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Cytotoxicity of chitosans with different acetylation degrees and molecular weights on bladder carcinoma cells

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ABSTRACT

The purpose of this research was to evaluate the cytotoxicity of chitosans with different degrees of acetylation (DA) and molecular weights (MW), as well as the effect of their positive ionic charges controlled by pH on bladder carcinoma cells (RT112 and RT112cp) using the tetrazolium salt colorimetric (MTT) assay. Our data showed that all chitosan samples were cytotoxic on RT112 and RT112cp cells with a higher cytotoxicity obtained at lower pH. Further, it was found that the toxicity increased with increasing DA. However, no significant difference in cytotoxicity between chitosans with different molecular weights was observed.

Annexin V-FITC staining test was then used to study and quantify the induction of apoptosis. Data shows that chitosans induce apoptosis of RT112 and RT112cp cells with the same dependence with DA.

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1. Introduction

In the recent years, the use of natural compounds with chemopreventive properties has attracted much interest in chemotherapy and treatment of cancers. A good example is the use of chitosan, a naturally occurring polymer that has been often tested in pharmaceutical and biomedical applications because of its promising properties such as biocompatibility, biodegradability and lower toxicity toward mammalian cells [1]. Chitosan was found to have various biological activities including antimicrobial activity [2], antioxidant activity [3], immuno-enhancing effects [4] and antitumor activity [3].

Chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine obtained from chitin, a biopolymer extracted principally from shrimp and crab shell waste. The term chitosan usually refers to a family of chitin derivatives obtained after partial deacetylation to varying acetylation degrees (DA < 50%) but also different molecular weights (MW). Such polymers become soluble in acidic conditions due to the protonation of the $-NH_2$ group at the C-2 position of glucosamine units [1]. According to many research, its unique polycationic property is responsible of its interesting physical and biological properties. It was also reported that chitosan could have variable biological activities according to its DA and MW [5–7].

According to the preparation process, there are two types of chitosans: (1) chitosans derived from chitin by deacetylation under heterogeneous conditions; (2) chitosans derived from a highly deacetylated chitosan (or poly-D-glucosamine) by partial reacetylation under homogeneous conditions [8,9]. The main difference between these two types of chitosan is the fraction of polymer solubilized in acidic conditions and the presence of aggregates evidenced by dynamic light scattering especially when DA increases [10,11]. In fact, deacetylation reaction performed under heterogeneous conditions gives an irregular distribution of N-acetyl-D-glucosamine and D-glucosamine residues and some blockwise acetyl group distribution along polymeric chains which control their solubility and degree of aggregation; this microstructure could have variable influence toward biological activities (even if two samples have the same average DA). The physico-chemical properties of such chitosans appear different from those of randomly acetylated chitosans obtained under homogeneous conditions [10]. Until now, in biological activity studies, such characteristics for chitosan have not been considered seriously even if chitosan has attracted considerable interest in many fields.

Antitumor activity of chitosan and its derivatives has been studied by many authors [12–16]. While several studies have reported the importance of chitosan for their anticancer activity, there was

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no clear information describing the relationship between chitosans characteristics and their observed activities. Thus, further studies are needed to better understand the mechanisms involved with chitosan-based tumor stasis.

In the present work, antitumor studies were performed on bladder cancer, a malignancy affecting the lining of the urinary bladder, which is estimated to be the seventh most common malignant neoplasm and the eighth leading cause of cancer-related death worldwide [17]. Previous studies demonstrated the effect of chitosan on bladder carcinoma cells using both *in vitro* and *in vivo* assays [18–20].

In this study, effect of chitosan characteristics (MW and DA) on its cytotoxicity was evaluated. Thus, fifteen well-defined chitosans with wide range of acetylation degrees and molecular weights (DA up to around 60% and different MW) prepared under homogeneous conditions, were used in this study. Our approach is original as we use homogeneous chitosans with defined characteristics and a random distribution of acetyl groups along the polysaccharide chain preventing formation of insoluble particles and having a good solubility in acidic conditions.

2. Materials and methods

2.1. Chitosan samples

Several chitosan grades were used throughout this study (Table 1). Chitosans were prepared by homogeneous reacetylation of highly deacetylated chitin in a large range of chitosan DA (2–61 mol%) and different average molecular weights (42.5–135 kDa) as indicated in our previous paper [7].

All the samples were fully soluble in acidic conditions and characterized by nuclear magnetic resonance (NMR) and steric exclusion chromatography (SEC) as indicated in previous papers [21,22].

2.2. Cell culture

Human bladder cancer cell line RT112 was obtained from Cell Lines Service (Eppelheim, Germany). Cisplatin resistant RT112 cells (RT112-cp) were kindly provided by B. Köberle (Institute of Toxicology, Clinical Center of University of Mainz, Mainz, Germany). RT112 and RT112-cp cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine (Invitrogen Life Technologies, Cergy Pontoise, France) and supplemented with 10% (v/v) fetal calf serum, 2.5 U/ml penicillin, and 2.5 mg/ml streptomycin (Invitrogen, Life Technologies). Cells were cultured at 37 °C in a 5% CO_2-humidified atmosphere and tested to ensure absence of mycoplasma contamination.

Table 1
Characteristics of the samples obtained by homogeneous reacetylation to cover a wide range of degrees of acetylation and molecular weights: $x = 0, 1, 2, 3$ are referred to initial and partially hydrolyzed chitosan samples with different initial molecular weights. A, B, C, D, E, F referred to samples with different degrees of reacetylation obtained from the same initial or partially hydrolyzed chitosan (named 0A, 0B, 0C, 0D, 0E and 0F).

Samples	DA (mol%) from ^{1}H NMR	M_w (g mol^{-1}) from SEC
0D	2	110,000
1D	2	77,000
2D	2	73,000
3D	2	42,500
0A	12	135,000
1A	12	94,000
2A	12	75,000
0B	24	127,000
1B	24	95,000
2B	22	85,000
3B	24	51,000
1C	41	84,000
2C	42	70,000
1E	56	75,000
1F	61	75,000

2.3. MTT tetrazolium salt colorimetric assay

Bladder cancer cells (3×10^3 cells/well) were seeded in 96-well plates and incubated in a 5% CO_2 incubator for 24 h at 37 °C, before treatment with chitin derivatives. Each chitosan sample was prepared at 1 mg/ml or ~6 mM under stirring in aqueous solution containing the stoichiometric amount of HCl. Successive dilutions of initial chitosan solution were then made up with RPMI (pH 7.5) medium to get 50, 100, 500 and 1000 μM concentrations expressed in monomeric unit of chitosan. For assays conducted at lower pH (6.1 and 6.5), pH of the RPMI medium was adjusted using 1 M HCl and HEPES as buffer (20 mM).

RT112 and RT112cp cells were treated with chitosan solutions of various concentrations during 2 h allowing chitosan penetration into cells as demonstrated separately. Then, the initial medium was replaced by fresh RPMI medium at pH = 7.5 for a total of 24, 48 and 72 h preventing cell death when tests are carried out at pH = 6.5 or lower.

After incubations, 10 μl of tetrazolium dye (MTT) (at 5 mg/ml in phosphate-buffer; PBS) was added to each well and incubated for 2 h. The plate was then centrifuged at 1800 × *g* for 5 min at 4 °C. After careful removal of the medium, 100 μl of DMSO was added to each well and plates were shaken. (Viable cells were determined by measuring the absorbance at 570 nm) Absorbance was recorded on a microplate reader (Sunrise; Tecan, Lyon, France) at the wavelength of 570 nm. The effect of each chitosan sample on growth inhibition was assessed as percent cell viability, where vehicle-treated cells were taken as reference of 100% viable.

2.4. Clonogenic assay of cells in vitro

RT112 and RT112cp bladder cancer cell lines (2×10^3 cells/well) were plated in six-well plate and incubated for 24 h at 37 °C, before treatment with chitosan. Initial solutions of chitosan were diluted with RPMI medium (pH = 6.1) to get 500 μM of final concentration. The cells were incubated in presence of chitosan for 2 h; then, the environing medium was replaced by the RPMI medium. After incubation for 48 h at 37 °C in a humidified incubator, cultures in presence and absence of chitosan were observed using an inverted microscope at 200× magnifications (Nikon, Tokyo, Japan). The harvesting of cells was performed using trypsinization. Then, cell suspension were diluted at 1/1000 and seeded into six-well plates for clonogenic assay according to the method elaborated by Franken et al. [23]. Plates were placed in the incubator until cells in control dish have formed large clones (7 days). Colonies were then fixed and stained using a mixture of 6.0% glutaraldehyde and 0.5% crystal violet to evidence the fraction of surviving cells.

2.5. Apoptosis detection

FITC-coupled Annexin V was obtained from Miltenyi Biotec (Paris, France) and labeling of cells was performed following the manufacturer's instructions. Data acquisitions were performed with the Accuri C6 cytometer (BD Biosciences, Le Pont de Claix, France). Parameters from 2×10^4 cells were acquired using the C-Flow software. Data were analyzed with FCS Express 3 software (De Novo Software).

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