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DNA damage induced by micellar-delivered doxorubicin and ultrasound: comet assay study

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Abstract

To minimize adverse side effects of chemotherapy, we have developed a micellar drug carrier that retains hydrophobic drugs, and then releases the drug by ultrasonic stimulation. This study investigated the DNA damage induced by doxorubicin (DOX) delivered to human leukemia (HL-60) cells from pluronic P-105 micelles with and without the application of ultrasound. The comet assay was used to quantify the amount of DNA damage. No significant DNA damage was observed when the cells were treated with 0.1, 1 and 10 wt% P-105 with or without ultrasound (70 kHz, 1.3 W/cm^2) for 1 h or for up to 3 h in 10 wt% P-105. However, when cells were incubated with 10 mg/ml free DOX for up to 9 h, DNA damage increased with incubation time (P□ 0:0011). Exposure of cells to the same concentration of DOX in the presence of 10-wt% P-105 showed no significant DNA damage for up to 9 h of incubation. However, when ultrasound was applied, a rapid and significant increase in DNA damage was observed (P□ 0:0001). The application of ultrasound causes the release of DOX from micelles or causes the HL60 cells to take up the micelle encapsulated DOX. Our experiments indicated that the combination of DOX, ultrasound and pluronic P105 causes the largest DNA damage to HL-60 cells. We believe that this technique can be used for controlled drug delivery. q2000 Published by Elsevier Science Ireland Ltd.

Keywords: Micellar drug delivery; Doxorubicin; Comet assay; Ultrasound

1. Introduction

The main disadvantage of conventional chemotherapy is that the drug is delivered throughout the body causing various side effects. The anti-neoplastic agents, used to eradicate cancer cells in chemotherapy, are often able to interact with other healthy host cells causing unwanted effects. One technique used to overcome these unwanted interactions is to sequester the therapeutic drugs in a package and release them at the tumor site. We have previously shown that pluronic micelles prevent encapsulated drugs from interacting with HL-60 cells [1,2]. Copolymers of the pluronic family are being used as drug delivery vehicles to deliver chemotherapeutic agents to cancer cells [3,4]. Pluronic copolymers are characterized by a triblock of polyethylene oxide(PEO)-polypropylene oxide (PPO)-polyethylene oxide (PEO). When pluronic polymers are dissolved in water they form micellar structures; the PPO portion forms the hydrophobic core of the micelles, while the PEO chains form its hydrophilic corona. The PEO chains prevent the recognition of these polymeric vehicles by the reticulo-endothelial system (RES) and insure their long circulation time in the blood [5]. Polymeric micelles are advantageous for several reasons. In addition to having long circulating times in the blood and other biological fluids [5,6], these micelles are the appropriate size to escape renal excretion while allowing for extravasation at the tumor site [4]. The chemotherapeutic drug can be easily incorporated into the micelle core by a simple act of

mixing, thus avoiding the complexity associated with the conventional methods of covalently bonding the drug to the polymeric carriers [7]. Our previous studies have shown that DOX and other anthracycline drugs are sequestered in the core of the P105 micelles at 37C [2].

Upon reaching the desired location, the micelle content needs to be released in order for the drug to perform its therapeutic effects. We are investigating the use of ultrasound to trigger drug release. Ultrasound is advantageous because it can be used to control temporal and spatial release of the drug. Previous studies have shown that ultrasound can be used to facilitate the transport of drugs into cells [2,8]. Ultrasound has been reported to enhance the action of some chemotherapeutic agents [9±16]. The goal of this study was to investigate the effect of ultrasound and doxorubicin (DOX) delivered in pluronic P-105 micelles on the amount of DNA damage in human leukemia (HL-60) cells. The drug is currently being used to treat acute leukemias, malignant lymphomas and solid tumors, including breast and liver carcinomas [17]. DOX and other anthracycline drugs are topoisomerase I and II inhibitors [18] that intercalate with DNA, affecting many of the cell functions including the synthesis of DNA and RNA. DOX causes single- and double-strand breaks to occur in the DNA, which lead subsequently to cell death. The comet assay [19] was used to quantify DNA damage caused by DOX when introduced in free form, or when encapsulated in P-105 micelles, in the presence and absence of low frequency ultrasound.

In this study, HL-60 cells were chosen because of the ease of delivering ultrasound to a volume of cell suspensions as opposed to the surface of the cell culture ¯ask. We recognize that this ultrasonic technique could not be used to treat non-localized cancers like leukemia, but the HL-60 cells are used in this study as a simple model of a cancer cell.

2. Materials and methods

2.1. Cells

HL-60 cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 6 mM l-glutamine and 7.5% sodium bicarbonate. The culture was maintained in 75-ml plastic tissue flasks at 37C in humidified 5% CO_2 and was passaged every 3±4 days. For each experiment, 160 ml of cells were cultured for 3 days until they reached a density of about 10^6 cells/ml. The cells were concentrated by centrifugation (to a final concentration of 1 x 10^7 cells/ml) and were then resuspended in 10 ml of supplemented RPMI medium before treatment. Cell viability was assessed using Trypan blue exclusion.

2.2. Drugs

Doxorubicin in HCl (Sigma, St Louis, MO) was dissolved in phosphate-buffered saline (PBS) and sterilized by filtration through a 0.2 mm filter.

2.3. Drug incorporation inside pluronic micelles

Pluronic P-105 was obtained from BASF (NJ). To prepare a stock solution, P-105 was dissolved in a PBS solution to a ®nal concentration of 20-wt% (20 g P-105 in 80 ml of PBS). The solution was then sterilized by filtration through a 0.2 mm ®lter. DOX was introduced into pluronic P-105 micellar solutions by mixing stock solutions at room temperature. A previous study has shown that DOX will accumulate in the hydrophobic core when mixed with pluronic P105 [20]. Final DOX concentration was 10 mg/ml.

2.4. Ultrasonication

Ultrasound power was generated by a Sonicor SC100 ultrasonicating bath (Sonicor Instruments, Copaique, NY) operating at 70 kHz. The power density was controlled by adjusting the input voltage using a variable ac

transformer (variac), and the insonation intensity in the bath was controlled by adjusting the variac voltage running the Sonicor bath. The insonation intensity as a function of applied voltage was determined using a calibrated hydrophone (Bruel and Kjaer model 8103, Decatur, GA). The acoustic intensity generated by the Sonicor bath increased with applied voltage and the intensity dropped below detection level when less than 60 V ac were applied from the variac. For subsequent experiments, the variac voltage was adjusted to produce an intensity of 1.3 W/cm^2. Since we were not studying the effect of hyperthermia induced by sonication, the temperature of the bath was maintained at 378C using a recirculating thermostatic bath.

2.5. Comet assay

The comet assay, performed as described by Fairbairn et al., was used to quantify DNA damage in HL-60 cells by measuring the fraction and length of broken nuclear DNA strands [19]. Large amounts of DNA damage as measured by the comet assay is indicatory of cell death, either by necrosis or by apoptosis [21]. Cell suspensions were incubated and sonicated with various combinations of DOX, pluronic P-105, and ultrasound. Incubated (non-sonicated) cells were assayed at 0, 1, 2, 3, 6 and 9 h while sonicated cells were assayed at 0, 30, 60, 120 min. These experiments were performed in pairs; the first pair had the drug introduced in free form in PBS while in the second pair the drug was dissolved in a solution of up to 10 wt% P-105. Details of the assay are given below.

Two hundred microliters of 10^6 cells/ml suspension were mixed with 600 ml of 1% low melting point agarose at 378C (0.75% final concentration) and immediately layered on custom frosted slides which feature a clear centered window [22]. The slides were placed on a chilled plate to solidify the agarose. The slides were then bathed in freshly prepared lysing solution (2.5 M NaCl, 10 mM Trisbase, 0.1% sodium sarcosinate, (pH 12.3)) in the dark for 1 h at room temperature. After lysing, the slides were placed in alkaline electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH 12.3) for 30 min, allowing for salt equilibration and further DNA unwinding. Electrophoresis was performed at 20 V and 400 mA for 10 min. The slides were then immersed in a bath of distilled water for 5 min in order to reanneal the DNA. Then they were stained with propidium iodide (2.5 mg/ml) for 20 min and covered with a glass cover slip before analysis.

The image of the electrophoresed DNA appears like a comet, with undamaged DNA in the head, and fragmented DNA migrating to form a tail. Comet images were analyzed at 400 £ using a Zeiss epifluorescence microscope with attached CCD camera and Gen II intensifier. Computer software kindly provided by Dr Peggy Olive (British Columbia Cancer Research Center) was used to score individual parameters for each cell. The main parameters of the comet imaging, as performed using this computerized technique are total DNA, % of DNA in the comet and tail, tail length and tail moment. The latter is the single most relevant index of DNA damage because it considers both the tail length and fraction of DNA in the comet tail. At least ®fty comets were read from each slide with two replicate slides for each sample. Each experiment was repeated three times.

2.6. Statistical methods

The results are represented as means ^ SD for tail moments. Treated cells at different incubation or sonication times were compared to untreated controls using ANOVA (MINITAB). Two sided P-values are reported as signi®cant, very signi®cant, or very highly signi®cant (P , 0:05, 0.01 or 0.001, respectively).

3. Results

3.1. Pluronic and ultrasound controls

To determine if incubating and ultrasonicating cells with various concentrations of pluronic P-105 had any effect on the DNA of HL-60 cells, we ®rst incubated and sonicated the cells with 0.1, 1 and 10 wt% P-105 for 1 h.

Next, we ultrasonicated the cells for 3 h at a frequency of 70 kHz and a power density of 1.3 W/cm^2 in the presence and absence of 10 wt% P-105. There was no signi®cant difference in the DNA damage between the control (no pluronic) and the cells that were exposed to P-105 only (P ⍰ 0:68), ultrasound only (P ⍰ 0:28), and the combination of both 10% P-105 and ultrasound for 1, 2 and 3 h (see Fig. 1) (P ⍰ 0:12). In addition, the cell viability was not affected as a result of the P-105 treatment or ultrasound treatment (see Fig. 2).

3.2. Doxorubicin only

When cells were incubated with 10 mg/ml free DOX, the DNA damage increased with incubation time. Fig. 1 shows that there was moderate damage in the ®rst 3 h, followed by very signi®cant DNA damage after 6 and 9 h of incubation with free DOX (P ⍰ 0:0011). The cell viability was 89 and 65% after 6 and 9 h, respectively.

3.3. Doxorubicin in P-105

When the cells were exposed to the same concentration of DOX in the presence of 10% P-105 and in the absence of ultrasound, there was no signi®cant DNA damage (Fig. 1) for up to 9 h (P ⍰ 0:2618). Fig. 2 shows that the cell viability was not affected after 9 h of incubation with the encapsulated agent. The damage was insigni®cant at all time points. DOX is sequestered inside the hydrophobic core of the pluronic micelles, and thus there is little or no interaction between DOX and the HL-60 cells [2,8]. The

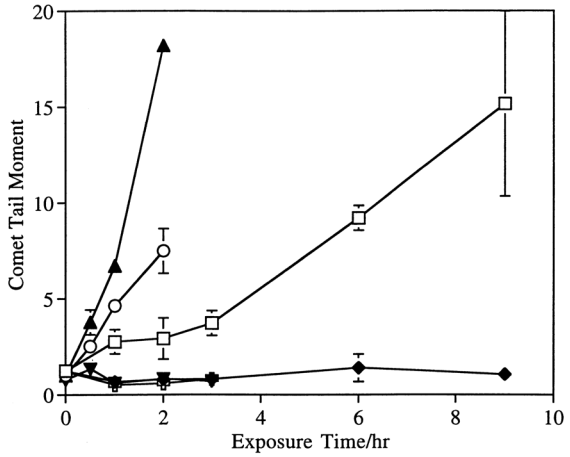

Fig. 1. The DNA damage represented by the magnitude of the comet tail moment for HL-60 cells exposed to the following conditions. A, 10 mg/ml DOX in PBS; V, 10 mg/ml DOX in 10% pluronic; W, 10 mg/ml DOX in PBS with ultrasound; O, 10 mg/ml DOX in 10% pluronic with ultrasound; 1 , PBS with ultrasound only; P, 10% pluronic with ultrasound. Error bars represent standard deviations. Where error bars are not observed, the size of the error bars is smaller than the size of the symbol.

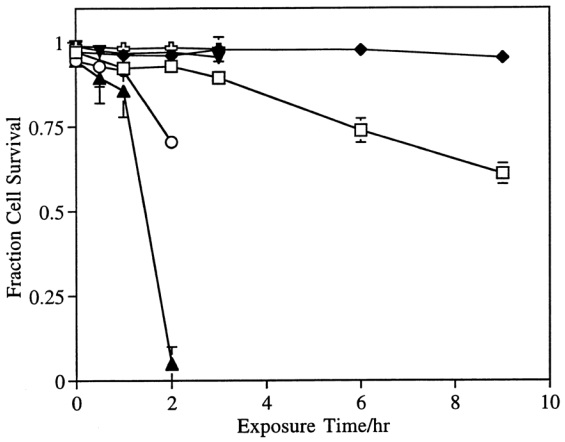

Fig. 2. The HL-60 cell viability determined by Trypan blue exclusion after exposure to the following conditions. A, 10 mg/ml DOX in PBS; V, 10 mg/ml DOX in 10% pluronic; W, 10 mg/ml DOX in PBS with ultrasound; O, 10 mg/ml DOX in 10% pluronic with ultrasound; 1, PBS with ultrasound only; P, 10% pluronic with ultrasound. Error bars represent standard deviations. Where error bars are not observed, the size of the error bars is smaller than the size of the symbol.

data are consistent with the hypothesis that insignificant amounts of DOX enter the cytoplasmic membrane when cells are exposed to DOX-loaded micelles in the absence of ultrasound.

3.4. Effect of sonication

When HL-60 cells were exposed to free DOX along with ultrasound, the damage was very highly signi®cant (P 0:0001). The data in Fig. 1 show that the damage was more rapid than when the cells were exposed to free DOX without ultrasound. However, the most signi®cant DNA damage was observed when the drug was delivered through micellar solution under the in¯uence of ultrasound. The DNA damage was also very highly signi®cant (P 0:0001) compared to the control or the damage caused by encapsulated drug with no ultrasound. The DNA damage increased linearly with time without any apparent lag time, suggesting that DOX is taken up quickly upon the application of ultrasound. Ultrasound greatly increased the rate of DNA damage from both free and encapsulated DOX, but the rate of damage was signi®cantly greater when DOX was introduced from micelles. The cell viability (Fig. 2) also decreased with time, and 96% of the cells were dead after 2 h exposure to the combination of DOX, P105 and ultrasound. The results also show that a strong synergistic effect exists between the drug, 10wt% P-105 and ultrasound.

4. Discussion

The main disadvantage associated with systemic chemotherapy for localized tumor treatment is that the drug is delivered throughout the body causing unwanted side effects. A plausible solution to overcome these unwanted effects is to use molecular vehicles to sequester the therapeutic drug in a package and release it to the tumor site at the appropriate time. This way the drug will have minimum interactions with healthy cells in the body, thus reducing many of the unwanted side effects associated with conventional chemotherapy. The main challenge is to completely sequester the drug, but yet be able to release it upon demand in both time and space. This study shows that the combination of ultrasound and micellar drug carriers can significantly increase the damage done to the DNA of HL-60 cells. The interaction of anticancer drugs with HL-60 cells can be prevented by encapsulating the

drug in pluronic P-105 micelles. These results suggest that pluronic micellar drug delivery vehicles may be able to meet the demand of both sequestration and controlled rapid release in a clinical setting.

The comet assay was used to quantitate the amount of DNA damage immediately after treating the cells. The comet assay tail moment measurements have been shown to correlate directly to the percent of viable cells as monitored using Trypan Blue exclusion for several human lymphoblast cell lines [21]. The study has shown that a tail moment exceeding 15 corresponds to low cell viabilities as measured by Trypan Blue exclusion.

The data presented in this paper confirm previous cell viability results that P-105 is not toxic to HL-60 cells reported by Munshi et al. [1]. Using Trypan Blue exclusion, our study also showed that pluronic P-105 is non-toxic to HL-60 cells at concentrations up to 10 wt% in the presence or absence of ultrasound (Fig. 2). Our results agree with the data presented by Loverock et al. [15] who showed that 1 h of exposure of ultrasound (2.6 MHz at 2.3 W/cm^2) rendered DOX significantly more toxic to Chinese hamster lung fibroblasts. When the same cell line was exposed to ultrasound alone, the cell viability was not affected. In another study, Saad and Hahn [10,11] showed that at higher power densities (1 W/cm^2 and 2.025 MHz ultrasound) the cytotoxic effect of DOX was increased. However, unlike our study, Saad and Hahn used ultrasound to induce hyperthermia. Ning et al. [16] demonstrated that ultrasound-induced hyperthermia, in addition to enhancing the antitumor activity, accelerated the release of DOX from long circulating liposomes. They reported that by increasing the temperature from 37 to 418C, the rate of release of DOX was increased six-fold after 1 h of sonication at 2 W/cm^2 [16]. Although our experiments were performed under isothermal conditions, this hyperthermic effect can be added to the other advantages of ultrasonic power when this technology is applied to clinically.

With respect to possible mechanisms of enhanced DNA damage, our first hypothesis is that ultrasound is causing release of the DOX from the micelles, and a subsequent increase in the amount of DOX that enters HL-60 cells. We suspect that pluronic also plays a role in this mechanism. Rapoport et al. reported that sonication for 1 h of HL-60 cells in the absence of pluronic P-105 did not increase the uptake of DOX [2]. However, in the presence of various concentration of P-105, ultrasound significantly increased the amount of DOX uptake. The data presented herein show that ultrasound without DOX does not cause significant (if any) DNA damage. Ultrasound with DOX, but in the absence of P-105 significantly increased DNA damage, but that damage was further enhanced by the presence of P-105.

While other research groups have previously reported ultrasonic enhancement of chemotherapy, our group is the first to both decrease unwanted interactions and then deliver an even more potent therapy upon demand via ultrasound. The use of ultrasound is advantageous since the technique is non-invasive and can be focused on shallow or deep soft tissues throughout the body. It may have particular application to treatment of breast or other localized cancers of internal organs. Extension of this research to the treatment of anchorage-dependent breast and liver cancer cells is in progress.

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