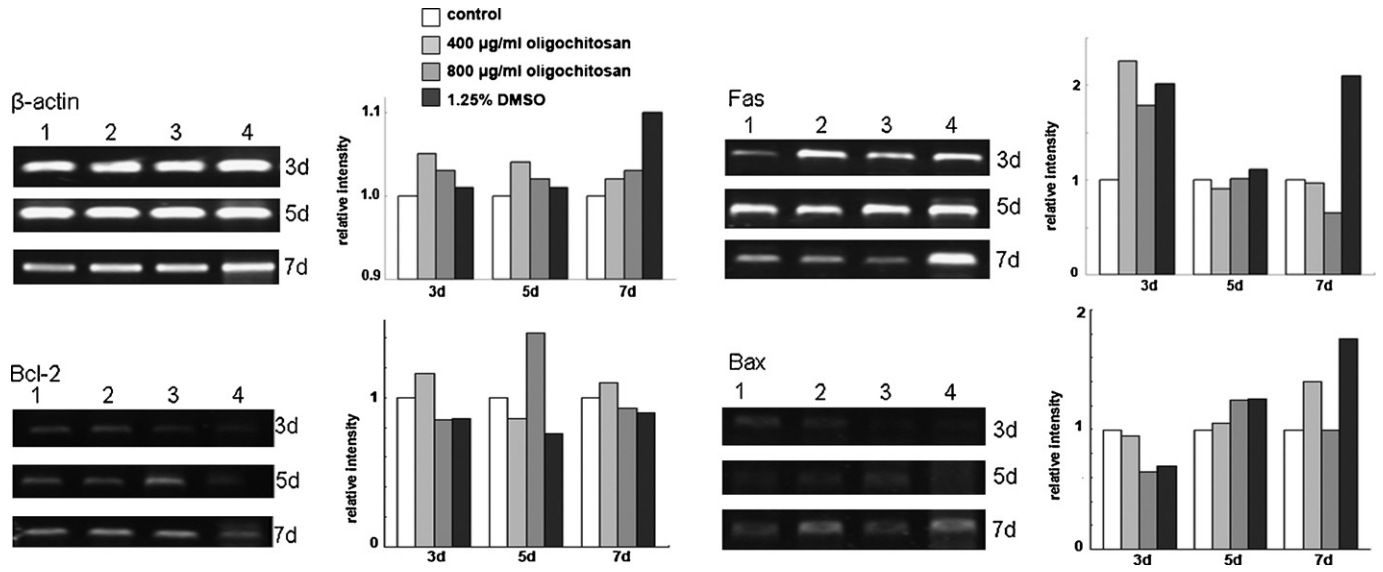

Fig. 3. Gene expression of Fas, Bax and Bcl-2 detected by RT-PCR after incubation with oligochitosan for 3, 5, 7 days. Lane 1: control; lane 2: oligochitosan 400 μg/ml; lane 3: oligochitosan 800 μg/ml; lane 4: 1.25%DMSO. The right figures were the statistic analysis of the genes expression.

cells are p53-negative, a further study to address this issue should be carried out to identify the primary target molecules of oligochitosan in regulating p21 function.

3.3. Oligochitosan-induced changes on gene expression of some molecules involved in extrinsic and intrinsic pathways

The gene expression of Bcl-2, Bax and Fas were detected by RT-PCR. After treated with oligochitosan, the gene expression of Fas increased clearly on 3 days, but the change of expression on 5, 7 days was not obvious. On the opposite, the gene expression of Bax had no clear difference between the control blank and oligochitosan treatment on 3 days, but it was up-regulated dramatically on 5, 7 days. However, the gene expression of Bcl-2 remained constant, as shown in Fig. 3.

3.4. Oligochitosan-induced changes on protein expression of Bcl2, phospho-Bcl-2, Bax and FADD

Though the gene expression of Bcl-2 had no obvious change, the protein expression was clearly down-regulated after treatment with oligochitosan for 5 and 7 days. Meanwhile, the phospho-Bcl-2 (ser70) increased obviously. In agreement with gene expression, the protein expression of Bax was improved evidently after treatment with oligochitosan for 5 and 7 days. The protein expression of FADD increased a little after incubation with oligochitosan for 3 and 5 days, on the 7 days, oligochitosan treatment promoted the expression of FADD prominently, as shown in Fig. 4.

Apoptosis proceeds via two distinct biochemical cascades (Barton, Davies, Balkwill, & Burke, 2005). The intrinsic or mitochondrial pathway is triggered by ionizing radiation or cytotoxic drugs and is initiated by cytochrome *c* (cyt *c*) release from the

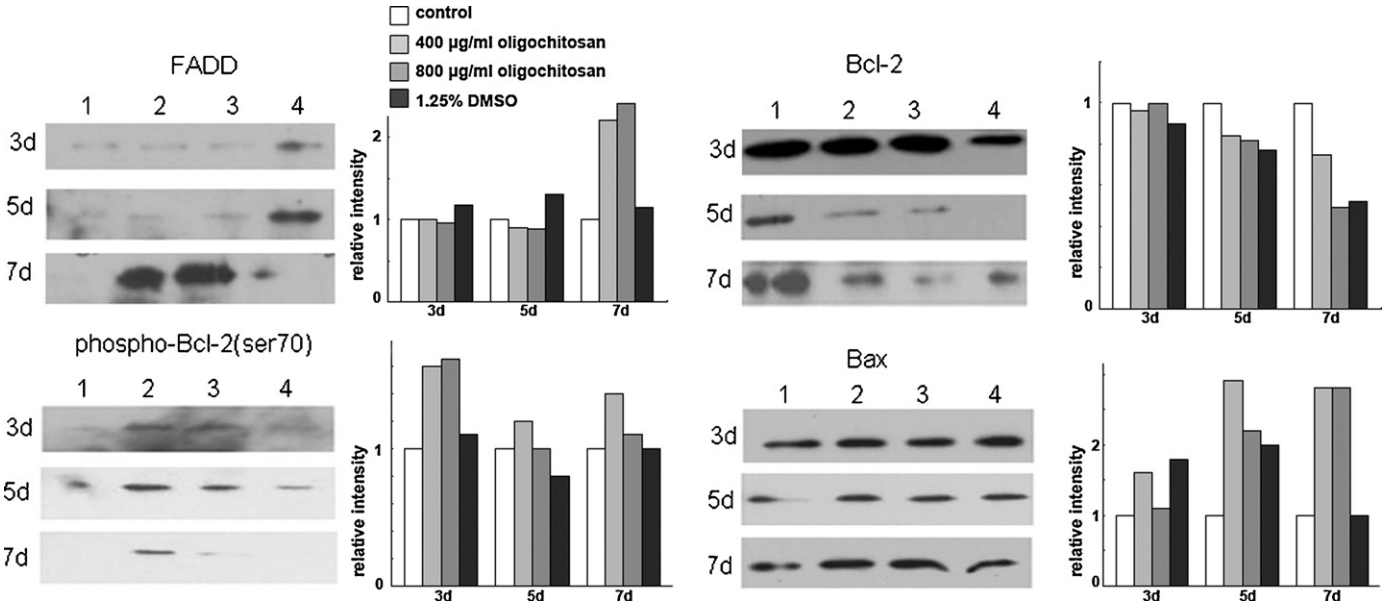

Fig. 4. Protein expression of FADD, Bax, Bcl-2 and phospho-Bcl-2 (ser70) detected by Western blotting after incubation with oligochitosan for 3, 5, 7 days. Lane 1: control; lane 2: oligochitosan 400 μg/ml; lane 3: oligochitosan 800 μg/ml; lane 4: 1.25%DMSO. The right figures were the statistic analysis of the proteins expression.

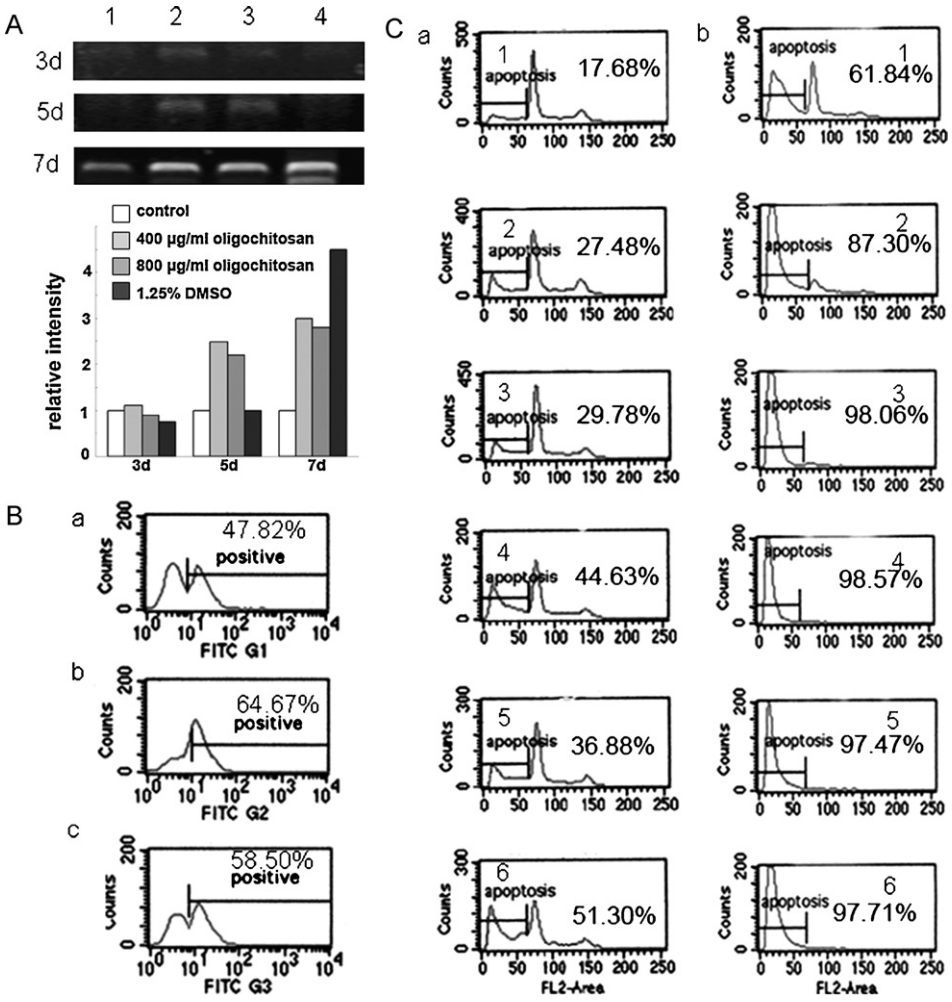

Fig. 5. Determination of apoptosis after transfection with galectin-9. (A) Gene expression of galectin-9 induced by oligochitosan and 1.25% DMSO. Lane 1: control; lane 2: oligochitosan 400 μg/ml; lane 3: oligochitosan 800 μg/ml; lane 4: 1.25%DMSO. The down figure was the statistic analysis of the galectin-9 expression. (B) The efficiency of electroporation transfection was determined by the detection of GFP using FACS. a: pEGFP-N1 transfection; b: pEGFP-N1-galectin-9s transfection; c: pEGFP-N1-galectin-9l transfection. (C) Apoptosis of HL-60 after transfection was determined by flow cytometric analysis. 1: pEGFP-N1 transfection only; 2: pEGFP-N1 transfection + oligochitosan 400 μg/ml; 3: pEGFP-N1-galectin-9 s transfection only; 4: pEGFP-N1-galectin-9 s transfection + oligochitosan 400 μg/ml; 5: pEGFP-N1-galectin-9l transfection only; 6: pEGFP-N1-galectin-9l transfection + oligochitosan 400 μg/ml.

mitochondria. In contrast, the extrinsic apoptotic pathway is receptor-mediated and includes the recruitment of procaspase-8 to the death inducing signaling complex (DISC) of cell surface death receptors. The DISC contains the Fas-associated death domain (FADD), an adapter protein with a death domain effector sequence that binds to a homologous sequence within procaspase-8.

To determine which pathway is involved in the oligochitosan-induced cell apoptosis, we detected the expression of Fas and FADD, which presents the extrinsic pathway, Bcl-2 and Bax, which presents the intrinsic pathway by RT-PCR and Western blotting. The results demonstrated that both the extrinsic and intrinsic pathway participated in the apoptosis of HL-60 induced by oligochitosan. After incubated with oligochitosan for 3 days, the gene expression of Fas increased clearly, while the changes of Bcl-2 and Bax were not obvious. However, on the 5 and 7 days, the expression of Bax increased prominently. Meanwhile, the protein expression of FADD, Bax and phospho-bcl-2(ser70) improved clearly, and the Bcl-2 decreased dramatically. Accompanied with the changes of these proteins, the rates of apoptosis increased in a dose and time dependent manner induced by oligochitosan. Further more, the cleavage of PARP was up-regulated clearly after oligochitosan supplementation.

3.5. Induction of apoptosis by transfection of pEGFP-N1-galectin-9S and -9L

Oligochitosan up-regulated the gene expression of galetin-9 obviously on 3, 5 and 7 days, shown in Fig. 5A. After electroporation transfection, the efficiency of galectin-9 expression was determined by the detection of GFP using FACS, shown in Fig. 5B. The transfected cells were allowed to recover for 24 h before incubated with oligochitosan for 3 and 5 days. Then the apoptosis was determined by the PI-staining using flow cytometry. After being incubated with oligochitosan for 3 days, the apoptosis of transfected cells was clearly higher than the cells treated with oligochitosan only. Meanwhile, the high expression of galectin-9 resulted in higher rate of apoptosis than transfected with p-EGFP-N1 only. Almost all of the transfected cells were apoptotic on 5 days (Fig. 5C).

It has been proved that galectin-9 plays the pro-apoptotic role in some kinds of cancer cells (Hirashima et al., 2004), so we detected whether galectin-9 play cross-promotion role with oligochitosan in the apoptosis of HL-60 cells. The results revealed that the over-expression of galectin-9 improved the apoptosis of HL-60 induced by oligochitosan, meanwhile, oligochitosan also strengthened the pro-apoptotic ability of galectin-9. Taken together, these findings

demonstrated that oligochitosan and galectin-9 played a synergistic role in apoptosis of HL-60.

4. Conclusion

According to the results above, we conclude that both the extrinsic and intrinsic pathways participate in the apoptosis of HL-60 induced by oligochitosan. Further more, oligochitosan arrest cell cycle at G0/G1 phase through up-regulating the expression of p21. Galectin-9 is involved in the apoptosis and plays a synergistic effect with oligochitosan. These findings provide the basis for in-depth drug targeted studies, while the pro-apoptotic ability of oligochitosan raises the potential use as an anti-leukemia agent.

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