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Chitosan exerts anticancer activity through induction of apoptosis and cell cycle arrest in oral cancer cells

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Abstract: Chitosan, a multipurpose biomaterial, has been shown to exert effects against several types of cancer including oral cancer. However, the mechanisms underlying the anticancer activities of chitosan on oral squamous cell carcinoma (SCC) cells remain largely unknown. The present study aimed to compare the effects of low-molecular-weight chitosan (LMWC) and cisplatin on oral SCC Ca9-22 and non-cancer keratinocyte HaCaT cell lines. Cell viability and cell cycle profiles were measured by MTT assay and laser scanning cytometry, respectively. Apoptosis was examined by TUNEL assay and electron micros-copy, followed by analysis of caspase activity. LMWC exhibited cytotoxic effects on Ca9-22, but not HaCaT cells, whereas cisplatin induced apoptosis in both types of cells. Exposure of Ca9-22 cells to LMWC led to G1/S cell cycle arrest and an increase of TUNEL-positive cells accompanied by an early apoptotic cell morphology and subtle increases of caspase activity.

Short-term LMWC exposure was less cytotoxic to HaCaT cells than to Ca9-22 cells, and anticancer activity was exerted through induction of apoptosis and cell cycle arrest, suggesting that LMWC could be a promising natural anticancer agent with fewer side effects on normal cells. (J Oral Sci 56, 119-126, 2014)

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Introduction

The 5-year survival rate for patients with oral squamous cell carcinoma (OSCC) has remained at approximately 50% for the last several decades, and a high proportion of affected patients present with advanced disease. The type of treatment chosen and the prognosis for patients with oral SCC are influenced by the stage of disease at diagnosis. Chemotherapy with cisplatin, 5-fluoro-uracil (5-FU) and docetaxel for oral cancer is associated with serious adverse reactions (1), and therefore intensive research has been focused on the discovery of anticancer agents from natural sources that exert only minimal toxicity on normal cells. Chitosan is an N-deacetylated derivate of chitin, which is present naturally and abundantly in crab and shrimp shells. The degree of deacetylation (DD) is

determined by the number of removed N-acetyl units. Chitosan has been widely used for multiple applications because it is a non-toxic biocompatible, biodegradable, and adsorptive material (2). A previous study has shown that low-molecular-weight chitosan (LMWC) exerts a cytotoxic effect on oral cancer cells (2). Although a higher concentration of LMWC in comparison to cisplatin was needed in order to kill cancer cells, it was relatively less cytotoxic to non-cancer cells.

Most anticancer agents use specific pathways to exert their cytotoxic actions. Identification of molecules activated by chitosan would be useful for understanding the mechanisms responsible for the cytotoxic effects of chitosan on cancer cells. It has been shown that chitosan induces apoptosis and activates caspase-3 (3), as well as activating the extrinsic apoptosis pathway through activation of caspase-8 (4). It has also been hypothesized that necrosis is the mechanism responsible for cell death when cancer cells are exposed to chitosan nanoparticles (5). Inhibition of the tumor cell cycle related to p21/Cip, p27/Kip and proliferating cell nuclear antigen (PCNA) has also been noted (6). Nevertheless, the mechanisms underlying the effects of LMWC on oral cancer cells observed previously have not been fully explored in detail (2). We therefore performed the present study to explore the anticancer effects of LMWC on oral cancer cells in comparison with cisplatin in terms of cytoxicity, cell cycle inhibition and apoptosis.

Materials and Methods

Stock solutions of LMWC and cisplatin
We prepared 5 mL of a 2% w/v stock solution of LMWC (Sigma Cat. No. 44,886-9, Milwaukee, WI, USA) using 1% glacial acetic acid, followed by filtering, and then stored it at room temperature until use. We also prepared a 10 mg/mL cisplatin (Wako, Osaka, Japan) stock solution in dimethyl sulfoxide (DMSO) just before use.

Cell culture
Ca9-22 and HaCaT cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO Cat. No. 11965-092, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Caisson Laboratories Inc., Logan, UT, USA) with 100 IU/mL penicillin, 100 mg/mL streptomycin (Caisson Laboratories) and 25 µg/mL fungizone (Biobasic Inc., Ontario, Canada) at 37°C in a humidified atmosphere containing 5% CO_2. Ca9-22 (assigned as JCRB0625) is a cell line derived from gingival carcinoma that was established at the Faculty of Dentistry, Tokyo Medical and Dental University (7). When cells reached conflu-

ence, they were trypsinized and the suspended cells were used for experiments. Cell morphology with/without LMWC or cisplatin was analyzed using a phase-contrast microscope at an original magnification of ×10.

Viability assay
Cell viability was tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measures the activity of mitochondrial dehydrogenase in the cells. A 5 mg/mL solution of MTT (Cat. No. M2128, Sigma-Aldrich Co., St Louis, MO, USA) in 0.9% NaCl was freshly prepared and added to each well. Three hours after incubation, the formed formazan crystals were dissolved in isopropanol and the plate was placed on an orbital shaker for 1 h at room temperature. The resulting color was measured spectrophotometrically at 490 nm using a microplate reader (Benchmark, BioRad, Hercules, CA, USA). Cells untreated with LMWC or cisplatin were used as controls. Percentage cell viability was calculated by dividing the absorbance of individual wells by the mean absorbance of control wells from three independent experiments done in triplicate. Cell proliferation instead of cytotoxicity was indicated if cell viability was higher than 100%. The cytotoxicity of LMWC and cisplatin was expressed as the IC_{50} value. IC_{50} values were determined using the dose-response curves for LMWC or cisplatin on cell viability (GraphPad Software Inc., La Jolla, CA, USA).

Caspase activity analysis
Cells were lysed and then tested for protease activity by addition of a caspase-specific peptide that is conjugated with a chromophore p-nitroaniline (pNA). Substrates used for measuring caspase-3, caspase-8, and caspase-9 activity were DEVD (Asp-Glu-Val-Asp), IETD (Ile-Glu-Thr-Asp), and LEHD (Leu-Glu-His-Asp), respectively. Cleavage of the peptide by the caspase releases pNA, resulting in a yellow color that can be quantified spectrophotometrically at 405 nm, in accordance with the manufacturer's instructions for the Caspase-3 Colorimetric Assay (Cat. No. BF3100, R&D Systems Inc., Minneapolis, MN, USA), Caspase-8 Colorimetric Assay (Cat. No. BF4100, R&D Systems) and Caspase-9 Colorimetric Assay (Cat. No. BF10100, R&D Systems). Where indicated, Caspase-8 inhibitor (Ac-IETD-CHO, Peptide Institute, Inc., Osaka, Japan) or Caspase-9 inhibitor (Ac-LEHD-CHO, Peptide Institute) at 100 µM was added 1 h before exposure to LMWC or cisplatin.

Caspase activity was calculated using the formula (mean absorbance experimental group/mean absorbance control) × 100%. The % caspase activity for the experi-
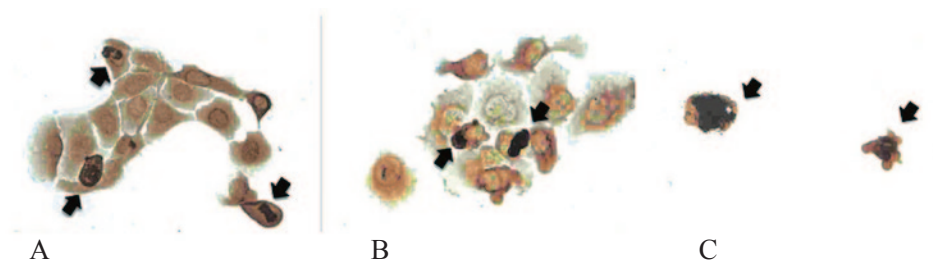
Fig. 1 Morphology of apoptotic cells in the TUNEL assay. Arrows indicate cells showing all the characteristics of apopotosis: (A) condensation of chromatin and cytoplasm, (B) cytoplasmic fragments with or without condensed chromatin (apoptotic bodies), and (C) chromatin fragments (micronuclei).

mental group was then compared to the control as a mean of three independent experiments performed in triplicate.

TUNEL assay

A suspension containing 10^5 cells was inoculated into a slide chamber (Lab-Tek 177445, Thermo Fisher Scientific Inc., Waltham, MA, USA). TUNEL assay was performed using an In Situ Cell Death Detection Kit, POD (Roche Diagnostic, Mannheim, Germany) in accordance with the manufacturer's instructions. After counterstaining with methyl green, apoptotic cells were detected by light microscopy (Fig. 1). The number of TUNEL-positive cells was counted among a total of 250 cells in randomly chosen microscopic fields at a final magnification of ×300 The percentage of apoptotic cells was calculated using the formula: (number of TUNEL-positive cells/250) × 100 (8).

Scanning electron microscopy (SEM)

Ca9-22 cells were cultured on glass coverslips and exposed to LMWC or cisplatin. After 24 h, the cells were fixed with 2.5% glutaraldehyde and then dehydrated using a graded ethanol series. The cells were air-dried and then vaccum-dried, then finally sputter-coated with gold. The surface morphology of the cells was analyzed using a Zeiss Scanning Electron Microscope EVO MA 10 (Carl Zeiss Microscopy GmbH., Jena, Germany).

Transmission electron microscopy (TEM)

Ca9-22 and HaCat cell suspensions were cultured in 60-mm culture dishes and exposed to LMWC or cisplatin. After 24 h, the cells were harvested and subjected to TEM analysis using JEOL JEM-1010 (JEOL Ltd., Tokyo, Japan).

Cell cycle analysis

The DNA content of the cells in each phase of the cell cycle was analyzed by laser scanning cytometry (LSC).

Cells were washed with PBS and centrifuged at 1,500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended with PBS and transferred to a new 1.5-mL tube, followed by centrifugation at 9,500 rpm for 1 min at 4°C. The supernatant and LMWC were carefully removed, without disrupting the cell pellet. The cells were gently resuspended and fixed with a 5-fold volume of cold 75% ethanol in PBS. The cell suspension was incubated for 15 min at room temperature, followed by centrifugation at 7,500 rpm for 5 min at 4°C. The cell pellets were resuspended in a 3-fold volume of 0.05 mg/mL propidium iodide (PI) containing 0.2 mg/mL RNase A, and then incubated at 37°C for 30 min. A cell suspension (20 μL) was smeared onto a glass slide. The smear was carefully covered with a coverslip, and the edge of the coverslip was sealed with transparent nail polish to avoid evaporation. Cell cycle analysis was performed using LSC (LSC101; Olympus, Tokyo, Japan). The excitation wavelength for PI was 488 nm. The software generated a DNA histogram showing the percentages of the cell population in each of the phases of the cell cycle (9).

Data analysis

All data from this study were stored digitally in a computer. Data analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Descriptive analysis of data was presented as mean and standard deviation. Determination of the IC_{50} concentration was performed using a linear regression equation for the cytotoxicity curve on the GraphPad Prism 5. Furthermore, mean differences between the experimental and control groups were analysed statistically using unpaired two tailed t-test when comparing two experimental groups and one-way ANOVA followed by Newman-Keuls Multiple Comparison Test, when comparing more than two groups. Significance was determined at $P < 0.05$.
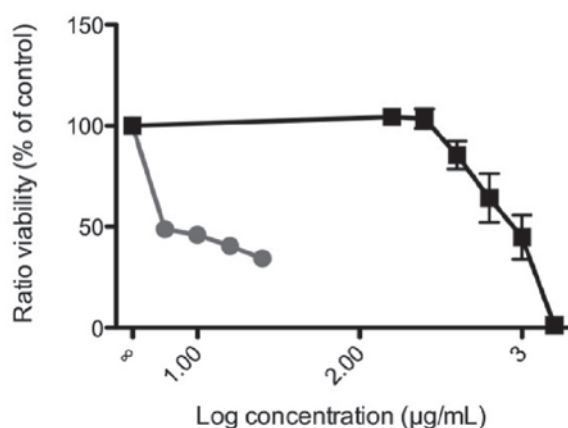
Fig. 2 Effects of LMWC and cisplatin on Ca922 cells. Ca9-22 and HaCaT cells were seeded at 5.0×10^5 cells/100 μL into 96-well plates and preincubated for 1 h, after which various concentrations of LMWC (150, 200, 250, 300, 1,000, and 5,000 μg/mL) and cisplatin (5, 10, 15, and 20 μg/mL) were added. The cells were incubated for 24 h before the viability test. Proliferation was indicated if cell viability was >100%. The results are the means ± SD of three independent experiments performed in triplicate. Bars = SD.
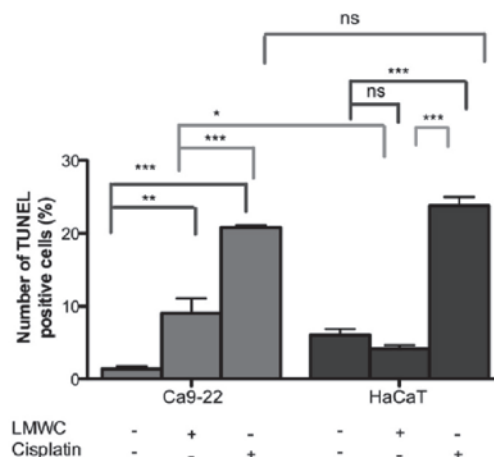

Fig. 3 Apoptosis contributes to cytotoxicity of Ca9-22 cells resulting from exposure to LMWC. A cell suspension containing 10^5 cells was inoculated into a slide chamber. Cells were preincubated for 1 h at 37°C before exposure to chitosan, then incubated for another 24 h in medium containing LMWC or cisplatin (800 μg/mL and 8 μg/mL, respectively) before TUNEL assay.

Results

The effects of LMWC and cisplatin exposure on Ca9-22 cells were examined by MTT assay. As shown in Fig. 2, the viability of Ca9-22 cells declined in a dose-dependent manner after exposure to LMWC. Linear regression analysis of the viability data for LMWC and cisplatin exposure was performed using GraphPad Prism 5. The IC_{50} value for LMWC in Ca9-22 cells was 800 ± 131.45 μg/mL, while that for cisplatin was 8 ± 0.029 μg/mL. The difference was statistically significant at $P = 0.00051$. These results indicate that cisplatin was highly cytotoxic to Ca9-22 cells with an IC_{50} value one-hundredth that of LMWC.

Next, in order to compare the cytotoxicity of LMWC between cancer cells and non-cancer cells, HaCaT cells, which are immortalized but not tumorigenic cells derived from dermal keratinocytes, were used as controls. Ca9-22 and HaCaT cells were treated with LMWC or cisplatin at the respective IC_{50} doses (800 μg/mL and 8 μg/mL) (10). The viability of HaCaT cells was 40% higher than that of Ca9-22 cells after exposure to LMWC (89.7 ± 16.6% vs 53.3 ± 5.2%), and the difference was statistically significant (Fig. 2). In contrast, the viabilities of Ca9-22 and HaCaT cells after exposure to cisplatin were not significantly different (49.0 ± 6.6% and 48.9 ± 5.5%, respectively) (Fig. 2).

To examine whether LMWC exerted cytotoxicity through induction of apoptosis, TUNEL assay was used to assess apoptosis. As shown in Fig. 3, exposure of Ca9-22 cells to LMWC increased the proportion of apoptotic cells (9.0 ± 3.6%), whereas exposure to cisplatin resulted in a greater proportion of apoptotic cells (20.8 ± 1.4%, $P < 0.001$), 14 times higher than that induced by LMWC. Furthermore, cisplatin caused apoptosis in 23% of HaCaT cells, indicating that it was a strong inducer of apoptosis in both Ca9-22 and HaCaT cells ($P < 0.001$) (Fig. 3). LMWC, on the other hand, did not increase the proportion of apoptotic HaCaT cells (Fig. 3). These results indicated that LMWC significantly induced apoptosis in Ca9-22, but not in HaCaT cells. Although exposure to LMWC (800 μg/mL) reduced cell viability by 50% (Figs. 2 and 3), it rendered only 9% of the cells apoptotic, suggesting that apoptosis at least partly contributed to LMWC-mediated cytotoxicity in Ca9-22 cells.

SEM analysis confirmed that cell-surface morphological changes typical of apoptosis occurred in Ca9-22 cells exposed to LMWC and cisplatin, such as cell membrane blebbing and disappearance of microvilli (Fig. 4, A and B); untreated Ca9-22 cells showed normal morphological features (data not shown). In line with this, TEM analysis of Ca9-22 cells after exposure to LMWC demonstrated cell shrinkage, margination of nuclear chromatin, and prominent mitochondria (Fig. 4C), whereas cells exposed to cisplatin demonstrated cell shrinkage, nuclear fragmentation, cytoplasmic vacuolation and prominent
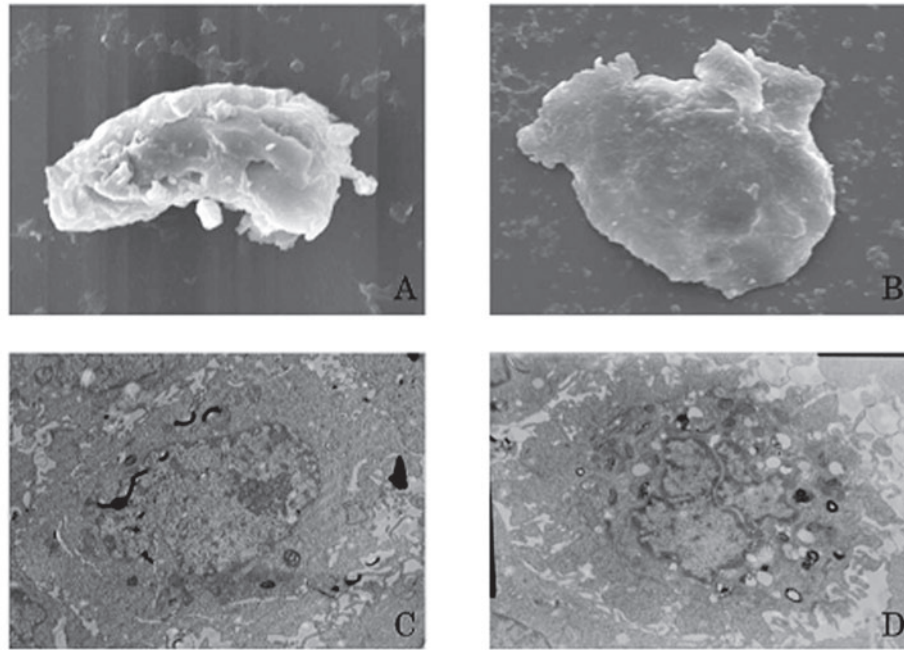
Fig. 4 Scanning electron micrograph of Ca9-22 cells cultured for 24 h in the presence of LMWC (A) or cisplatin (B) *in vitro* (SEM, ×200). Transmission electron micrograph of Ca9-22 cells cultured for 24 h in the presence of LMWC, showing cell shrinkage, margination of nuclear chromatin and prominent mitochondria (C); or in the presence of cisplatin, showing cell shrinkage, nuclear fragmentation, cytoplasmic vacuoles and prominent mitochondria (D) *in vitro* (TEM, ×5,000).
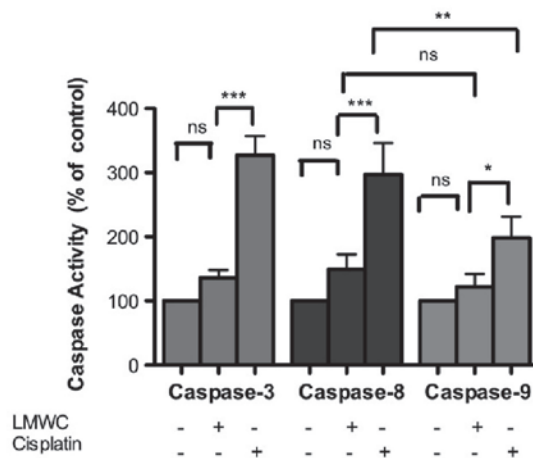

Fig. 5 LMWC does not significantly induce caspase activity in comparison to cisplatin. Ca9-22 and HaCaT cell suspensions containing 2.0×10^6 cells were preincubated in a 60-mm culture dishes for 1 h, and then treated with 800 μg/mL LMWC or 8 μg/mL cisplatin. Untreated cells were used as controls. Analyses were performed 24 h after treatment. Results are mean ± SD of three independent experiments performed in triplicate. ANOVA with Newman-Keuls Multiple Comparison Test was performed to analyze the association of means between groups, where *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, and ns = not significant.
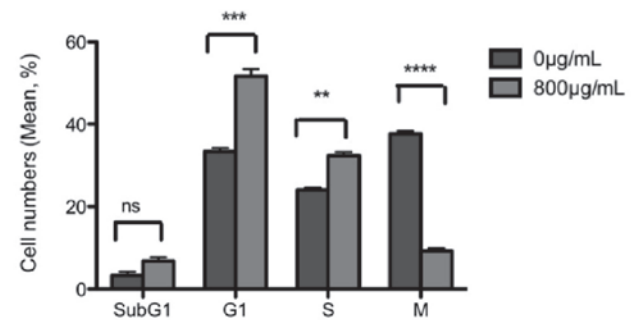

Fig. 6 Exposure to LMWC results in cell cycle arrest at G1 and S phase. Bar diagram showing distribution of the Ca9-22 cell population in each phase of the cell cycle. Differences in mean ± SD between cell cycle phases were compared with those in control cells and analyzed by *t*-test. Bars show SD, where **$P < 0.01$, ***$P < 0.001$, and ns = not significant.

mitochondria (Fig. 4D). Thus, LMWC-exposed Ca9-22 cells exhibited morphological changes typical of the early phase of apoptosis.

To examine the apoptotic pathways activated by LMWC, caspase-3, caspase-8 and caspase-9 activities were measured after exposure of Ca9-22 cells to LMWC or cisplatin. Cisplatin, but not LMWC, significantly increased caspase-3 activity (Fig. 5). Cisplatin also increased caspase-8 activity more strongly than caspase-9 activity (297% and 198%, respectively), whereas LMWC increased the activities of caspase-8 and caspase-9 to 149.1 ± 23.5% and 122.2 ± 19.9%, respectively, although

these increases were not significant (data not shown). Exposure of Ca9-22 cells to cisplatin in the presence of caspase-8 and caspase-9 inhibitors reduced caspase-3 activity by 75% and 50%, respectively (data not shown). Since caspase-3 activity in LMWC-exposed cells was not functional, the result of the assay was not would not have been justified.

Next, the effects of LMWC on cell cycle progression in Ca9-22 cells were analyzed by LSC, since the results presented above suggested that apoptosis only partially contributed to the cytotoxic effect of LMWC on Ca9-22 cells. Consistent with this assumption, the sub-G1 cell population after exposure to LWMC did not increased significantly (only 4-7%) (Fig. 6). However, LMWC exposure resulted in a significant increase in the G1 and S phase populations (from 33% to 51%, and from 24% to 32%, respectively), indicating that LMWC induced G1/S cell cycle arrest in Ca9-22 cells.

Discussion

In this study we demonstrated that LMWC exhibited cytotoxic effects on Ca9-22, but not HaCaT cells, whereas cisplatin induced apoptosis in both types of cells. The IC_{50} of cisplatin on Ca9-22 cells was one hundredth that of LWMC, indicating that cisplatin had a much more potent anticancer effect than LWMC. However, at the same concentration, cisplatin was also cytotoxic to HaCaT cells. These findings are similar to those of a previous study in which trimethyl-chitosan (TMC) was shown to exert a cytotoxic effect at concentrations higher than 741 µg/mL, and thus exposure to chitosan at 3.2 µg/mL would not have exerted any effects (11,12). The high concentration of chitosan needed to cause cytotoxicity of cancer cells in the present *in vitro* study may not be problematic in future *in vivo* studies because chitosan is a safe natural agent with an oral toxicity higher than 16 g/day/kg (13). The effect on HaCaT cells we observed has also been described in a previous study showing that chitosan was able to induce mitogenic activity in fibroblasts and keratinocytes (14).

The opposite effects of LMWC on the Ca9-22 and HaCaT cell lines might have been due to a difference in the mechanisms of cytotoxicity. The stronger cytotoxic effect of chitosan on Ca9-22 cells might be related to the highly positively charged amino groups in the chitosan molecule being attracted to the cancer cell membrane, which has a greater negative charge than that of normal cells (15). Thus, to induce cytotoxicity *in vitro*, chitosan might directly attack cancer cells through interaction with the tumor cell membrane or extracellularly via a specific receptor, or via endocytosis (11). Alternatively, chitosan might cause membrane disruption through electrostatic interaction, leading to an increase of the inflammatory cytokines IL-6 and IL-8. These cytokines have been shown to have mitogenic effects on normal fibroblasts and keratinocytes (16). Cancer cells might lose their ability to respond to the membrane-damaging effects of chitosan, such as the release of such inflammatory cytokines. These cytotoxic effects suggest that chitosan might be a promising and safer therapeutic option for treatment of oral cancer, with fewer side effects. However, future studies involving *in vivo* experiments, drug formulation and clinical trials will be necessary.

Exposure of Ca9-22 cells to LMWC increases the proportion of TUNEL-positive cells and the subG1 cell population. Determination of apoptotic cells in this study was based on the number of TUNEL-positive cells per 250 cells counted in a randomly chosen microscopic field, and the counting was performed twice. The results of the TUNEL assay showed that LMWC had an antagonist effect on HaCaT cells. Analysis of apoptotic cells using LSC also demonstrated only a minor increase in the subG1 cell population after LMWC exposure. Estimation of the subG1 cell population further confirmed that apoptosis might not be the major mechanism of cell death occurring after exposure to LWMC, since the proportion of apoptotic cells did not differ significantly from that among control cells.

Exposure of Ca9-22 cells to LMWC resulted in a subtle increase of caspase expression. Analysis of caspase activity confirmed that apoptosis might not the major mechanism of cell death induced by LMWC (Fig. 5). Although we found only a subtle increase of caspase activity relative to the control, use of a more sensitive detection kit might have yielded different results. Attempts were made to confirm the apoptosis pathway responsible for the slight degree of apoptosis. Cisplatin-exposed cells clearly showed a requirement for caspase-8, but we were unable to determine the type of apoptotic pathway operating after exposure to LMWC. These results confirmed a previous study showing that cisplatin induced apoptosis in Ca9-22 cells through the extrinsic pathway (17). Our study confirmed that cisplatin is a potent inducer of apoptosis and requires the presence of caspase-8 for this effect. However, cancer cells might require higher concentrations of caspase-8 inhibitors to fully escape apoptosis induced by cisplatin.

The results of SEM and TEM confirmed the absence of a necrotic cell population exhibiting cell membrane disruption (Fig. 4), while morphological characteristics of apoptotic cell death including membrane blebbing and cell shrinkage were observed. SEM showed that the

degree of membrane blebbing of LMWC-exposed cells was less than that of cells exposed to cisplatin (18). This might explain the only marginally increased caspase activity in Ca9-22 cells after exposure to LMWC. Normally, numerous microvilli would be present on the surface of the cells (19). No apoptotic bodies were detected by SEM in Ca9-22 cells exposed to LMWC. A previous study of cells exposed to various inducers of apoptosis showed that some cells had marginal blebbing, which is considered to be an early process of apoptosis related to microfilament activity. These blebs result from changes in the contractility of actomyosin through only partial activation of caspase-3.

Whereas SEM revealed only marginal membrane blebbing, TEM revealed changes in the cytoplasm of Ca9-22 cells exposed to LMWC (Fig. 4). Ca9-22 cells exposed to cisplatin showed nuclear fragmentation and numerous mitochondria, whereas after LMWC exposure they showed only nuclear margination with prominent mitochondria. Further analyses are still needed, since the effect of LMWC on Ca9-22 cells might be dose- and time-dependent. No morphologic features of late apoptosis were seen in this study, suggesting that a longer period may be needed in order for LMWC to induce apoptosis.

Exposure of Ca9-22 cells to LMWC led to G1/S cell cycle arrest, and an increase of cells in G1 phase was evident in comparison to control cells ($P < 0.001$) (Fig. 6), thus indicating that LMWC arrested this population of cells in G1 phase. This might have involved changes in the expression of proteins regulating this phase, especially those that prevent cells from entering S phase, and might take place in a p53-independent setting (20). The presence of DNA damage sometimes requires a rapid response that does not involve transcription or translation, and therefore protein needs to be synthesized faster (21). When the checkpoint is presented in early G1/S phase, there is possibility that the Cdk-4/Cdk-6-Cyclin complex would be inhibited by CKI p15 and p27 induced by TGF-β. This would prevent RB phosphorylation independently of p53 (22,23). Alternatively, G1 arrest might also occur when the checkpoint is in the mid to late G1 phase, because of prevention of RB phosphorylation (mid phase) or inhibition of cyclin E-Cdk2 activity (late phase) (24). It has been shown that in mammalian cells exposed to UV radiation, ATM/ATR is activated to cause Cdc25A phosphorylation through Chk1/Chk2, resulting in ubiquitination of Cdc25A. This would decrease the concentration and activity of Cdc25A, resulting in cyclin E-Cdk2 inactivation and arrest of cells in G1 phase (24). Therefore, signaling between TGF β-p15/p27-Cdk4/Cdk6 or ATM/ATR-Chk1/Chk2-Cdc25A-Cdk2 might be involved in the G1 arrest resulting from LMWC exposure (24). However, further studies to investigate this issue will be needed.

There was an increase in the proportion of Ca9-22 cells in S phase after exposure to LMWC, in comparison with the control ($P < 0.01$) (Fig. 6), indicating that LMWC induced S-phase arrest in this population of cells. Generally, the regulation of S-phase arrest does not involve a specific role for p53 or p21, and therefore the mechanism might involve ATM-Chk2-Cdc25A-Cdk2 signaling. This would result in inhibition of DNA synthesis in S phase for several hours (20). A further study to investigate this possibility will be needed. The number of cells in G2/M phase was markedly decreased after exposure to LMWC ($P < 0.0001$) (Fig. 6). As possible DNA damage in Ca9-22 cells after exposure to LMWC might result in cell cycle arrest in G1 and S phase, mitosis would be prevented. It will be important to perform a further study to clarify the molecules involved in the cell cycle arrest induced by LMWC. The primary aim of anticancer treatment is to induce permanent cell cycle arrest that results in cellular senescence, which is a major process of aging as well as an important anticancer mechanism (25). G1- and S-phase cell cycle arrest resulting from exposure to LMWC might be initiated through an increase in the expression of TGF β. This molecule might activate Smads 2/3 and Smad 4, which further activate p15 and p21 and finally activate the production of reactive oxygen species (ROS), resulting in cell senescence (25). Further research to identify markers of cellular senescence should be performed to clarify this issue. Also, investigation of cells that are not attached to the culture dish after exposure to chitosan might provide clues to cell fate and offer a deeper insight into the anticancer mechanism of LMWC.

Short-term exposure to LMWC was less cytotoxic to HaCaT cells than to Ca9-22 cells, and anticancer activity occurred through induction of apoptosis and cell cycle arrest, suggesting that LMWC is a promising natural product that could be applicable as an anticancer agent, exerting fewer side effects on normal cells. However, many more studies including *in vivo* experiments, drug formulation and, finally, clinical trials will be required. Furthermore, the possibility of combined treatment with chitosan and other conventional anticancer drugs would yield useful data on the potential use of chitosan for anticancer treatment.

Acknowledgments

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