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Cytotoxic Activity of Aminoderivatized Cationic Chitosan Derivatives

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Abstract—Chitosan derivatives were prepared by dialkylaminoalkylation and reductive amination followed by quaternization. In this study, the cytotoxic activity of the chitosan derivatives was investigated and a relationship between structure and activity is suggested. The cationic chitosan derivatives elicited dose-dependent inhibitory effects on the proliferation of tumor cell lines.

Introduction

A main goal of cancer research is to completely prevent recurrence following surgery and to increase survival time. Cytotoxic anticancer chemotherapeutic agents generally produce severe side effects, while reducing host resistance to cancer and infections, especially through the destruction of lymphoid and bone marrow cells. As a result, many cancer patients die of pneumonitis, septicemia, uremia, or other secondary diseases, rather than from the cancer itself.[1] Most anticancer chemotherapeutic agents have detrimental side effects that are not conducive to prolonging the lives of cancer patients. Therefore, it is important to find new, powerful anticancer agents that are nontoxic and biocompatible. Polysaccharides, such as lentinan, pachymaran, schizophyllan, hemicellulose, and mannan, have been shown to have various immunostimulative and antitumor activities.[2] In particular, lentinan and schizophyllan have been used as immunotherapeutic agents to treat cancer in humans and in experimental tumor systems.[3] Chitosan is a cationic polymer that has an amino group in its chemical structure, unlike other polysaccharides. Many functions of chitosan can be attributed to its cationic structure, such as its antitumor, antimicrobial, antiinflammatory, and antihypercholeste- rolemic activities.[4,5] When ingested, chitosan has fewer side effects than synthetic drugs, and it is also environmentally friendly. However, the effect of chitosan itself is relatively weak compared to those of synthetic drugs. Therefore, in a previous study, we increased the positive charge density of chitosan by dialkylaminoalkylation and reductive amination followed by quaternization.[6] In this study, the cytotoxic activity of these chitosan derivatives was investigated and a relationship between structure and activity is suggested.

Materials

NH_2-, diethylaminoethyl (DEAE)-, dimethylaminoethyl (DMAE)-, and dimethylaminoisopropyl (DMAiP)-chitosan with 0.60 ± 0.03 degrees of substitution were prepared and then quaternized (q), as described in a previous report (Scheme 1).[6]

Culture and cellular proliferation studies

Dulbecco's modified Eagle's medium (GIBCO, N.Y., USA) supplemented with 10% fetal bovine serum (GIBCO, NY, USA) was used as the basal cell culture medium. HepG2, HeLa, COLO, and CCL-13 cell lines were originally purchased from the Cell Bank of the Chinese Academy of Science, Shanghai, China.

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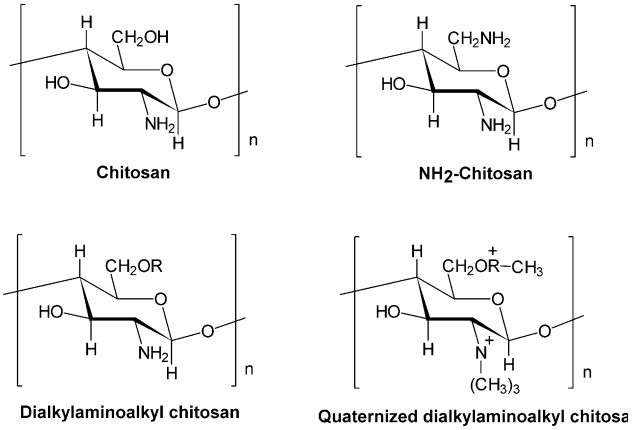

Scheme 1. The cationic chitosan derivatives (DMAE-chitosan; R = DMAE, DMAiP-chitosan; R = DMAiP, DEAE-chitosan; R = DEAE).

HepG2, HeLa, COLO, and CCL-13 cells were cultured for 1 day in a tissue culture dish (35×10 mm) at an initial concentration of 7×10^4 cells/mL. The next day, the culture medium was replaced with medium containing the desired concentrations of the chitosan derivatives (25–100 μg/mL) or control medium. Viable cells were identified and counted using the tryphan blue dye exclusion test on the second day of culture.

Cytotoxic activity of chitosan derivatives

The results presented here suggest that some chemically modified derivatives of chitosan have the potential to suppress the growth of HepG2, HeLa, and COLO tumor cells. Figure 1 shows that these chitosan derivatives elicited dose-dependent inhibitory effects on the proliferation of the HepG2 cell line. As shown in Figure 1, the DEAE-chitosan derivatives were highly tumor-

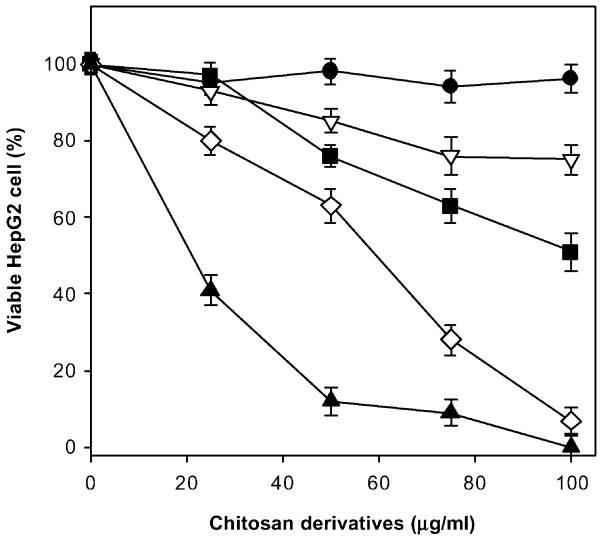

Figure 1. Cytotoxic activities of the chitosan derivatives with various concentrations against HepG2 cell line. Symbols: ● chitosan, ▽ NH_2-chitosan, ■ quaternized NH_2-chitosan, ◇ DEAE-chitosan, ▲ quaternized DEAE-chitosan. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

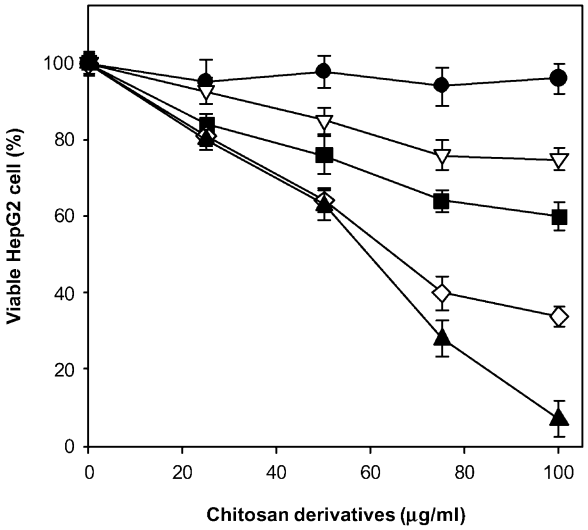

Figure 2. Cytotoxic activities of the chitosan derivatives with different hydrophobicity against HepG2 cell lines. Symbols: ● chitosan, ▽ NH_2-chitosan, ■ DMAE-chitosan, ◇ DMAiP-chitosan, ▲ DEAE-chitosan. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

suppressive and the NH_2-chitosan derivatives showed moderate activity. Of note, qDEAE-chitosan killed ca. 90% of HepG2 cells at a concentration of only 50 μg/mL. The IC_{50} values (day 2) for qDEAE-, DEAE-, qNH_2-chitosan were 22, 60, and 100 μg/mL, respectively. Tumor suppression increased significantly as the cationic charge of the chitosan derivatives increased via aminoderivatization followed by quaternization, indicating that there is an electrostatic interaction between the chitosan derivatives and the negatively charged functional residues on the tumor cell surface. Release of the materials absorbing light at 260 nm (mainly DNA and RNA) may be a direct measure of cell lysis. In the

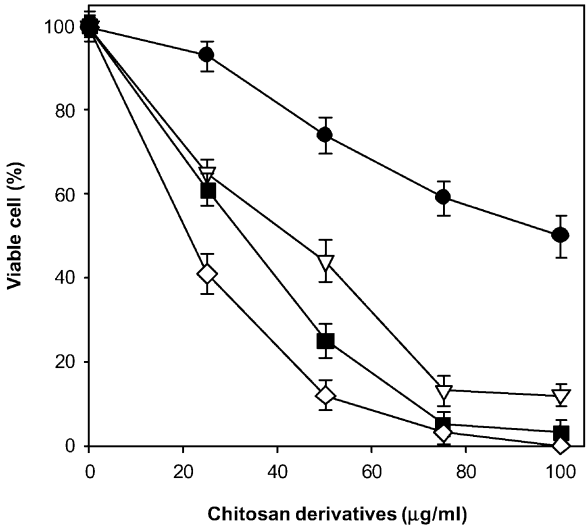

Figure 3. Cytotoxic activities of quaternized DEAE-chitosan against different cell lines. Symbols: ● CCL-13, ▽ COLO, ■ HeLa, ◇ HepG2. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

Table 1. Cytotoxic activity of chitosan, qNH_2-, DEAE-, and qDEAE-chitosan against different cell lines

Panel of cell lines	Cell line	Compd/cytotoxicity (IC_{50}, μg/mL)[a]			
		Chitosan	qNH_2-chitosan	DEAE-chitosan	qDEAE-chitosan
Diploid	CCL-13	na	302 (±28)	225 (±25)	110 (±12)
Colon cancer	COLO	na	172 (±13)	115 (±17)	41 (±7)
Cervix cancer	HeLa	na	118 (±15)	72 (±12)	31 (±4)
Liver cancer	HepG2	na	100 (±14)	60 (±8)	22 (±4)

[a]Values are means of three experiments, standard deviation is given in parentheses (na = not active).

absence of qDEAE-chitosan, we observed no release from the intact cells. When the cells were exposed to qDEAE-chitosan (100 μg/mL), the materials absorbing light at 260 nm were clearly released from the cells. Complete lysis of the cell in contact with qDEAE-chitosan was confirmed via a light microscopic picture showing the loss of cellular membrane integrity (data not shown). The in vitro tumor-suppressive activity of qDEAE-chitosan was as potent as that of antibiotics, such as adriamycin (IC_{50} 5–100 μg/mL)[7,8] and cecropin B (IC_{50} 10–300 μg/mL),[9] and much higher than values reported for other polysaccharides (IC_{50} ~1000 μg/mL).[9] Figure 2 shows the suppression of HepG2 cell proliferation in the presence of the chitosan derivatives with different substituents.

According to Figure 2, the cytotoxic activity of the chitosan derivatives increased with the number of carbons in the alkyl group in the order chitosan < NH_2-chitosan < DMAE-chitosan < DMAiP-chitosan < DEAE-chitosan. That is, the cytotoxic activity increased with increasing hydrophobicity, indicating that there is a hydrophobic interaction between the chitosan derivatives and the functional residues on the tumor cell surface. These hydrophobic interactions may induce the aggregation of anions on the polymer surface. Although the electrostatic ionic interaction between the negatively charged groups of the tumor cells and the positively charged amino groups of the chitosan derivatives is the major factor affecting the cytotoxic activity of the chitosan derivatives, the experimental results clearly show the existence of secondary binding forces, that is, hydrophobic interactions between hydrophobic regions on the tumor cells and the chitosan derivatives.

For qDEAE-chitosan at a concentration of only 50 μg/mL, tumor cell proliferation was suppressed from 60 to 90% in the three cell lines tested in the order COLO < HeLa < HepG2 (Fig. 3). Of these cancer cell lines, the IC_{50} values were lowest in the HepG2 cell line (Table 1). Moreover, qDEAE-chitosan seems to be selective for aneuploid tumor cells, since low toxicity was observed against diploid cells (CCL-13). Therefore, this study supports the use of qDEAE-chitosan in further preclinical and clinical studies involving a broad spectrum of malignant tumors.

The higher-order structure of polysaccharides is important for their physiological activities. Pachymarun from *Poria cocos* and laminarin are both (1→3)-β-D-glucans, but neither possesses antitumor properties because both have single helical structures. Yet pachymaran and carboxymethylpachymaran have marked antitumor activity.[10–12] All have triple helical structures. Such structure–activity relationships suggest the existence of biological systems that recognize the configurational structures of polysaccharides. The higher order structure of active chitosan derivatives and the details of their mechanisms of action in the host are now under investigation, especially to clarify the entity intervening between polysaccharides and tumors.

Acknowledgements

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