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High molecular weight water-soluble chitosan protects against apoptosis induced by serum starvation in human astrocytes

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Abstract

The effect of high molecular weight water-soluble chitosan (WSC) on serum starvation-induced apoptosis in human astrocytes (CCF-STTG1 Cells) was investigated. WSC, having an average molecular weight of 300 kDa and a degree of deacetylation over 90%, can be produced using a simple multi-step membrane separation process. Serum starvation led to growth arrest, rounding up of cells and appearance of p53 bands. Prolonged (48 h) incubation in serum starved medium led to cell detachment and death. WSC significantly protected the serum starvation-induced cellular rounding up and protected the serum starvation-induced cell death as tested by flow cytometry. WSC also protected serum starvation-induced p53 activation as determined by Western blot. These results suggest that WSC may prevent serum starvation-induced apoptosis of CCF-STTG1 cells via p53 inactivation.

Keywords: High molecular weight water-soluble chitosan; Human astrocytes; Serum starvation; Apoptosis; p53

1. Introduction

Apoptosis, or programmed cell death, is an important physiologic process in the normal development and homeostasis of multicellular organisms [1,2]. Derangements of apoptosis can have deleterious consequences as exemplified by several human disease states, including acquired immunodeficiency syndrome, neurodegenerative disorders, and cancer [3,4]. Apoptosis, which was originally described for its role in normal homeostatic cell turnover in healthy adult tissues [5], is a form of cell death associated with nuclear and cytosolic condensation and the formation of apoptotic bodies [6]. Apoptosis has been reported to be induced by stimuli such as oxidants, various xenobiotics, heat shock, glucocorticoid therapy, irradiation, and growth factor withdrawal [7,8]. Indeed, many growth factors play dual roles as mitogens and survival factors [9,10]. In quiescent cultures, growth factors maintain cell viability even at very low concentrations [11]. After complete withdrawal of growth factors, some cell lines exhibit features that are characteristic of physiologic cell death in vivo. Neuronal death that occurs in neurodegenerative diseases may be due to apoptosis [4]. Glial cells comprising astrocytes and microglia constitute more than 90% of the total cell population in the adult brain, and support neurons by providing trophic factors and the regulation of immunological processes [12]. Astrocytes have a wide range of functions critical for maintaining a balanced homeostatic environment in the central nervous system.

The tumor suppressor p53 is a phosphoprotein involved in the positive and negative regulation of the cell cycle. The p53 gene is located on chromosome 17 and the protein product is a transcriptional activator that can cause cell-cycle delay to allow for DNA repair after cellular damage, or it can promote cell death via apoptosis [13,14]. In many cells, following exposure to DNA damaging agents, wild-type p53 levels increase 5–60-fold as a result of stabilization of the p53 protein. p53-deficient cells or cells express-

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Abbreviations used: WSC, high molecular weight water-soluble chitosan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide; PI, propidium iodine; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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ing mutant p53 fail to undergo apoptosis in response to DNA damage [15,16].

Chitosan, a non-acetylated or partially deacetylated chitin (a linear homopolymer of β-(1-4)-linked *N*-acetylglucosamine) has been proposed as biomaterial because of its apparent satisfactory biocompatibility. In mammals, chitosan has been reported to stimulate non-specific resistance against *Escherichia coli* infection and suppression of the growth of Meth A tumor in syngeneic Balb/c mice [17] and stimulation of nitric oxide production in RAW 264.7 macrophages [18]. An easily water-soluble chitosan has a higher reactivity than water-insoluble chitosan [19].

In the present study, we investigated whether WSC inhibits serum starvation-induced apoptosis in human astrocyte CCF-STTG1 cells. We showed that WSC significantly inhibited the serum starvation-induced apoptosis through inactivation of p53 in CCF-STTG1 cells.

2. Materials and methods

2.1. Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), sodium EDTA, RNase and propidium iodine (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human anti-p53 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Epitope is within residues 213–217 of human p53 and residues 206–211 of mouse p53. Reacts with human, mouse and rat p53. Reacts selectively with mutant p53 in flow cytometry, immunofluoresence and immunoprecipitation. Reacts with both mutant and wild-type p53 in Western blotting and paraffin sections. RPMI 1640, calcium chloride and fetal bovine serum (FBS) were purchased from GIBCO (Burlington, Ontario, Canada). Tissue culture plates of 96 wells and 35-mm diameter dishes were purchased from Nunc (Naperville, IL, USA). High molecular weight water-soluble chitosan was obtained from JA KWANG Co. (Ansung, Korea).

2.2. Cell cultures

A human astrocyte cell line, CCF-STTG1 cells were obtained from the Cancer Bank, Daejon, Korea. Cells were seeded in 35-mm culture dishes or microplates (1×10^6 cells/ml) and grown in RPMI 1640 supplemented with 10% heat inactivated FBS, 1% nonessential amino acids, 1% glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. The cells were maintained in a humidified atmosphere of 95% O_2–5% CO_2 at 37°C. Serum starvation was carried out by changing the culture medium to fresh RPMI 1640 supplemented with only 0.1% FBS.

2.3. Preparation of WSC

The WSC was dissolved in distilled water and then filtered through 0.2 μm filter membrane. The WSC stock solution (1 mg/ml) was stored at −20°C. Immediately before use, the WSC was diluted in distilled water.

2.4. Morphological assessment

Cells were cultured in serum-starved medium alone or in serum-starved medium containing WSC during 48 h and then photographed by microscope (×40) (Olympus, Japan).

2.5. MTT assay

The number of living cells in 96-well plates was determined by the MTT assay [20]. MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/ml. From this stock solution, 10 μl per 100 μl of medium was added to each well, and plates were incubated at 37°C for 2 h. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. The dark blue crystals that are the metabolized product of MTT were extracted by DMSO. Absorbence at 540 nm was determined and used for the measurement of the proportion of surviving cells.

2.6. Flow cytometry

Proportions of cell cycle phase were analyzed by flow cytometry. In brief, the cells were fixed with 70% ethanol at 4°C for 60 min. After being washed with PBS, the cells were treated with 0.5 ml of RNase and then with 1 ml of PI (100 μg/ml in PBS) solution in dark at 4°C for 30 min. After being washed and passed through nylon mesh, the samples were kept on ice until measured. The DNA histogram was obtained with a flow cytometry cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). Ten thousand events from the gated subpopulation were recorded separately.

2.7. DNA extraction and electrophoresis

The characteristic ladder pattern of DNA break was analyzed by agarose gel electrophoresis. Briefly, DNA from the CCF-STTG1 cells (1×10^6 cells/each group) was isolated by Wizard Genomic DNA purification kit (Promega Co, Wisconsin Medicine, WI, USA) and serial ethanol precipitation. Isolated genomic DNA (10 μg) was subjected on 1.5% agarose electrophoresis at 100 V for 1 h. DNA was visualized by staining with ethidium bromide under UV light.

2.8. Western blot analysis

Whole cell lysates were made by boiling cells in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 20% glycerol, 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature

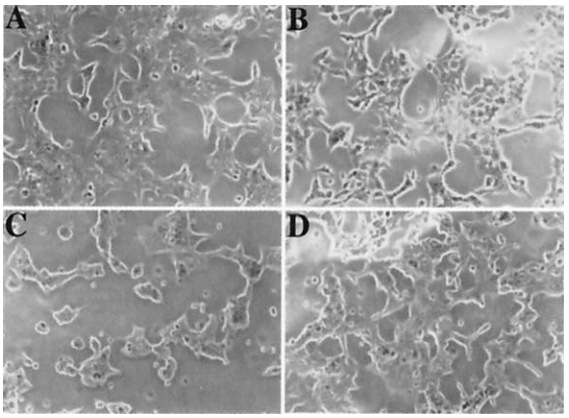

Fig. 1. Visualization of morphological change in CCF-STTG1 cells. The cells (1×10^6 cells/ml) were incubated with normal medium alone (A), normal medium plus WSC (10 μg/ml) (B), serum starved-medium alone (C) or serum starved-medium plus WSC (10 μg/ml) (D) for 48 h and photographed by microscope (×40). Each data represents one of three independent experiments.

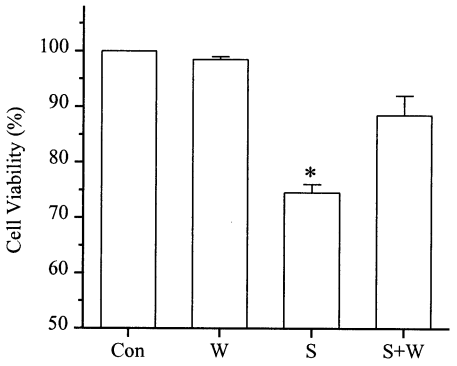

Fig. 2. Effect of WSC on the cell viability in CCF-STTG1 cells. The cells (1×10^6 cells/ml) were incubated with normal medium alone (Con), normal medium plus WSC (10 μg/ml) (W), serum starved-medium alone (S) or serum starved-medium plus WSC (10 μg/ml) (S + W) for 48 h. The cell viability (Con, 100%; W, 98.5 ± 0.5%; S, 74.5 ± 1.5%; S + W, 88.5 ± 3.5%) was measured in three independent experiments in triplicate using MTT assay. $*P < 0.05$ versus medium alone control.

and incubated with anti-human p53 antibody. After being washed in PBS-Tween-20 three times, the blot was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp, Piscataway, NJ, USA).

2.9. Statistical analysis

Collected data were expressed as mean ± S.E. Statistical analysis was performed by the Student's *t*-test to express the difference from control group.

3. Results

3.1. Effect of WSC on serum starvation-induced rounding up of cells

Serum starvation causes cellular rounding up and the formation of apoptotic bodies. This morphological change started to appear about 10 h after initiation of serum starvation. In this study, we investigated the ability of WSC on serum starvation-induced cellular rounding up in CCF-STTG1 cells. The cells were cultured either in serum-starved (with only 0.1% FBS) medium alone or in serum-starved medium that contained WSC (10 μg/ml) during 48 h and then morphological change was assessed. As shown in Fig. 1, WSC inhibited serum starvation-induced cellular rounding up. The visualized morphological change by microscope was conspicuous.

3.2. Effect of WSC on the cell viability

Prolonged incubation (≥48 h) in serum starved medium led to cell detachment and death. The effect of WSC on serum starvation-induced cell death in CCF-STTG1 cells, as assessed by the MTT test, is shown in Fig. 2. When the cells were incubated in serum starved-medium for 48 h, cell viability decreased by 74.5 ± 1.5% compared with controls (100%). When the cells treated with WSC (10 μg/ml) were exposed to serum starved-medium, cell death was significantly inhibited compared with the effect of serum starved-medium alone.

3.3. Effect of WSC on serum starvation-induced apoptosis

In order to induce the serum starvation-induced apoptosis, CCF-STTG1 cells were incubated for 48 h in medium supplemented with only 0.1% FBS. Serum starvation-induced apoptosis of CCF-STTG1 cells was determined by flow cytometry. As shown in Fig. 3, when cells were incubated in serum starved-medium, apoptosis increased by 34% compared with controls. When the cells treated with WSC (10 μg/ml) were exposed to serum starved-medium, apoptosis of CCF-STTG1 cells was almost completely inhibited compared with the effect of serum starved-medium alone. In addition, DNA fragmentation in a ladder pattern indicates internucleosomal chromatin cleavage, which is a characteristic of apoptosis. As was expected, a ladder of fragmented DNA was detected in serum starvation cells (Fig. 4). Pretreatment of CCF-STTG1 cells with WSC (10 μg/ml) inhibited the serum starvation-induced DNA fragmentation. This data coincides with flow cytometry data.

3.4. Effect of WSC on serum starvation-induced p53 activation

Serum starvation led to appearance of p53, and apoptosis that follows DNA damage is dependent upon, or enhanced by, the induced expression of p53. Whether WSC inhibits serum starvation-induced activation of p53 was assessed by

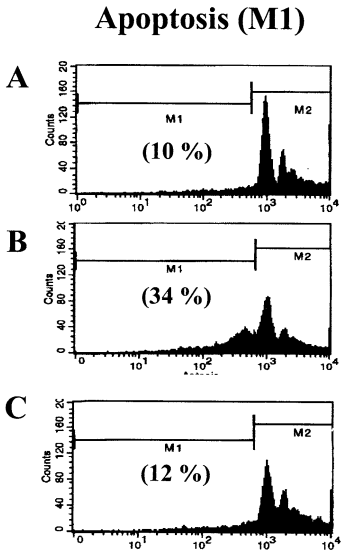

Fig. 3. Effect of WSC on the cell cycle distribution of CCF-STTG1 cells. The cells (1×10^6 cells/ml) were incubated with normal medium alone (A), serum starved-medium alone (B) or serum starved-medium plus WSC (10 μg/ml) (C) for 48 h. The cells were stained with PI solution and analyzed for DNA content by flow cytometry (see Materials and methods). Each data represents one of three independent experiments.

Western blot using whole cell extracts. Fig. 5 shows that serum starvation-induced activation of p53 was blocked by co-treatment of WSC (10 μg/ml).

4. Discussion

In the present work we demonstrate that WSC were able to protect against apoptosis in human astrocytoma cells induced by serum starvation. Chitosan can be produced by biomass and considered as a polyamine $(-NH_2)_n$ but it has a limitation in applications due to its insolubility in most solvents including water. Other commercial chitosans, having a degree of deacetylation below 70%, are a mixture of various low molecular weight of chitosan and can be partially soluble in organic acid. Therefore, additional purification process is needed. WSC, having an average molecular weight of 300 kDa and a degree of deacetylation over 90%, can be produced using a simple multi-step membrane separation process [21]. The initial chitosan has an ionic form ($-NH_3^+$) in the structure [22]. The free ions in the ionic form of the initial chitosan solution were removed by ion exchange methods before coating with collagen. At present, the precise physiological significance of inhibitory effect on apoptosis by WSC is unclear. However, during the last many years, prevention of apoptosis has received decreasing various brain diseases. Neuronal death caused by neurotrophic factor deprivation or various brain insults was shown to be mediated via apoptosis [23]. This has led to the suggestion that the neuronal death that occurs in neurodegenerative diseases such as Alzheimer's disease (AD) may be due to apoptosis. AD is characterized by gradual degeneration of neurons in various brain regions [12]. One potential therapeutic approach for the treatment of AD patients is to inhibit progression of the disease by inhibiting the process of neuronal death. Apoptosis in astrocytes and microglia has been implicated in the pathogenesis of central nervous system (CNS) disease processes. High grade astrocytomas are the most common primary CNS malignancy in adults.

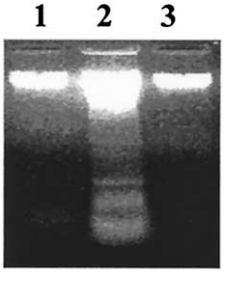

Fig. 4. Effect of WSC on the DNA fragmentation of CCF-STTG1 cells. The cells (1×10^6 cells/ml) were incubated with normal medium alone (Lane 1), serum starved-medium alone (Lane 2) or serum starved-medium plus WSC (10 μg/ml) (Lane 3) for 48 h. Twenty μg of DNA were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination.

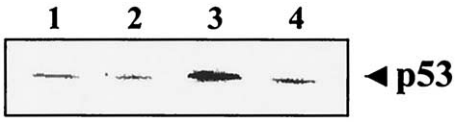

Fig. 5. Western blot analysis. Fifty μg of total protein were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by Western blotting using an anti-p53 polyclonal antibodies. Lane 1, normal medium alone; lane 2, normal medium plus WSC (10 μg/ml); lane 3, serum starved-medium alone and lane 4, serum starved-medium plus WSC (10 μg/ml). Arrows represent appearance of p53. Datum represents one of three independent experiments.

Apoptosis is an active process resulted in characteristic morphological changes to the cell including condensed regions of nuclear material, internucleosomal DNA cleavage, and membrane blebbing. The serum-deprived cells in this study demonstrated several characteristics of apoptotic cell.

WSC inhibited the serum starvation-induced cellular rounding up and loss of contact with neighboring cells. Serum starvation also induces the apoptosis or the programmed cell death through the activation of p53 [15,16]. p53 activation is known to play an important role in the G1 checkpoint after ionizing radiation where it facilitates DNA repair or promotes cell death via apoptosis [24]. Our data suggest that p53 is involved in apoptosis induced by serum starvation in CCF-STTG1 cells. Hence we showed that WSC can prevent serum starvation-induced apoptosis via blocking of p53 activation. Previous studies have shown that chitosan induce expression of TNF-α, a pro-apoptotic factor in monocytes [25] and induce frank apoptosis in p53-null HL-60 human tumor cells [26]. In our studies, however, WSC itself had no effect on apoptosis of CCF-

STTG1 cells (data not shown). The WSC we have used in this experiment is different from the chitosan used by Pae et al., but it needs more study for elucidating these points.

Although the present study did not directly demonstrate general cell death of astrocytoma, CCF-STTG1 cells because only morphological changes have been observed, our finding that WSC inhibits the serum starvation-induced apoptosis in the cells may suggest that WSC can promote neuroprotective effects in brain. Further studies are needed to clarify the WSC effects in vivo.

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