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Effects of Chitosan and Nanoparticles on Human Pancreatic Cancer Cells are Enhanced in Combination Treatments with the Chemotherapeutic Drugs

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Abstract. Chitosan has increasingly gained popularity in biomedical applications. Experimental results demonstrated that chitosan exhibited anti-microbial activities through its interaction(s) with microbial cell surface. We hypothesized that the properties of chitosan can be exploited to inhibit cancer cell proliferation and growth. In this study, we investigated the effects of chivtosan, chitosan in combination with nanoparticles (namely, nanogold and nanosilver particles), and chitosan in combinations with nanoparticles and/or three chemotherapeutic drugs (namely, Adriamycin, Methotrexate, and Cisplatin) on human pancreatic cancer PANC-1 cells. We found that chitosan, chitosan in combination with nanoparticles, and the three chemotherapeutic drugs exerted different inhibitory effects on the survival/proliferation of PANC-1 cells. The inhibitory effects of the drugs on the survival/proliferation of PANC-1 cells were greater when employed in combination with chitosan and nanoparticles. Western blot analysis revealed treatment with chitosan, chitosan in combination with nanosilver particles, and chitosan in combination with Adriamycin and nanosilver particles exerted differential effects on the expression of AKT, p-AKT, ERK, and p-ERK proteins (important cell survival/proliferation signals) in PANC-1 cells. In summary, these results suggested that chitosan and nanoparticles may have chemotherapeutic potential in the design of new and/or improved treatments for pancreatic cancer.

Keywords: chitosan, nanoparticles, chemotherapeutic drugs, pancreatic cancer.

1. Introduction

A putatively biocompatible material, chitosan is the deacetylated product of chitin and is a linear polysaccharide. Chitosan has been widely employed by researchers as an important and promising biomaterial in tissue engineering [1], wound healing [2], and drug delivery [3], because of its low cost, large-scale availability, anti-microbial activity, as well as its biodegradability and biocompatibility [4].

Amongst the most recent developments and advances in applications of nanoparticles in nanotechnology and biotechnology is the deployment of nanoparticles in cell cultures in drug discovery and drug delivery studies: these advances are considered ground-breaking in addition to the more established applications of nanoparticles as probes and in imaging [5]. Nanoparticles include all particles that possess at least one dimension that is less than 100 nm; the material origins of the particles can be organic, inorganic, metals, polymers, etc. Nanoparticles possess unique properties, more importantly, a large surface-to-volume ratio which accounts for the high surface reactivity for many of these particles. These favorable properties are

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being exploited in multiple applications, especially in recent biomedical ones [5]. Among the metal nanoparticles, nanogold and nanosilver particles have gained particular interest in biomedical applications because nanogold particles possess favorable biocompatibility and stability [6], and nanosilver particles possess antimicrobial properties [7]. Thus, it is not surprising nanoparticles such as nanogold and nanosilver may have utility in cancer therapy.

Pancreatic cancer, which is the fifth leading cause of cancer death worldwide, is a devastating disease associated with an extremely poor prognosis [8]. By the end of 2016 in the United States, it is estimated about 53,070 individuals will be diagnosed with, and 41,780 will die from pancreatic cancer [9]. Pancreatic cancer is considered one of the deadliest cancers in humans because it exhibits a very high tendency to aggressively invade the neighboring normal tissues, spreading rapidly and extensively [8]. More importantly, it has usually metastasized from the pancreas by the time it is first diagnosed as techniques for early diagnosis of pancreatic cancer is still sadly lacking [8]. Furthermore, despite the availability of various treatment modalities, such as surgery, chemotherapy and radiotherapy, median survival from diagnosis is around 3 to 6 months and 5-year survival is less than 5%. Additionally, it displays intrinsic resistance to conventional chemotherapy-radiation treatment strategies [8]. Thus, there is an urgent need to find new and/or improved treatments for pancreatic cancer [8].

Combining the knowledge of material science with that of biopharmaceutical sciences proved to be fruitful and promising in devising novel experimental strategies in discovering new and/or improved combination therapies for treating deadly cancers such as pancreatic cancer [5, 8]. These [5, 8] and other [10] considerations have prompted us to initiate the current series of studies to determine the feasibility of combining chitosan and nanoparticles with well-known chemotherapeutic agents such as Adriamycin, Methotrexate and Cisplatin in the design of new combination chemotherapies for treating pancreatic cancer. Therefore, we hypothesized that chitosan film can inhibit cancer cell growth because chitosan exhibits anti-microbial activities through its interaction(s) with microbial cell surface to alter cell permeability [11]. We also hypothesized that this inhibitory effect of chitosan is greater if we combine it with nanoparticles and/or chemotherapeutic drugs. This study, therefore, aims to investigate these hypotheses by examining the effects of chitosan, chitosan in combination with nanoparticles (namely, nanogold and nanosilver particles), and chitosan in combinations with nanoparticles and/or three chemotherapeutic drugs (namely, Adriamycin, Methotrexate, and Cisplatin) on human pancreatic cancer PANC-1 cells.

2. Materials and Methods

2.1. Chemical Regents and Antibodies

Chitosan (from shrimp shells, minimum 75% deacetylated, and molecular weight 190-375 kDa), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Adriamycin, Methotrexate, and Cisplatin were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Tetrachloroauric (III) acid ($HAuCl_4 \cdot 3H_2O$), trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and silver nitrate ($AgNO_3$) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The monoclonal antibodies against AKT, phospho-AKT (Ser473), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2) and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Goat polyclonal antibodies to rabbit IgG and rabbit polyclonal antibodies to mouse IgG were purchased from Abcam Inc. (Cambridge, MA, USA). All chemicals were of analytical grade unless otherwise stated.

2.2. Cell Culture

Human pancreatic cancer PANC-1 cells, obtained from ATCC (Manassas, VA, USA), were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and incubated at 37 ℃ and in 5 % (v/v) CO_2.

2.3. Preparation of Chitosan Films

The preparation of chitosan films was as described previously [12]. Briefly, a certain amount of chitosan was weighed and dissolved in 100 ml of 1% (v/v) acetic acid solution at room temperature overnight. The solution was filtered to remove insoluble particles in the chitosan solution, poured onto a plastic plate, and

then oven-dried at a constant temperature of 40 ℃ for 24 hours to form a solid film. The dry transparent film was carefully peeled off from the plastic plate, washed with 5% (w/v) NaOH aqueous solution until the pH reached about neutral and then repeatedly washed with distilled water. After that, the chitosan film was punched out in the form of circular sheets with ~15 mm in diameter. Subsequently, the circular membranes were sterilized in 70% (v/v) ethanol overnight and then exposed to ultraviolet light for 40 minutes on each side. Finally each circular piece of chitosan film was rinsed extensively with sterile phosphate-buffered saline (PBS) and then placed into a 24-well culture plate.

2.4. Preparation of Nanosilver and Nanogold Particles

To prepare nanosilver particles, $AgNO_3$ and $C_6H_5Na_3O_7 \cdot 2H_2O$ solutions were filtered through a 0.22 μm microporous membrane filter prior to being used for preparing nanosilver particles. Nanosilver particles were prepared according to the literature [13] by adding $C_6H_5Na_3O_7 \cdot 2H_2O$ solution to boiling $AgNO_3$ aqueous solution. The prepared concentration of nanosilver particles was about 108 μg/ml and their size was about 60 nm as characterized by scanning electron microscopy [12].

To prepare nanogold particles, $HAuCl_4 \cdot 3H_2O$ and $C_6H_5Na_3O_7 \cdot 2H_2O$ solutions also were filtered through a 0.22 μm microporous membrane filter prior to being used for preparing nanogold particles. Nanogold particles were prepared according to the literature [14] by adding $C_6H_5Na_3O_7 \cdot 2H_2O$ solution to boiling $HAuCl_4 \cdot 3H_2O$ aqueous solution. The prepared concentration of nanogold particles was about 49 μg/ml and their size was about 34 nm as characterized by transmission electron microscopy [15].

2.5. Preparation of Nanosilver/Chitosan and Nanogold/Chitosan Scaffolds

A specified amount of nanosilver or nanogold solution was added into each well of the 24-well culture plate in which a sterile chitosan film had already been placed. After 12 hours, nanosilver or nanogold solution was aspirated with residual nanoparticles attached on the film, and sterile PBS was added twice into each well to wash the film.

2.6. Cell Survival/Proliferation Assay

Cell survival/proliferation was determined by using the modified MTT assay [16]. PANC-1 cells were seeded with equal density in each well of the 24-well plates with or without specified concentrations of chitosan film, nanosilver/chitosan scaffolds or nanogold/chitosan scaffolds on the bottom of each well and cultured as described above. After 1 hour (allowing cells attached to the bottom of each well), cells were treated with or without specified concentrations of the drugs investigated (Adriamycin, Methotrexate or Cisplatin). At the end of the incubation period, 100 μL MTT dye (0.5% (w/v) in PBS) was added into each well and the plate was incubated for an additional 4 hours at 37 ℃. The purple-colored insoluble formazan crystals in viable cells were dissolved using DMSO and the subsequent absorbance (designated as X) of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA).

The chitosan, nanosilver or nanogold particles by themselves had absorbance, thus, their absorbance had to be subtracted from the absorbance of live cells with different treatments. The control sets of wells were set up alongside those sets of wells in the plates as detailed in the preceding paragraph except that the control sets of wells did not contain any seeded cells. At the end of the specified culture period, 100 μL of MTT dye (0.5% (w/v) in PBS) was added into each well and the plates were incubated for an additional 4 hours at 37 ℃. The subsequent absorbance (designated as Y) of the content of each well was measured at 570 nm as described above. (X-Y) was taken as the absorbance attributed to viable cells in each well.

2.7. Western Blot Analysis

Expression of proteins of interests was determined by Western blot analysis essentially as described previously [10]. The expression of the protein of interest was determined using the chemiluminescence technique.

2.8. Statistical Analysis

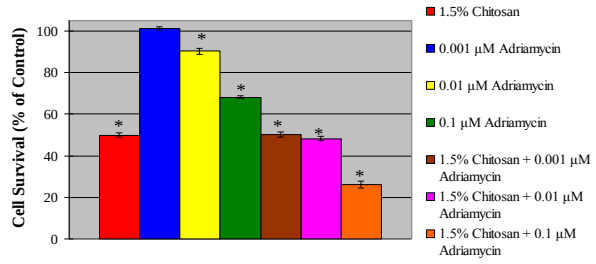
All measurements were repeated with 3 sets at a minimum of 6 samples for each set, and all data were recorded as the mean ± standard error of the mean (shown in Figures). Data analysis was carried out by one-

way analysis of variance (ANOVA), followed by post-hoc Student–Newman–Keuls test for multiple comparisons using the software KaleidaGraph version 4 (Synergy Software, Reading, PA, USA). Significance level was set at $p<0.05$.

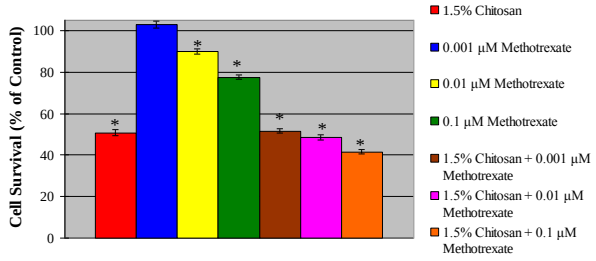
3. Results

3.1. Effects of Chitosan, Chitosan in Combination with Different Concentrations of Chemotherapeutic Drugs on Survival of PANC-1 Cells

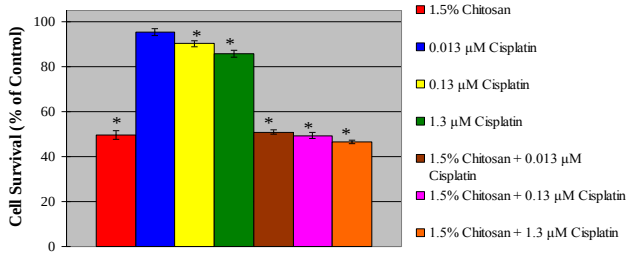
When PANC-1 cells were treated with chitosan for 72 hours, their survival/proliferation decreased to 50% of that of the untreated PANC-1 cells (Fig. 1). Exposure of PANC-1 cells for 72 hours to either Adriamycin or Methotrexate alone at concentrations at or below 0.01 μM did not affect their survival/proliferation (Fig. 1A and 1B). However, at 0.1 μM, Adriamycin and Methotrexate induced 32% and 23% decreases in PANC-1 cell survival/proliferation, respectively (Fig. 1A and 1B). Even at the highest concentration (1.3 μM) we studied, Cisplatin only lowed PANC-1 cell survival/proliferation by less than 15% compared to that of the untreated cells (Fig. 1C), indicating that at the concentration range employed, Cisplatin was less effective than Adriamycin and Methotrexate in lowing the survival/proliferation of PANC-1 cells; the rank order of the effectiveness of the three drugs in lowering the survival/proliferation of PANC-1 cells was: Adriamycin > Methotrexate > Cisplatin. Combining drugs with chitosan enhanced the inhibitory effects on survival/proliferation of PANC-1 pancreatic cancer cells.



(A)



(B)



(C)

Fig. 1: Effects of 1.5% chitosan, different concentrations of Adriamycin, Methotrexate, Cisplatin, and 1.5% chitosan in combination with different concentrations of Adriamycin, Methotrexate, Cisplatin on survival of PANC-1 cells.

3.2. Effects of Chitosan, Chitosan in Combination with Nanoparticles and/or 0.1 μM Adriamycin on Survival of PANC-1 Cells

Because Adriamycin was the most effective among the three drugs investigated in lowering the survival/proliferation of PANC-1 cells (Fig. 1), we chose it for further study in combination treatments. We therefore examined the effects of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM

Adriamycin on survival/proliferation of PANC-1 cells in culture for up to 14 days. As shown in Fig. 2, all the treatments induced decreases in the survival/proliferation of PANC-1 cells. After 14 days, chitosan with nanosilver and 0.1 μm Adriamycin was the most effective treatment combination in inhibiting PANC-1 cell survival/proliferation: there were almost no live cells after this treatment on the 14th day of culture (Fig. 2). Chitosan with nanosilver was the second most effective combination treatment in lowering the survival/proliferation of PANC-1 cells (Fig. 2). Chitosan alone was the least effective treatment in lowering the survival/proliferation of PANC-1 cells: nevertheless, it still exerted significant inhibitory effect on the cells (Fig. 2). On the other hand, treatment with chitosan and nanosilver particles showed greater effect than treatment with chitosan and nanogold particles, indicating that nanosilver particles were more cytotoxic to PANC-1 cells than nanogold particles (Fig. 2).

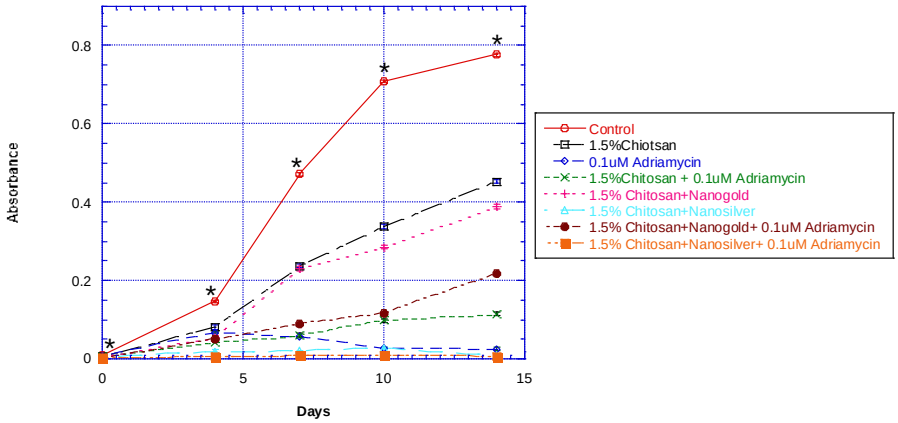


Fig. 2: Effects of 1.5% chitosan and 1.5% chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of PANC-1 cells.

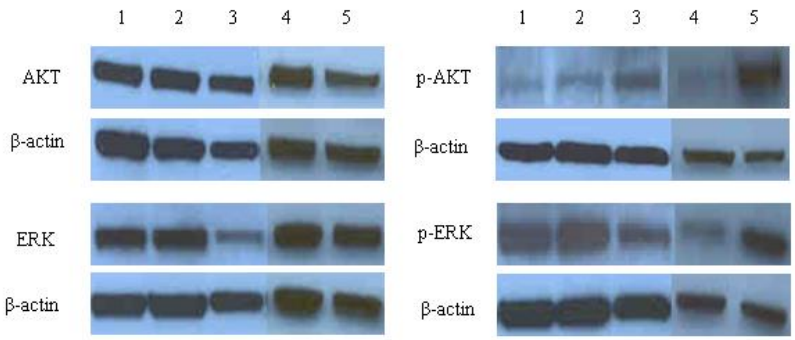


Fig. 3: Effects of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on the AKT, p-AKT, ERK, and p-ERK protein expression in PANC-1 cells. Lane 1, lysate of control PANC-1 cells; lane 2, lysate of PANC-1 cells treated with 1.5% chitosan; lane 3, lysate of PANC-1 cells treated with 1.5% chitosan in combination with nanosilver particles; lane 4, lysate of PANC-1 cells treated with 1.5% chitosan in combination with 0.1 μM Adriamycin; lane 5, lysate of PANC-1 cells treated with 1.5% chitosan in combination with nanosilver particles and 0.1 μM Adriamycin.

3.3. Effects of Chitosan, Chitosan in Combination with Nanoparticles and/or 0.1 μM Adriamycin on the AKT, p-AKT, ERK, and p-ERK Protein Expression in PANC-1 Cells

To further elucidate the molecular mechanisms underlying the effects of the combination drug treatments on PANC-1 cells, we examined the expression of cell survival signaling pathways (AKT and ERK signaling pathways) after the cells had been exposed to the treatments for 72 hours. The total AKT in PANC-1 cells remained almost unchanged after all treatments administered (Fig. 3). Treatment of PANC-1 cells with chitosan alone induced an increase in their expression of p-AKT, but their increases in p-AKT expression

were more pronounced when treated with chitosan combined with nanosilver particles or treated with chitosan combined with both nanosilver particles and 0.1 μM Adriamycin (Fig. 3). However, the effects of these treatments on the protein expression of ERK signaling pathway were different from the effects on the AKT signaling pathway in PANC-1 cells (Fig. 3). The combined treatment of chitosan with nanosilver particles resulted in a decrease in total ERK expression in PANC-1 cells (Fig. 3). Phosphorylated ERK protein expression was decreased when PANC-1 cells were treated with chitosan in combination with 0.1 μM Adriamycin. Treatment of these cells with chitosan alone and chitosan in combination with nanosilver particles and 0.1 μM Adriamycin led to an increase in their p-ERK expression. On the other hand, treatment of these cells with chitosan in combination with nanosilver particles did not induce any changes in their p-ERK expression (Fig. 3).

4. Discussion

To our knowledge this study is the first to report on the effects of chitosan and nanosilver and nanogold particles, with and without the combination treatment with chemotherapeutic drugs (namely, Adriamycin, Methotrexate, and Cisplatin) on human pancreatic cancer PANC-1 cells. In particular, this study investigated our hypothesis that the anti-cancer property of chitosan is enhanced if it is employed in combination treatment with nanogold or nanosilver particles and/or anti-cancer drugs. In accord with our hypothesis, we found chitosan and the nanoparticles exerted some anti-survival/proliferative effects on PANC-1 cells and the presence of the chemotherapeutic drugs tested markedly enhanced the anti-survival/proliferative effects of chitosan and the nanoparticles on PANC-1 cells (Fig. 1 and 2).

Treatment with chitosan alone lowered the survival/proliferation of PANC-1 pancreatic cancer cells (Fig. 1 and 2). While treatment with Adriamycin, Methotrexate or Cisplatin alone at concentrations tested below 0.1 μM did not significantly affect the survival/proliferation of PANC-1 cells (Fig. 1), at treatment concentration of 0.1 μM or higher, all three chemotherapeutic drugs, when used alone, induced significant decreases in the survival/proliferation of PANC-1 cells, with the rank order of Adriamycin > Methotrexate > Cisplatin (Fig. 1). Moreover, 1.5% chitosan in treatment combination enhanced this inhibitory effect of the three drugs (especially that of Adriamycin) in PANC-1 cells (Fig. 1). Additionally, in combination treatments with chitosan, both nanogold and nanosilver particles further enhanced the inhibitory effect of Adriamycin on the survival/proliferation of PANC-1 cells (Fig. 2).

Our observation that the combination inhibitory effects of chitosan, Adriamycin and nanosilver particles on the survival/proliferation of PANC-1 cells (Fig. 1 and 2) prompted us to investigate some of the cell survival/proliferation signaling mechanisms underlying the effects of chitosan, chitosan in combination with nanosilver particles, and chitosan in combination with nanosilver particles and Adriamycin on PANC-1 cells. Since AKT and ERK signaling pathways play a major role in survival and proliferation of many types of cells including cancer cells [8,10], we determined the effects of these treatments on the protein expression of the two signaling pathways in human pancreatic cancer PANC-1 cells by Western blot analysis. We found that when PANC-1 cells were treated with chitosan in combination with 0.1 μM Adriamycin, phosphorylated ERK protein expression was decreased, which contributed to the inhibition of proliferation. However, chitosan alone and chitosan in combination with nanosilver particles and 0.1 μM Adriamycin induced increases in the expression of p-AKT and p-ERK in PANC-1 cells (Fig. 3). It is of interest to note that the combined treatment of PANC-1 cells with chitosan and nanosilver particles resulted increases in the expression of p-AKT but did not induce any changes in their p-ERK expression (Fig. 3). Nevertheless, phosphorylated ERK protein expression was decreased when PANC-1 cells were treated with chitosan in combination with 0.1 μM Adriamycin whereas treatment of these cells with chitosan and chitosan in combination with nanosilver particles and 0.1 μM Adriamycin led to an increase in p-ERK expression (Fig. 3). Consequently, our observations could not be explained simply by assuming that the inhibitory effect on proliferation of PANC-1 cells could only be correlated with the reduction of p-AKT and p-ERK protein levels in the cells [17-19]. The activation of the apoptotic pathways by the combination treatment may play a role here in PANC-1 cells. It suffices to say only future studies will clarify these mechanistic issues.

5. Acknowledgment

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