Effect of Chitosan and Nanoparticles on Cancer Cells Treated with Therapeutic Drugs: Comparison of Cell Survival

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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 growth. In this study, we investigated the effects of chitosan, chitosan in combination with nanoparticles (nanogold and nanosilver particles), and chitosan in combinations with nanoparticles and/or three therapeutic drugs (Adriamycin, Methotrexate, and Cisplatin) on human brain glioblastoma U87 cells, human pancreatic cancer PANC-1 cells; the results were compared with results of similar treatment on normal human fibroblast BJ cells.

We found that chitosan, chitosan in combination with nanoparticles, and the three therapeutic drugs had different inhibition effects on the growth of these three cell types as indicated by MTT assay. The inhibition effects of drugs were greater when combined with chitosan and nanoparticles. Taken together, these results suggested that chitosan and nanoparticles may have chemotherapeutic potential in the design of new treatments for glioblastoma and pancreatic cancer.

1. INTRODUCTION

Chitosan is the deacetylated product of chitin and is a linear polysaccharide composed of randomly distributed β-(1, 4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). As a biocompatible material, chitosan has been widely used by researchers as an important and promising biomaterial in
tissue engineering (Pok et al. 2013, Sionkowska and Planecka 2013), wound healing (Wijekoon et al. 2013, Khodja et al. 2013), and drug delivery (Chronopoulou et al. 2013, Huang et al. 2013) because of its low cost, large-scale availability, anti-microbial activity, as well as biodegradability and biocompatibility (Khor and Lim 2003).

Recently employing nanoparticles in cell cultures for treatments and therapeutic uses is among the newest developments other than probing and imaging in nanotechnology and biotechnology (Gao et al. 2012). 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 lead to high surface reactivity for many of these particles. These favorable properties are being exploited in many directions in science and technology, more so recently in biomedical applications (Gao et al. 2012). Among the metal nanoparticles, nanogold and nanosilver particles have gained particular interest in biomedical applications because nanogold particles possess favorable biocompatibility and stability (Gu et al. 2004) and nanosilver particles possess antimicrobial properties (Chen and Schluesener 2008).

Glioblastoma is the most common primary brain tumors in adults. It is the highest grade (grade IV as defined by the World Health Organization) astrocytoma and is characterized by increased proliferation and invasion into the surrounding normal tissues (Behin et al. 2003, Dutra-Oliveira et al. 2012). Despite some recent improvement in the treatment of this malignancy, the prognosis of patients with glioblastoma remains extremely poor. Over the past 30 years, the median survival time of patients with malignant glioblastoma has been improved from 6 months to 14.6 months (Arko et al. 2010). Pancreatic cancer, which is the fifth leading cause of cancer death worldwide, is a devastating disease associated with an extremely poor prognosis (Jemal et al. 2011). By the end of 2013 in the United States, it is estimated about 45,220 individuals will be diagnosed, and 38,460 will die from the disease (Siegel et al. 2013). It is considered one of the most deadly cancers in humans because it is prone to aggressively invading surrounding tissue, spreading rapidly and extensively and it has usually metastasized when it is diagnosed (Guzman et al. 2009). 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%. Moreover, it displays intrinsic resistance to conventional chemo-radiation treatment strategies (Duffy et al. 2003). Therefore, there is an urgent need to find new treatments directed to glioblastoma and pancreatic cancer.

Combinations of material science in biological field proved to be fruitful and promise to hold great potential in biomedical developments (Gao et al. 2012). In our previous studies, we found that chitosan film and chitosan in combination with nanoparticles could promote wound healing (Lu et al. 2008) and proliferation of keratinocytes (Zhang et al. 2009, Lu et al. 2010). 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 (Raafat et al. 2008). We also hypothesized that the inhibition effect is greater if we combine chitosan with nanoparticles and/or therapeutic drugs.

The objectives of this study were to evaluate the effects of chitosan, chitosan in combination with nanoparticles (nanogold and nanosilver particles), and chitosan in
combinations with nanoparticles and/or three therapeutic drugs (Adriamycin, Methotrexate, and Cisplatin) on human brain glioblastoma U87 cells and human pancreatic cancer PANC-1 cells; the results were compared with results of similar treatment on normal human fibroblast BJ cells by MTT assay.

Adriamycin, Methotrexate and Cisplatin are three widely used drugs in cancer chemotherapy: Adriamycin is one of the most frequently used anticancer agents utilized in the treatment of a variety of cancers including small cell lung cancer, breast cancer, sarcoma, lymphoma, and acute leukemia; Methotrexate is an antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases; Cisplatin is used to treat various types of cancers including sarcomas, some carcinomas, lymphomas, and germ cell tumors.

2. MATERIALS AND METHODS

2.1 Chemical reagents 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 (HAuCl4•3H2O), trisodium citrate (C6H5Na3O7•2H2O) and silver nitrate (AgNO3) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The monoclonal antibody 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 to rabbit IgG and rabbit polyclonal to mouse IgG were purchased from Abcam Inc. (Cambridge, MA, USA). All chemicals were of analytical grade unless otherwise stated.

2.2 Preparation and dilution of stock solution of drugs

Stock solution of 10 mM Adriamycin or Methotrexate was prepared with DMSO and further dilutions (0.001, 0.01 and 0.1 μM) were prepared with medium. Cisplatin stock solution of 13 μM was prepared with DMSO, and further diluted solutions (0.013, 0.13 and 1.3 μM) were prepared with medium. The stock solutions were sterilized and filtered using a 0.22 μm microfilter under laminar flow hood and stored in a freezer. All dilutions were prepared fresh before addition to the cells.

2.3 Cell culture

The human astrocytoma U87 cells obtained from ATCC (Manassas, VA, USA) were cultured in modified Eagle's medium (MEM) supplemented with 10% (v/v) FBS and incubated at 37°C and 5% CO2; the human pancreatic cancer PANC-1 cells obtained from ATCC were cultured in RPMI 1640 medium supplemented with 10% FBS (v/v) and incubated at 37°C and 5% CO2; the human foreskin fibroblasts BJ cells obtained from ATCC were cultured in MEM supplemented with 10% (v/v) FBS and incubated at 37°C and 5% CO2.
2.4 Preparation of chitosan films

Chitosan of 0.25, 0.5 or 1.5% (by weight) was weighed and dissolved in 100 ml 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°C 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 pH reached about neutral and then repeatedly washed with distilled water. After that, the chitosan film was punched out in the form of circles 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.

2.5 Preparation of nanosilver, nanogold particles

To prepare nanosilver particles, AgNO$_3$ and C$_6$H$_5$Na$_3$O$_7$•2H$_2$O solutions were filtered through a 0.22 µm microporous membrane filter, and nanosilver particles were prepared according to the literature (Kamat et al. 1998) by adding C$_6$H$_5$Na$_3$O$_7$•2H$_2$O solution to boiling AgNO$_3$ aqueous solution, concentration of Ag nanoparticles was about 108 µg/ml, their size was about 60 nm.

To prepare nanogold particles, HAuCl$_4$•3H$_2$O and C$_6$H$_5$Na$_3$O$_7$•2H$_2$O solutions also were filtered through a 0.22 µm microporous membrane filter prior to use. Nanogold particles were prepared according to the literature (Turkevich et al. 1951) by adding C$_6$H$_5$Na$_3$O$_7$•2H$_2$O solution to boiling HAuCl$_4$•3H$_2$O aqueous solution, concentration of Au nanoparticles was about 49 µg/ml, their size was about 30 nm.

2.6 Preparation of nanosilver/chitosan and nanogold/chitosan scaffolds

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 and sterile PBS was added into each well to wash the film.

2.7 Cell viability assay

Cellular viability was determined by using the MTT assay (Mossman, 1983). Cells were seeded with equal density in each well of the 24-well plate and cultured as described in the previous cell culture subsection. At the end of the incubation period, MTT dye (0.5%, w/v, in PBS) was added to each well and the plate was incubated for an additional 4 hours at 37°C. The purple-colored insoluble formazan crystals in viable cells were dissolved using dimethyl sulfoxide (DMSO) and the subsequent absorbance of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA) (Dukhande et al. 2006).
2.8 Statistical analysis

All measurements were repeated with 3 sets at a minimum of 6 samples of each set, and all data were recorded as mean ± standard error of the mean (shown in figures).

3. RESULTS AND DISCUSSION

3.1 Effect of chitosan, chitosan in combination with different concentrations of therapeutic drugs on survival of U87 cells

We liked to first establish how the chitosan alone would affect the cell culture of U87 cells. Fig. 1(A) showed the absorbance, thus the cell survival, of U87 cells growth on a plate in 72 hours vs. the same culture grown with the same setup except having a layer of chitosan at the bottom of the plate. As shown, various concentrations of chitosan film exhibited differential growth inhibition of U87 cells. In fact, low concentration of chitosan (0.25%) had the highest inhibitive effect, while the higher concentrations (0.5 and 1.5%) showed more moderate inhibition. It is yet to be able to rationally explain why lower concentration (0.25%) of chitosan film exhibit higher growth inhibition to U87 cells, but chitosan film would generate inhibition effect to U87 cell-growth is unquestionable and the effect of 0.5 and 1.5% chitosan film was nearly negligible. Once the chitosan effect to U87 cells was established (Fig. 1(A)), it was decided that the 1.5% chitosan would be used to carried out the combined effect of chitosan with drugs and/or with nanoparticles to the target cells, due to its structural advantage and ease of handling.

![Graph showing the effect of chitosan on U87 cells at different concentrations in 72 hour culture](image-url)
Fig. 1(B) Effect of chitosan, Adriamycin, and chitosan with different concentrations of Adriamycin on U87 cells in 72 hour culture

Fig. 1(C) Effect of chitosan, Methotrexate, and chitosan with different concentrations of Methotrexate on U87 cells in 72 hour culture

Fig. 1(D) Effect of chitosan, Cisplatin, and chitosan with different concentrations of Cisplatin on U87 cells in 72 hour culture
As shown in Fig. 1, chitosan induced approximately 50% decrease in survival of U87 cells. Exposure of U87 cells for 72 hours to both Adriamycin and Methotrexate at concentration below 0.01 μM did not affect their survival as determined by the MTT assay (Figs. 1(B) and (C)).

However, at concentration of 0.1 μM, both Adriamycin and Methotrexate induced nearly 50% decrease in survival of U87 cells. Cisplatin showed less inhibitive effect on U87 cells. At the highest concentration (1.3 μM) we studied, U87 cell survival decreased less than 20% of those of untreated cells (i.e., control) (Fig. 1(D)). The rank order of the effects of the three drugs was: Adriamycin > Methotrexate >> Cisplatin.

All three drugs enhanced the inhibitive effect when used in combination with chitosan; Adriamycin in combination with chitosan was the most effective among the three drugs. Thus, we used chitosan (1.5%) in combination with 0.1 μM Adriamycin to conduct subsequent experiments to observe the total inhibitive effect of chitosan, Adriamycin, and nanoparticles on all the cell types in this study.

3.2 Effect of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of U87 cells

We examined the effects of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of U87 cells up to 14 days. As shown in Fig. 2, all the treatments induced decreases in cell survival of U87 cells. There were apparent differences in the effects exerted by different treatments. After being treated for 14 days, chitosan with nanosilver and 0.1 μm Adriamycin was the most effective. Chitosan with nanogold and 0.1 μm Adriamycin was the second most effective; chitosan alone was the least effective. Therefore, these results suggested that a combination of chitosan, nanoparticles, and Adriamycin was more effective than chitosan, and Adriamycin alone,
or chitosan with nanoparticles. Chitosan with nanosilver particles showed greater effect than chitosan with nanogold particles indicating that nanosilver particles were more cytotoxic to U87 cells than nanogold particles.

3.3 Effect of chitosan, chitosan in combination with different concentrations of therapeutic drugs on survival of PANC-1 cells

In order to assess if the effects of chitosan and the three therapeutic drugs on U87 cells were similar to other cancer cells, we also explore the effects of chitosan and these three therapeutic drugs on human pancreatic cancer PANC-1 cells. When PANC-1 cells were treated with chitosan for 72 hours, their survival decreased to 50% of that of untreated (i.e., control) PANC-1 cells (Fig. 3).

Exposure of PANC-1 cells for 72 hours to either Adriamycin or Methotrexate at concentration below 0.01 \( \mu \text{M} \) did not affect their survival (Figs. 3(A) and (B)). However,
Fig. 3(C) Effect of chitosan, Cisplatin, and chitosan with different concentrations of Cisplatin in 72 hour culture

at concentration 0.1 μM, Adriamycin and Methotrexate induced 32% and 23% decreases in PANC-1 cell survival, respectively. For Cisplatin, at the highest concentration (1.3 μM) we studied, PANC-1 cell survival decreased less than 15% compared to those of the untreated cells (control) (Fig. 3(C)), indicating that at the concentration range employed, Cisplatin was less effective than Adriamycin and Methotrexate in lowering the survival of PANC-1 cells; the ranking order of the effectiveness of the three drugs was: Adriamycin > Methotrexate > Cisplatin. Combining drugs with chitosan enhanced the inhibition effect to PANC-1 (pancreatic cancer) cells, the effect was similar to those of U87 cells (brain glioblastoma), and the combination effect was approaching to the linear “additive” scenario.

Fig. 4 Effect of chitosan and chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on PANC-1 cells
3.4 Effect of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of PANC-1 cells

We also examined the effects of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of PANC-1 cells up to 14 days. As shown in Fig. 4, all the treatments induced decreases in cell survival of PANC-1 cells. After 14 days, chitosan with nanosilver and 0.1 μM Adriamycin was the most effective in inhibition of PANC-1 cell-growth: there were almost no live cells after treatment at 14 days. Chitosan with nanosilver was the second most effective treatment; chitosan alone was the least effective treatment nevertheless showed significant inhibition. Chitosan with nanosilver particles showed greater effect than chitosan with nanogold particles indicating that nanosilver particles were also more cytotoxic to PANC-1 cells than nanogold particles to PANC-1 cells. It also showed that treatment of chitosan in combination with nanosilver and 0.1 μM Adriamycin was more effective on inhibition of PANC-1 cells than on U87 cells (Fig. 4 with Fig. 2).

3.5 Effect of chitosan, chitosan in combination with different concentrations of therapeutic drugs on survival of BJ cells

To determine if the effect of chitosan and the three therapeutic drugs on cancer cells were similar to normal cells, we further examined the effects of chitosan and these three therapeutic drugs on the normal human fibroblast BJ cells. As shown in Fig. 5, exposure of BJ cells to chitosan alone induced approximately 70% decrease in cell survival, which implies that chitosan alone had higher inhibition effect to the normal human fibroblast than the previous human brain glioblastoma (U87) and pancreatic cancer cells (PANC-1). Otherwise, these three therapeutic drugs exerted similar differential inhibitory effects on the growth of BJ cells as were on the previous cell types. The ranking order of the inhibitive effect of the three drugs was Adriamycin > Methotrexate > Cisplatin, and the inhibitory effects of the drugs were enhanced when the cells were treated with drugs in combination with chitosan, as were the previous two cell types.

Fig. 5(A) Effect of chitosan, Adriamycin, and chitosan with different concentrations of Adriamycin in 72 hour culture
3.6 Effect of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of BJ cells

In contrast to the results of U87 and PANC-1 cells, the normal human fibroblast BJ cells reacted differently with the treatments in the prolonged 14 day culture. As shown in Fig. 6, either chitosan or chitosan with nanogold particles exhibited any stress to the growth of BJ cells after 14 days; chitosan has depressed the cell growth initially as compared to the control, but the cells recovered rapidly after one week of acclimation time and the addition of nanogold particles helped the cells recover more rapidly, implying that the nanogold particles simulated growth of BJ cells under our experimental conditions which was not the case for Ag nanoparticles.
Adriamycin alone suppressed BJ cell-growth as it would to the cancer cells, except that its effect to the normal human fibroblast was not as substantial. When Adriamycin was paired with chitosan, the effectiveness of growth depression was drastically enhanced, but depression enhancement alleviated when Adriamycin was paired chitosan and Au nanoparticles. This result was consistent with the earlier observation that Au nanoparticles stimulated BJ cell-growth.

Ag nanoparticles suppressed cell-growth in general. It was most effective when Ag nanoparticles were used in combination with chitosan and Adriamycin, the cells showed almost no growth after 14 days as compared to those in the control culture.

4. CONCLUSIONS

In this study we demonstrated the anti-proliferative effect of chitosan and nanoparticles on the human brain glioblastoma U87 cells and human pancreatic cancer PANC-1 cells. The combination of chitosan with nanoparticles (Au and Ag) and/or the three therapeutic drugs resulted in a greater inhibitory effect, Adriamycin was the most effective among the three drugs. Thus, when chitosan and nanoparticles were used in combination with drugs in treatment, the approach may enable lower dosage of drugs to be used, hence reduced toxicity.

We also found that chitosan with nanosilver particles showed greater inhibitory effect than chitosan with nanogold particles on the growth of the two cancer cell types investigated; more importantly, nanogold particles stimulated cell-growth for normal human fibroblast BJ cells indicating good biocompatibility. Chitosan alone slightly suppressed normal human fibroblast BJ cells in the initial acclimation period but would stimulate cell-growth in the prolonged cell culture of 14 days.
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