

Chitosan Induces Apoptosis via Caspase-3 Activation in Bladder Tumor Cells

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Recently, because of its low toxicity and biological effects, chitosan has been widely used in the medical and pharmaceutical fields, e.g., for nasal or oral delivery of peptide or polar drug delivery. Here, we report a growth-inhibitory effect of chitosan on tumor cells. The growth inhibition was examined by WST-1 colorimetric assay and cell counting. We also observed DNA fragmentation, which is characteristic of apoptosis, and elevated caspase-3-like activity in chitosan-treated cancer cells. The findings suggest that chitosan may have potential value in cancer therapy.

Key words: Chitosan — Apoptosis — Caspase — Bladder tumor

Chitosan, a non-toxic natural polymer,^{1,2} has been used as an absorption enhancer for poorly absorbable drugs.^{3–6} It has a hypocholesterolemic effect,^{1,7,8} an immunomodulating function,⁹ and a hypoglycemic effect,¹⁰ and it reduces body weight¹¹ and improves serum urea, creatinine and cholesterol levels in patients with chronic renal failure.¹² It also shows a growth-inhibitory effect on tumor cells,^{13,14} inhibition of tumor-induced angiogenesis¹⁵ and an inhibitory effect on tumor metastasis.¹⁶ However, the cellular mechanisms of these biological activities remain unknown.

Dietary fiber decreases the risk of cancers of the colon and rectum.¹⁷ The anti-tumor effect of dietary fiber is due to inhibition of cell proliferation and induction of apoptosis.^{17,18} Anticancer agents, which are well known apoptosis inducers, pass through the cell membrane and interact with cellular targets, then show cytotoxicity.^{19,20} However, there are apoptotic agents, polypeptides or polysaccharides for example, which do not permeate through the plasma membrane but only interact with the cell membrane.^{21–23} Chitosan, one of the dietary fibers which is not hydrolyzed by human digestive enzymes, may be one of these.

In the present study, we examined the growth-inhibitory effect of chitosan on human bladder tumor cells. Using DNA fragmentation analysis by agarose gel electrophoresis, the DNA nick end labeling method²⁴ and detection of fragmented DNA in individual cells (comet assay²⁵), it was found that chitosan induced apoptosis in cultured cancer cells. This effect of chitosan was not mediated by immune cells because our culture system included no immunological system. We also found that chitosan activates caspase-3, a common mediator of the apoptotic pathway that plays a dominant role in the death signaling initiated by various antitumor agents.^{19,26,27} Therefore, chitosan may interact with the cell membrane to trigger apoptosis.

This is the first study to show that chitosan directly inhibits cell proliferation by inducing apoptosis, and we suggest that chitosan may be applicable for cancer therapy.

MATERIALS AND METHODS

Chemicals Chitosan was from Mind-Ace Co. (Miyazaki). Adriamycin was purchased from Kyowa Hakko (Tokyo). The WST-1 cell counting kit was obtained from Wako Chemicals (Osaka). Hoechst 33342 dye was purchased from ICN (Aurora, OH). Low gelling temperature agarose (type VII) was from Sigma Chemical Co. (St. Louis, MO). The *In situ* Apoptosis Detection Kit was from TaKaRa Biochemicals (Kyoto). The DAB (diaminobenzidine) substrate kit was purchased from Funakoshi (Tokyo). The “EnzChek” Caspase-3 assay kit was from Molecular Probes (Eugene, OR). Caspase-3 inhibitor (Z-DEVD-CMK) was obtained from Bachem AG (Bubendorf, Switzerland).

Cell line and cell culture Human bladder tumor cells 5637 were obtained from the American Type Culture Collection. Cells were grown at 37°C in an atmosphere of 5% CO₂/95% air. The 5637 cells were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

Growth inhibition assay The effect of chitosan on cell growth was assayed by the WST-1 method. Briefly, 3×10³ cells which had been seeded in a 96-well microculture plate were incubated for 24 h under the above conditions. The original growth medium was replaced with a drug-containing one, and cells were incubated for another 3 days, after which, 10 μl of WST-1 solution was added and incubation was continued for 2 h. The number of surviving cells was measured with a microplate reader (Bio Rad, Hercules, CA) at a reference wavelength of 600 nm and test wavelength of 450 nm. Cell viability was determined as % of the control.

Morphological analysis of chitosan-treated cells Cellular morphological change was assessed by staining nuclei

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of cells with Hoechst 33342 as described by Hasegawa *et al.*²⁸⁾ Cells seeded at 4.0×10^5 cells/5 ml in complete culture medium were incubated under maintenance conditions for 24 h, after which they were treated with 100 $\mu\text{g}/\text{ml}$ of chitosan or 100 ng/ml of adriamycin. After 3 days of incubation, attached cells were collected and resuspended in phosphate-buffered saline (PBS). Collected cells were stained with 10 μM Hoechst 33342 for 10 min at room temperature, then analyzed with a fluorescence microscope (AX-70, Olympus, Tokyo), with excitation at 360 nm. Viable cells revealed blue, round nuclei and apoptotic cells showed fragmented or condensed nuclei.

DNA fragmentation analysis Cells seeded at 10^6 cells/10 ml in complete culture medium were incubated under maintenance conditions for 24 h, after which the medium was replaced with fresh medium containing various concentrations of chitosan or adriamycin. Incubation with the drug continued for 72 h, after which cells were scraped off and assayed. DNA fragmentation was assayed by a conventional method²⁹⁾ with some modifications. Briefly, cells were lysed in 1 ml of cell lysis buffer containing 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.1% SDS (sodium dodecylsulfate) and 0.1 mg/ml proteinase K. After 1 h of incubation at 55°C, 10 μl of 10 mg/ml RNase was added, and incubation continued for 1 h at 37°C. Then, DNA was precipitated overnight at -20°C with ethanol. After centrifugation, the pellet was resuspended in 25 μl of 10 mM Tris-Cl pH 8.0/1 mM EDTA. DNA was separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

DNA nick end labeling (TUNEL) method Cells were grown on poly-L-lysine-coated slide glasses and treated with drugs. After 3 days of incubation, cells were fixed in 0.2% picric acid/4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 2 or 3 days at room temperature, then washed with 10% acetic acid 3 times to remove precipitated chitosan and left for 1 day at room temperature. Endogenous peroxidase was inactivated by covering the cells with 0.3% H_2O_2 for 15 min at room temperature. The cells were rinsed with 0.01 M PB/0.9% NaCl and immersed in the Labeling Safe Buffer containing the TdT Enzyme of the *In situ* Apoptosis Detection Kit, and then incubated in a humid atmosphere at 37°C for 60 min. They were then treated with anti-FITC (fluorescein isothiocyanate) HRP (horseradish peroxidase) conjugate for 30 min at 37°C and stained with the DAB substrate kit.

Alkaline comet assay The alkaline comet assay was performed as described by Shibuya *et al.*²⁵⁾ Briefly, 10^5 cells were mixed with 1.5 ml of 1% low temperature gelling agarose in PBS containing 20 μm microspheres (Coulter Co., Miami, FL) at a density of $10^4/\text{ml}$ at 40°C. Fifty microliters of the agarose mixed with each sample was immediately layered on a superfrosted glass microscope slide (Matsunami Glass Ind. Ltd., Kishiwada) and covered

with gelbond film (Pharmacia Biotech AB, Uppsala, Sweden). The slide was kept on ice for 1 min to allow the agarose to gel, then the gelbond film with the attached layer of agarose gel was removed. Thereafter, the gelbond film was placed on 20 μl of 1% agarose with the same microspheres mentioned above, kept on ice for 1 min, then removed and lysed in a solution containing 0.03 N NaOH, 1 M NaCl and 0.5% Sarcosyl, pH 12.5 for 50 min. The agarose layer containing each sample was washed 3 times with 0.03 N NaOH and 1 mM EDTA. The gelbond films attached to the agarose layer were placed in a horizontal gel electrophoresis apparatus. Fresh 0.03 N NaOH/2 mM EDTA was added to the chamber as the gelbond films soaked up the buffer, then electrophoresis was conducted at 0.5 V/cm for 25 min. After electrophoresis, the samples were rinsed in PBS and stained for 30 min with 2.5 $\mu\text{g}/\text{ml}$ of propidium iodide in PBS in the dark. The gelbond films were rinsed in PBS and covered with glass on microscope slides. The slides were viewed with a fluorescence microscope (AX-70, Olympus), under green light using a 530 nm reflector.

Preparation of cell extracts and assay of enzyme Caspase-3 activities in cell lysates were measured using the "EnzChek" Caspase-3 assay kit. Treated cells were collected, washed twice with ice-cold PBS, suspended in Cell Lysis buffer, which is a component of the kit, then subjected to a freeze-thaw cycle. According to the instruction manual, the lysates were centrifuged at 5000 rpm for 5 min in a microcentrifuge. The transferred supernatants were subjected to the assay of caspase-3 activity. The fluorescence was measured using a Fluoromark plate reader (Bio Rad) with excitation at 355 nm and emission at 460 nm. The protein concentration was determined with a Bio Rad protein assay dye reagent.

Membrane lipid peroxidation Lipid peroxidation in cells was measured by the thiobarbiturate (TBA) method.³⁰⁾ Briefly, treated cells were collected and resuspended in 0.7 ml of saline. Then 0.5 ml of the cell suspension was transferred into a brown tube, 4 ml of 1/12 N H_2SO_4 was added and mixed well, and then 0.5 ml of 10% phosphotungstic acid was added. After mixing well, centrifuging and discarding the supernatant, the pellet was used for the lipid peroxidation assay. The peroxidized lipid yielded malondialdehyde (MDA) upon heating with TBA and the former gave a red pigment which was detected fluorometrically. The amount of MDA was normalized to the amount of total protein of each cell group.

RESULTS

Growth-inhibitory effect of chitosan In the WST-1 assay, cell viability was reduced dose-dependently to 50–60% of the control by 72 h treatment with chitosan, up to the concentration of 10 $\mu\text{g}/\text{ml}$ (Fig. 1A). At chitosan con-

centrations above 10 $\mu\text{g/ml}$, the viable cell number was still reduced dose-dependently in the trypan blue dye exclusion test (Fig. 1B). As chitosan is barely soluble in water, precipitated chitosan polymer may interfere with the WST-1 colorimetric assay. Therefore, we directly counted the cell numbers when $>50 \mu\text{g/ml}$ of chitosan was used.

Induction of apoptosis by chitosan It was suggested that chitosan shows a growth-inhibitory effect on tumors by activating the immune system and accelerating lymphokine production.³¹⁾ The present system included no immune cells, so the growth-inhibitory effect of chitosan here should be due to direct cytotoxicity. However, after 3 days of incubation with chitosan at a concentration of 100 $\mu\text{g/ml}$, most of the cells were still attached to the dish and only a few floating cells were observed. Therefore, chito-

san appears not to be highly toxic. To determine what kind of cell death chitosan induces, we studied the morphological change of 5637 cells, using Hoechst 33342 dye. Fig. 2 shows the nuclear fragmentation and chromatin condensation in chitosan-treated cells (Fig. 2B). These changes are characteristic of apoptosis. The same pattern was observed in adriamycin-treated cells (Fig. 2C).

To confirm the apoptosis induction by chitosan treatment, we studied the patterns of DNA fragmentation in 5637 cells, following 3 days exposure to chitosan (Fig. 3). The "DNA-ladder" began to be seen after 48 h incubation (data not shown). As shown in Fig. 3, DNA-ladder formation was not observed in chitosan dimer-treated cells. However, chitosan polymer induced DNA fragmentation as potently as adriamycin, a well known apoptosis inducer.

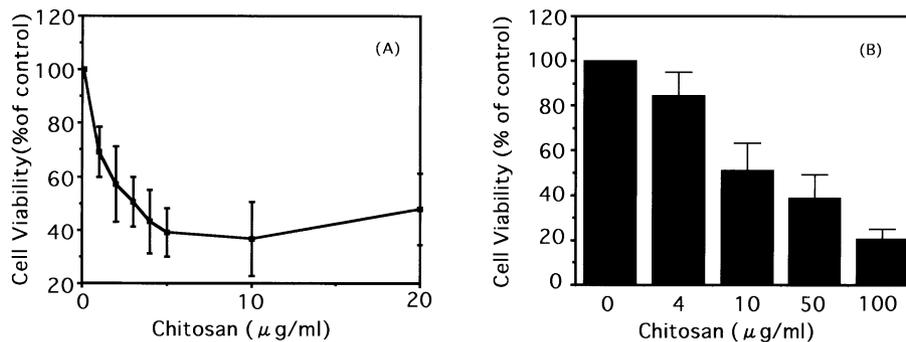


Fig. 1. Growth-inhibitory effect of chitosan on human bladder 5637 cells. A. WST-1 assay. Chitosan affected the viability of 5637 cells in a dose-dependent manner. Cells were treated with the indicated concentrations of chitosan and cell viability was assayed using a WST-1 cell counting kit as described in "Materials and Methods." Values represent the results of six independent experiments performed in triplicate and the values are expressed as the mean viable cells ($\pm\text{SD}$) as a percentage of controls. B. Cell count. Chitosan concentrations above 10 $\mu\text{g/ml}$ may interfere with the WST-1 colorimetric assay. Therefore, living cells were counted by use of the trypan blue dye exclusion test. Values are from 4 independent experiments performed in triplicate and represent the mean viable cells ($\pm\text{SD}$) as a percentage of controls.

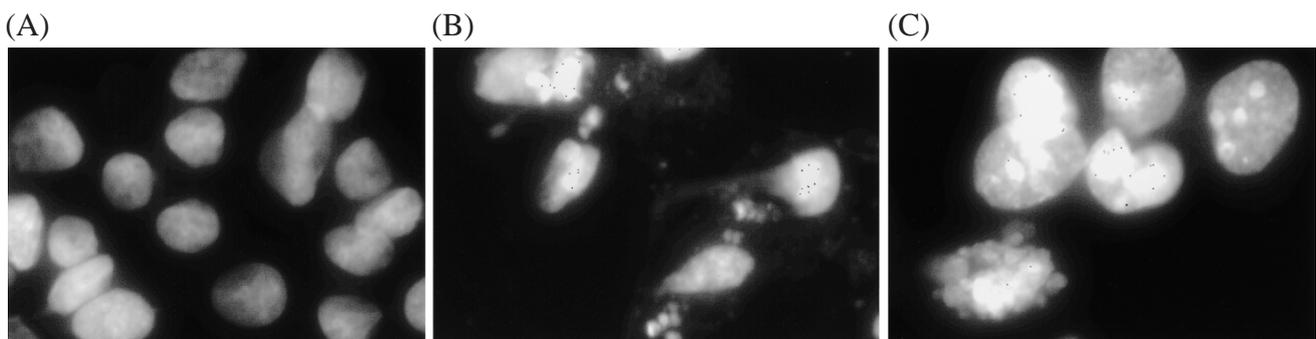


Fig. 2. Morphological analysis of 5637 cells treated with chitosan or adriamycin. Cells were treated with 100 $\mu\text{g/ml}$ of chitosan (B) or 100 ng/ml of adriamycin (C) for 3 days. Panel A shows the cells without treatment. After 3 days of incubation, cells were collected and stained with Hoechst 33342 as described in "Materials and Methods." Photographs were taken under 400 \times magnification, using an Olympus AX-70 fluorescence microscope.

To determine the frequency of apoptosis in attached 5637 cells treated with chitosan, we analyzed apoptotic cells *in situ* using the TUNEL method. As shown in Fig.

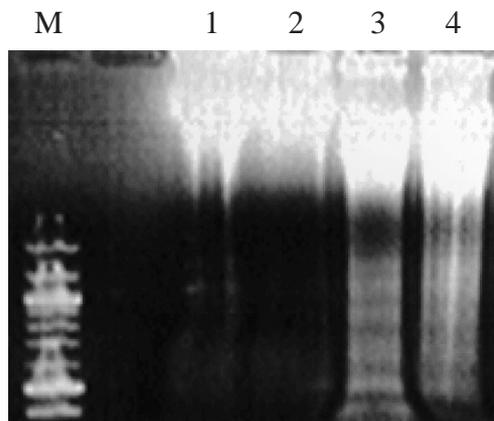


Fig. 3. DNA ladder formation. DNA fragmentation assays of the treated cells were performed as described in “Materials and Methods” (lane M, DNA size marker; lane 1, DNA from control cells; lane 2, DNA from chitosan dimer-treated cells; lane 3, DNA from 100 µg/ml chitosan-treated cells; lane 4, DNA from 50 ng/ml adriamycin-treated cells).

4C, the number of chitosan-treated cells was significantly lower than that of the control and more than half of the attached cells treated with chitosan were stained, while few cells without treatment were stained, suggesting that chitosan induced apoptosis in the attached cells. This apoptotic change was not observed in chitosan dimer-treated cells (Fig. 4B). DNA breakage was also detected using the alkali comet assay (single-cell electrophoresis assay). As shown in Fig. 5, DNA damage in individual cells treated with chitosan polymer showed the comet tail, which was imaged using fluorescence microscopy. The “comet tail” indicated that cellular DNA was fragmented because apoptotic change had occurred. The percentage of the tailing situations was calculated from the average of eight visual fields (Table I).

Caspase-3 activities To assess the events evoked by chitosan treatment, we examined the activities of caspase-3 in cultured 5637 cells. In cultures treated with chitosan, the number of cells was increased after 3 days of culture, but the total number of treated cells was less than that of control cells. After 3 days of incubation, the caspase-3 activity of chitosan-treated cells was increased 2-fold compared with control cells (Fig. 6). As shown in Fig. 6, chitosan dimer did not change the caspase-3 activity. Each fluorescence was normalized with respect to the soluble protein

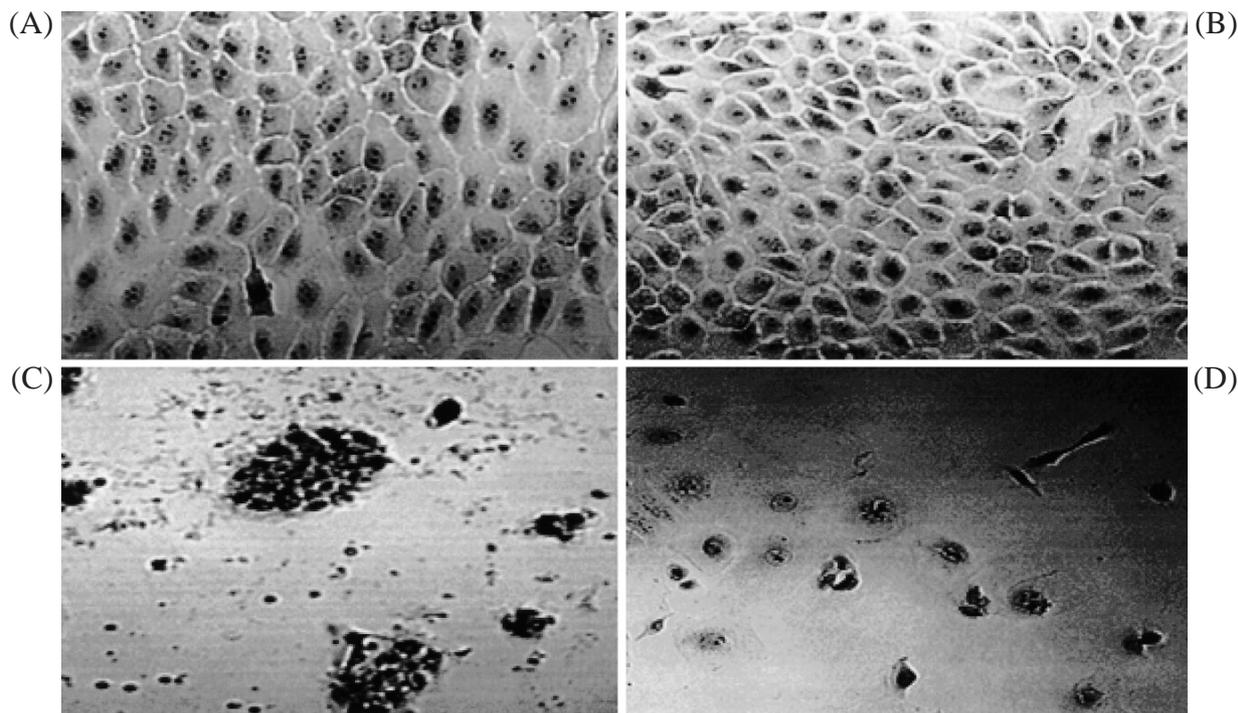


Fig. 4. Apoptotic cells show a positive brown TUNEL stain. Cells were treated with either vehicle (A), 100 µg/ml of chitosan dimer (B), 100 µg/ml of chitosan polymer (C) or 100 ng/ml of adriamycin (D) for 3 days. Photographs were taken under 100× magnification, using an Olympus BX50WI light microscope.

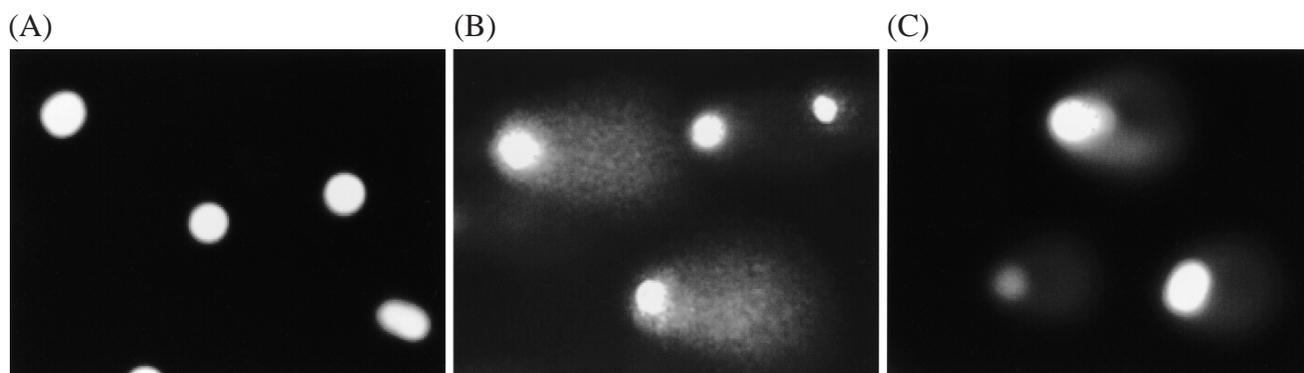


Fig. 5. Microscopic image of cells that had undergone alkaline comet assay. Panel A, control cells; panel B, 100 $\mu\text{g}/\text{ml}$ chitosan-treated cells; panel C, 100 ng/ml adriamycin-treated cells. Photographs were taken under 400 \times magnification, using an Olympus AX-70 fluorescence microscope.

Table I. Percentage of the Individual Cells That Showed Comet Tail Calculated from 8 Individual Visual Fields

Treatment	% of positive cells
Control	7
Chitosan (100 $\mu\text{g}/\text{ml}$)	49
Adriamycin (100 ng/ml)	73

Values are the averages from 2 independent experiments.

amount. Caspase-3 activity ratio was calculated as the caspase-3 activity of treated cells divided by that of the untreated cells.

Effect of caspase-3 inhibitor on chitosan-treated cells Caspase-3 inhibitor Z-DEVD-CMK alone did not affect the cell viability at the concentration of 20 μM , but the same concentration of Z-DEVD-CMK attenuated the growth-inhibitory effect of chitosan (Fig. 7, A and B). This suggested that caspase-3 was involved in the cell death (apoptosis induction) in the chitosan-treated cells.

Membrane lipid peroxidation Lipid peroxidation products may play an important role in neuronal death implicated in excitotoxicity and apoptosis.³²⁾ Several transcription factors are activated in response to oxidative stress via caspase activation. Therefore, we examined the lipid peroxidation products in chitosan-treated cells. Fig. 8 shows that lipid peroxidation products were not increased in the chitosan-treated cells compared with the cells without treatment. Membrane lipid peroxidation promotes membrane depolarization and calcium influx through *N*-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels,³³⁾ which finally leads to caspase activation. The present findings suggested that caspase-3 activation after chitosan treatment does not involve the generation of lipid peroxidation products.

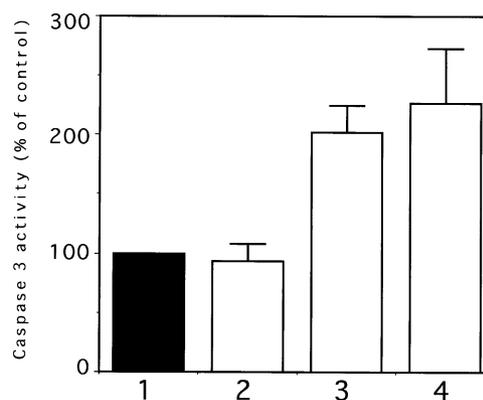


Fig. 6. Cells were treated and caspase-3 activity was assayed as described in "Materials and Methods" (column 1, control; column 2, 100 $\mu\text{g}/\text{ml}$ of chitosan dimer; column 3, 100 $\mu\text{g}/\text{ml}$ of chitosan polymer; column 4, 100 ng/ml of adriamycin). Chitosan polymer-treated cells show almost 2-fold higher caspase-3 activity than control cells. However, chitosan dimer did not affect the caspase-3 activity. One hundred ng/ml of adriamycin, a well-known apoptosis-inducing agent, elevated the caspase-3 activity similarly to the chitosan polymer. Values represent 6 independent experiments performed in duplicate and values are the means \pm SD.

DISCUSSION

Although chitosan is widely accepted as a non-toxic and biodegradable polymer, the present study showed that chitosan inhibited tumor cell growth dose-dependently by inducing apoptotic change in a human bladder tumor cell line. A previous study showed that chitosan, as a cationic polymer, interacted with the cell membrane, causing red blood cell lysis and melanoma cell growth inhibition.¹⁴⁾ In those experiments, however, the nature of the intracellular

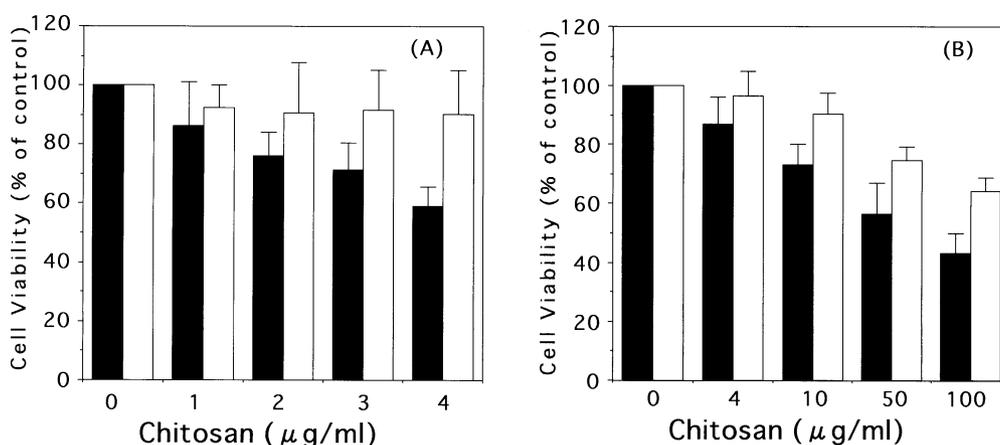


Fig. 7. Effect of caspase-3 inhibitor on tumor cell growth. A caspase-3 inhibitor, Z-DEVD-CMK, blocked the effect of chitosan on the viability of 5637 cells. Cells were treated for 3 days with vehicle (dimethylsulfoxide, DMSO), or 20 μM Z-DEVD-CMK in the presence of the indicated concentrations of chitosan. Cell viability was determined using a WST-1 cell counting kit (A) or the trypan blue dye exclusion test (B). Values shown are the means (±SD) of 3 independent assays performed in triplicate (■, without inhibitor; □, with inhibitor).

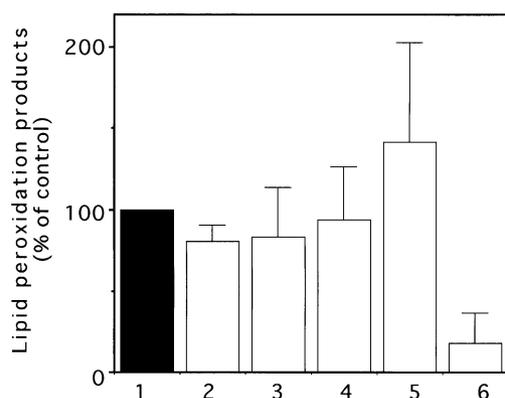


Fig. 8. Lipid peroxidation. Cells were treated with chitosan, adriamycin or allopurinol for 3 days, then lipid peroxidation was measured as the amount of MDA (see "Materials and Methods"). Column 1, control; column 2, chitosan dimer (100 μg/ml); column 3, chitosan polymer (100 μg/ml); column 4, chitosan polymer (100 μg/ml)+50 μM of allopurinol; column 5, adriamycin (100 ng/ml); column 6, adriamycin (100 ng/ml)+50 μM of allopurinol. Values are the mean (±SD) of three independent experiments performed in triplicate.

events caused by chitosan was unclear. We have shown in this paper that chitosan has a growth-inhibitory activity on human bladder tumor cells (Fig. 1). This growth inhibition was due to cell killing activity of chitosan, because chitosan-treated tumor cells showed nuclear fragmentation and chromatin condensation (Fig. 2), which are characteristic of apoptotic cells. Apoptosis was confirmed by the detec-

tion of internucleosomal DNA cleavage on agarose gel electrophoresis. The DNA extracted from chitosan-treated cells showed fragmentation after 3 days of treatment (Fig. 3).

To examine whether the fragmented DNA in the chitosan-treated cells was derived from the attached cells or from floating cells adhered to other cells, the TUNEL method was used. Fig. 4 shows that more than half of the attached cells treated with chitosan were labeled, which indicates DNA breakage. DNA fragmentation is characteristically observed in floating cells treated with other apoptosis inducers, such as adriamycin. The present findings indicate that chitosan induced apoptotic change in the attached cells, which suggests that apoptosis induction might occur in a different manner compared with that of adriamycin.

The alkaline comet assay has been introduced as a method for detecting DNA breakage in individual cells.²⁵⁾ This method has high sensitivity and is not time-consuming. As shown in Fig. 5, cells treated with chitosan or adriamycin showed a "comet tail" while control cells were round, without a tail. From the examination of individual fields under a microscope, almost half of the chitosan-treated cells showed a "comet tail" (Table I).

Chitosan has a positive charge, so it might chelate polypeptides or proteins included in the FBS. We examined the protein content of the culture medium with or without chitosan. The protein amount of the routinely used culture medium was reduced only 5–7% in the presence of 100 μg/ml chitosan (data not shown). Therefore, some of the growth factors presumably remained in the culture media, suggesting that DNA fragmentation may not be

due to serum deprivation. The chitosan-treated cells did not exhibit a shrunken shape, which is characteristic of apoptotic cells, but appeared slightly thinner.

Recently, many chemotherapeutic agents have been shown to activate apoptotic mechanisms which lead to drug-induced cell death, and the activation of caspases plays a crucial role in the biological events associated with apoptosis.²⁶⁾ In particular, caspase-3 has been reported as the executioner upon which many apoptotic pathways converge.^{20, 27)} Therefore, we examined the caspase-3 activity in the chitosan-treated cells using a caspase-3 specific substrate, Z-DEVD-AMC, which is cleaved to afford a fluorescent product. As shown in Fig. 6, caspase-3 activity in the chitosan-treated cells was markedly elevated. The relative caspase-3 activity in the chitosan-treated cells was increased about 2-fold over that of untreated cells. This clearly shows that the growth-inhibitory activity of chitosan can be attributed to apoptosis induction through caspase-3 activation. Chitosan dimer showed no effect on caspase-3 activity (Fig. 6), suggesting that the biological effect of chitosan is characteristic of the high-molecular-weight form, not the small-molecular sugar.

The growth-inhibitory effect of chitosan was reduced in the caspase-3 inhibitor-treated cells, which suggested that caspase-3 activation is required for chitosan-induced apoptosis. However, as shown in Fig. 7, caspase-3 inhibitor could not completely block the apoptosis induced by chitosan. This suggests that there may be a pathway other

than caspase-3 activation which contributes to the growth-inhibitory effect of chitosan.

A radiation study revealed membrane participation in the first step of radiation-induced programmed cell death.³⁴⁾ Lipid peroxidation, evoked by stimulants including radiation, resulted in impairment of the function of several different plasma membrane proteins,^{33, 34)} which could contribute to disruption of cellular homeostasis and cell death. This type of cell death has been characterized as apoptosis.^{32, 33)} Many different oxidative insults including various anticancer agents and radiation have been shown to induce apoptosis in various systems^{19, 34)} *in vitro* and *in vivo*. Since chitosan is highly positively charged, it may adhere to the cell membranes^{3, 5, 14)} and evoke some biological change in the cell membrane. The present findings suggest that chitosan does not induce lipid peroxidation in cell membranes. Therefore, chitosan could interact with the cell membrane to induce apoptosis through a signal transduction pathway(s) other than membrane lipid peroxidation. Further studies should be conducted to clarify the second messenger of the apoptosis pathway activated by chitosan.

In conclusion, the apoptosis induction by chitosan via activation of caspase-3 may be applicable for cancer treatment.

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