Original Article

Nano - drug Delivery of Apoptosis Activator 2 to AGS Cells by Liposomes Conjugated with Anti-TROP2 Antibody

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Abstract

Background: Gastric cancer is the second most common causes of cancer related death in the world and is responsible for two third of cancer related death in the developing countries. survival rate surgery is low and radiation therapy and chemotherapy as alternatives ways for treatment of gastric cancer are not very promising. Thus there is an urgent need for introducing novel treatment procedures and promising new anti-canceric drugs. **Aim:** In this study we used pre-prepared liposomes and after necessary manipulations ,these modified liposomes were used for delivery of apoptosis activator 2 to gastric adenocarcinoma cell line (AGS). **Materials and Methods:** we used pre-prepared liposomes and after necessary manipulations, these modified liposomes were used for delivery of apoptosis activator 2 to AGS cells and induced apoptosis was evaluated by related apoptotic DNA ladder, TUNNEL and Cell Death experiments. **Results:** Evaluation of apoptosis by Apoptotic DNA Ladder in liposome treated and untreated AGS cells by DNA laddering and fragmentation, TUNEL and Cell Death Detection confirmed that treatment of AGS cell lines with apoptosis activator 2 loaded liposomes which targeted cell surface TROP2 antigen in cancer cells significantly increased apoptosis in these cells. **Conclusion:** Nano drug delivery of apoptosis activator 2 to human gastric adenocarcinoma cell line with liposomes targeted TROP2 antigen is a possible way for smart killing of human gastric adenocarcinoma cells.

Keywords: Human Stomach Adenocarcinoma, Apoptosis, Cancer, Liposome, Tumor-associated calcium signal transducer 2

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Introduction

Gastric cancer is the second most common cause of cancer-related deaths in the world[1] and is responsible for two-thirds of cancer-related deaths in the developing countries.[2] Although surgery is one of the most common methods of gastric cancer treatment, its survival rate is less than 33%. Radiation therapy and chemotherapy as alternatives for surgery in the treatment of gastric cancer are not very promising. Thus, there is an urgent need for introducing novel treatment procedures and promising new anti-cancer drugs.[1] Recently, new treatment methods for gastric cancer have been proposed,[3-5] and among them targeted drug delivery system decreases adverse side effects of radiation therapy and chemotherapy and seems to be an attractive treatment alternative.[5] Liposomes are central key points in this kind of drug delivery systems. Liposomes have attracted considerable attention as drug delivery carriers because of their biocompatible and non-toxic nature which protects their cargo from degradation by plasma enzymes, and transports their load through biological membranes.[6,7] Apoptosis as a natural human body protecting phenomenon is the center of attention of a recent research.[8,9] Apoptosis activator 2 is one of the known apoptosis activator agents[10-12] and transferring of this compound to the cancer cell will induce apoptosis, which leads to cell death and may be used in the treatment of cancer. Adenocarcinoma gastric cells bearing different antigenic markers on their cell surface including Tumor-associated calcium signal transducer 2 (TROP2). Recent studies showed a supporting role for TROP2 signaling network in the growth of cancer.[13,14]

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As preparation of liposomes from their primary components demands specialized and expensive instruments, and synthesis of such liposomes may not be possible in many laboratories, in this study, we used commercially prepared empty liposomes. Provided empty liposomes were loaded with an apoptosis activator 2 and anchored with a biotinylated phosphatidyl ethanolamine. After conjugation with a biotinylated TROP2 antibody and attachment via avidin, cultured human Stomach Adenocarcinoma (AGS cells) was treated with these prepared, loaded, anchored, and targeted liposomes and induction of apoptosis was evaluated in these cells.

Materials and Methods

Coupling of biotin to antibody

TROP2 antibody (Abcam, UK) was dissolved in sodium bicarbonate buffer, 10 µl of biotin solution was added, maintained at room temperature in darkness for 4 hours and centrifuged in a spin filter at 12000*g* for 30 minutes. Absorbance of upper solution was measured at 354 and 280 nm for measuring molecular substitution ratio.[15,16] Creation of drug-encapsulated liposomes from pre-made empty liposomes. About 200 µl of apoptosis activator 2 solution (10 µM)[17] was added to a prepared liposome vial, kept at room temperature for 4 hours and after addition of double distilled water, the solution was agitated for 30 minutes.[18,19] Biotinilation of drug-encapsulated liposomes with biotinilated phosphatidyl ethanolamine: One ml of biotinilated phosphatidyl ethanolamine was added to chloroform and the solution was evaporated under rotary evaporator, and 1 ml of apoptosis activator 2 liposomes was added to this solution.[15] Conjugation of antibodies to liposomes. About 100 µl of biotinilated antibody was added to avidin solution (2 µg/ml) and after spin filter at 12000*g* for 30 minutes, it was added to the prepared apoptosis activator 2-loaded liposomes solution.[15]

Exposure of AGS to immno-liposomes

AGS cells obtained from Iranian Pasteur Institute (C131) in RPMI 1640 with 10% FBS and after subculture, 1×10^4 AGS cells were seeded to each well of 12-well cell culture plates (Falcon, USA) containing 2 ml RPMI 1640 with 10% of FBS and 10% of anti-anti antibiotic antimycotic solution (Gibco, Glasgow, UK) and after 72 hours, supernatant of the wells was removed and the cells were washed twice with PBS and 1% FBS, incubated over-night in 2 ml RPMI 1640 supplemented with 1% FBS and 15 µl of different concentration of selenite sodium, unconjugated and conjugated liposomes and sterile double distilled water as negative control. After 24 hours, supernatant of the wells was removed, their cells were washed twice with PBS and resuspended by adding trypsin /EDTA (Gibco, Glasgow, UK).[20,21] After centrifugation, the pellet cells were resuspended in 1 ml of HPSS salt solution and its volume was increased to 10 ml with 70% ethanol. The suspension was maintained at –20°C till the time of evaluating experiments.[22]

Evaluation of apoptosis by apoptotic DNA ladder

Evaluation of apoptosis by apoptotic DNA ladder was done by apoptotic DNA ladder kit according to its manual (Roche, Germany). Briefly, one of the 15 ml tubes containing AGS-treated cells preserved in 70% ethanol was removed from freezer and after thawing, centrifuged at 200*g* for 10 minutes. Sediment was resuspended in 1 ml culture media containing 1% FBS and centrifuged at 1500*g* for 5 minutes. The pellet cells, resuspended in 200 µl of PBS and 200 µl of Binding/Lysis Buffer supplied with the Kit was added to the cell suspension and after incubation, addition of isopropanol, centrifugation and subsequent washing, resultant DNA was dissolved in 200 µl of Kit's elution buffer. Positive control of the kit was used as positive control in Gel electrophoresis of DNA. Gel electrophoresis was done in a 2% gel and stained with SYBER Green I Nucleic Acid Gel Stain.

Evaluation of apoptosis by cell death detection ELISA

Evaluation of apoptosis was done by cell death detection ELISA kit according to its manual (Roche, Germany). Briefly, one of the 15 ml tubes containing AGS-treated cells preserved in 70% ethanol was removed from freezer and after thawing, centrifuged at 200*g* for 10 minutes. Sediment was resuspended in 1 ml culture media containing 1% FBS and centrifuged at 1500*g* for 5 minutes. The pellet cells were resuspended in 500 µl of kit's incubation buffer and incubated for 30 minutes at room temperature. The suspension was centrifuged at 200*g* for 10 minutes and after incubation, following washing steps, conjugation, and addition of substrate and further washing steps, the absorbance of suspension was measured at 405 nm with a correction at 490 nm.

Evaluation of apoptosis by *in situ* Cell Death Detection Kit, Fluorescein (TUNEL)

Evaluation of apoptosis by *in situ* Cell Death Detection Kit (TUNEL) (Roche, Germany) was done according to the manual of the kit. Briefly, one of the 15 ml tubes containing AGS-treated cells preserved in 70% ethanol was removed from freezer and after thawing, centrifuged at 200*g* for 10 minutes. Sediment was resuspended in 1 ml culture media containing 1% FBS and centrifuged at 1500*g* for 5 minutes. The pellet cells, were resuspended in PBS to the final concentration of 2×10^7 cells/ml, and 100 µl from this cell suspension was transferred into a V-bottom-shaped 96-well micro-plate. After addition of fixation solution and subsequent incubation, the plate was centrifuged at 300*g* for 10 minutes and after washing with PBS, the cells were resuspended in 100 µl of permeabilization solution for 2 minutes on ice. After washing with PBS, 50 µl of TUNEL reaction mixture was added. For negative control, (untreated AGS cells) only 50 µl of labeling solution was added. Non-treated, fixed and permeabilized cells incubated with DNaseI recombinant for 10 minutes at room temperature were used as positive control. After incubation in darkness, washing and transferring cells into 250 µl of PBS, samples were directly analyzed under Olympus ×7 fluorescent microscopes with WIB filter.

Results and Discussion

Induction of apoptosis by apoptosis activator 2 in AGS cells

Evaluation of apoptosis by apoptotic DNA ladder in liposome-treated and untreated AGS cells showed that DNA laddering and fragmentation were observed in cells treated with apoptosis activator 2-loaded liposomes [Figure 1]. For evaluation of anti-canceric properties of pre-prepared liposomes, they were loaded with apoptosis activator II and for illustration of cell death by necrosis in comparison with apoptosis, necrotic concentration of selenite sodium were used. DNA laddering which is one of the characteristics of apoptosis is obvious in line 6 and 7 and necrotic effect of 4 and 2.5 mM of selenite sodium is seen in line 2 and 3 of Figure 1.

Also, TUNEL test in AGS cells treated with 10 µM of apoptosis activator 2/ml of RPMI 1640 culture media supplemented with 1% of BSA showed a significant increase in apoptosis in comparison with non-treated AGS cells and selenite sodium control concentration (2.5 and 4 mM) which causes necrosis instead of apoptosis as seen in AGS cells[23] [Figure 2].

As it is shown in Figure 2, after 24 hours of treatment, maximum induction of apoptosis was achieved in wells that were treated by 4 mM selenite sodium and conjugated liposomes, but after 72 hours, although the amount of apoptosis increased in wells treated with conjugated liposomes, apoptosis and necrosis replaced each other in 4 mM selenite sodium- treated wells and within this time, in induction of apoptosis, the second place after conjugated liposomes was occupied by 1.5 mM concentration of selenite sodium. Analysis of apoptosis by Quantify Nucleosomes in cell cultures with the cell death detection ELISA showed that AGS cells treated with 10 µM of apoptosis activate 2/ml of RPMI 1640 culture media supplemented with 1% of BSA showed a significant increase in apoptosis in comparison with non-treated AGS cells. Also, in this experiment,

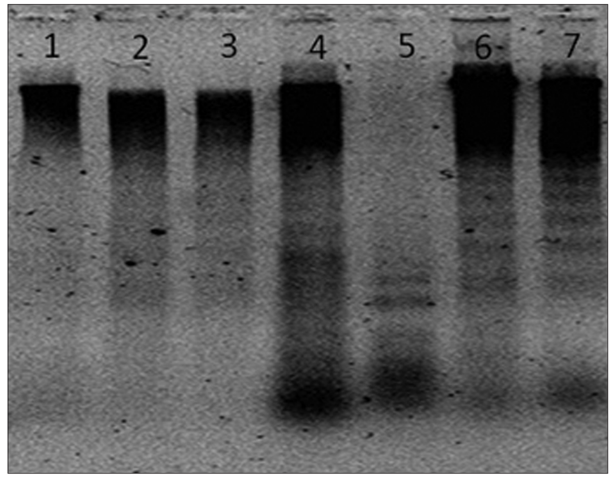

Figure 1: Line 1: Untreated AGS cells. Line 2 and 3: 4 and 2.5 mM selenite sodium. Line 4: Control positive of the kit. Line 6 and 7: TROP2 conjugated liposomes loaded with apoptosis activator of double-check experiments

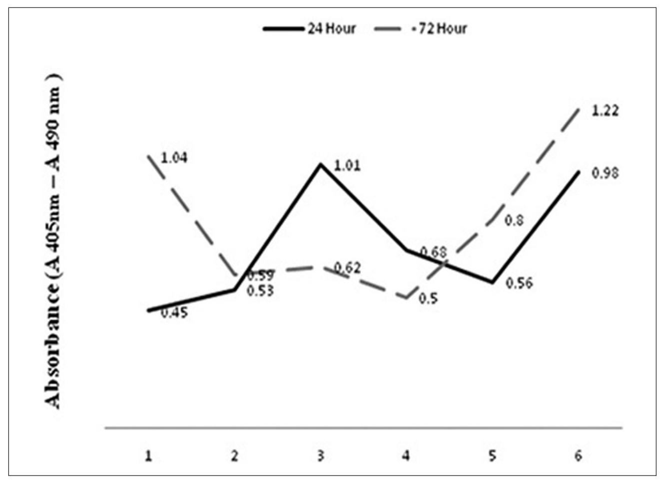

Figure 2: (1) 1.5 mM selenite sodium/ml of cell culture media. (2) 2.5 mM selenite sodium/ml of cell culture media. (3) 4 mM selenite sodium/ml of cell culture media. (4) Empty liposome. (5) untreated AGS. (6) Anti TROP2 conjugated liposomes loaded with apoptosis activator 2. Extent of apoptosis was measured by a nucleosome ELISA Cell Death Detection Kit as described in Materials and Methods section

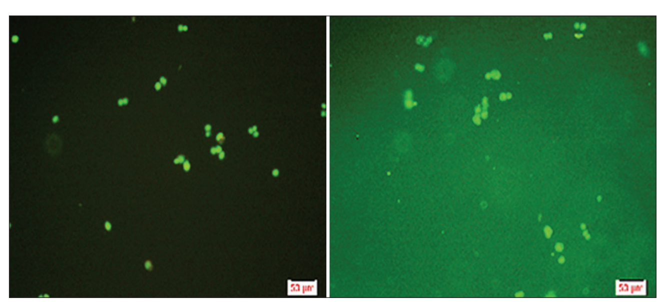

Figure 3: Induction of apoptosis by anti TROP2 conjugated liposomes loaded with apoptosis activator 2 on AGS cell line. A representative TUNEL staining of AGS cells transfected with anti TROP2-conjugated liposomes loaded with apoptosis activator 2 (right) and control positive of the test (left) were shown

cytotoxic concentrations (2.5 and 4 mM) of selenite sodium were used as control of cell death by necrosis[23] [Figure 3].

In conclusion, preparation of liposomes is an accurate and difficult process but recently provided commercial empty liposomes made a cross-cut way for using liposomes in clinical routine applications. In this study, we used pre-prepared liposomes and after necessary manipulations, these modified liposomes were used for delivery of apoptosis activator 2 to AGS cells and induced apoptosis was evaluated by related apoptotic DNA ladder, TUNNEL and Cell Death experiments. All of these experiments confirm that treatment of AGS cell lines with apoptosis activator 2-loaded liposomes which targeted cell surface TROP2 antigen in cancer cells significantly increased apoptosis in these cells, and by this way it is possible to deliver drug-loaded immunoliposomes to target cells.

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