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Anticancer Effect of Doxorubicin Loaded Heparin based Super-paramagnetic Iron oxide Nanoparticles against the Human Ovarian Cancer Cells

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***Abstract*—**This study determines the effect of naked and heparin-based super-paramagnetic iron oxide nanoparticles on the human cancer cell lines of A2780. Doxorubicin was used as the anticancer drug, entrapped in the SPIO-NPs. This study aimed to decorate nanoparticles with heparin, a molecular ligand for 'active' targeting of cancerous cells and the application of modified-nanoparticles in cancer treatment. The nanoparticles containing the anticancer drug DOX were prepared by a solvent evaporation and emulsification cross-linking method. The physicochemical properties of the nanoparticles were characterized by various techniques, and uniform nanoparticles with an average particle size of 110±15 nm with high encapsulation efficiencies (EE) were obtained. Additionally, a sustained release of DOX from the SPIO-NPs was successful. Cytotoxicity tests showed that the SPIO-DOX-HP had higher cell toxicity than the individual HP and confocal microscopy analysis confirmed excellent cellular uptake efficiency. These results indicate that HP based SPIO-NPs have potential uses as anticancer drug carriers and also have an enhanced anticancer effect.

***Keywords*—**Heparin, A2780 cells, ovarian cancer, nanoparticles, doxorubicin.

I. Introduction

OVER the past few decades, inorganic nanoparticles, whose structures exhibit significantly novel and distinct physical, chemical, and biological properties, and functionality due to their nanoscale size, have elicited much interest. Nanostructure materials are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications [1]. In medicine, nanotechnology has been explored for early detection, diagnosis and treatment of diseases [2].

Even with the biological and pharmaceutical advancement that the scientific community has achieved, cancer is still, without doubt, one of the biggest killers worldwide. The current focus in the development of cancer therapies is on targeted drug delivery to provide therapeutic concentrations of anticancer agents at the site of action and spares the normal tissues. Numerous investigations have shown that both tissue and cell distribution profiles of anticancer drugs can be controlled by their entrapment in submicronic colloidal systems (nanoparticles).

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The rationale behind this approach is to increase antitumor efficacy,while reducing systemic side-effects. Nanoparticulate drug delivery systems have attracted significant attention in the field of cancer nanotechnology. Over the past decade, various self-assembled nanoparticulate carriers such as liposomes, polymeric micelles, and nanoparticles have been widely explored to selectively deliver anti-cancer agents to tumor tissues for effective cancer therapy [3]. Swellable hydrophilic polymer nanoparticles, have recently gained much interests as promising nanoparticulate carriers due to their nanoscale size (50–200 nm) and high stability favorable for intravenous and intracellular drug delivery [4]. They also provide an aqueous interior space for incorporation of various bioactive macromolecules such as proteins.

Heparin is a highly sulfated natural glycosaminoglycan composed of repeating disaccharide units of pyranosyluronic acid and glucosamine residues. In addition to its well-known anticoagulant activity, heparin is involved in diverse physiological processes including cell proliferation, differentiation, and inflammation, through interacting with a number of proteins having heparin-binding domains [5]. Surprisingly, heparin also exerts various anti-cancer activities in the processes of tumor progression and metastasis.

A key problem for tumor treatment is the reducing sensitivity of tumor cells to cytotoxic drugs. Thus, many polymer nanospheres and nanoparticles have been introduced as drug-delivery systems to enhance the efficiency of anticancer drug delivery based on the ability to target specific locations in the body [6]. The most promising materials are magnetic nanoparticles. superparamagnetic iron oxide nanoparticles (SPIO-NPs, $Fe3O_4$), a biocompatible and super-paramagnetic nanomaterial with satisfactory chemical stability and low toxicity, are widely used for targeted-drug carriers with target-orientation and sustained-release properties [7].

This study determines the effect of naked and heparin-based super-paramagnetic iron oxide nanoparticles on the human cancer cell lines of A2780. Doxorubicin was used as the anticancer drug, entrapped in the SPIO-NPs. This study aimed to decorate nanoparticles with heparin, a molecular ligand for 'active' targeting of cancerous cells and the application of modified-nanoparticles in cancer treatment.

II. MATERIALS AND METHODS

A. Chemicals

Heparin sodium salt derived from porcine mucosa (Mw ¼12,000) was obtained from Wako Pure Chemical Industries (Osaka, Japan). MTT was purchased from Sigma Aldrich. Monoclonal antibodies including caspase-3, bax, bcl-2, NF-κB, survivin, and β-actin were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). SPIO-NPs (Fe_3O_4; State Key Lab of Bioelectronics, Nanjing, China) were well distributed in RPMI 1640 medium containing 10% (v/v) heat-inactivated new-born calf serum (Sijiqing, Hangzhou, China) by using ultrasound treatment in order to obtain SPIO-NPs colloidal suspension.

B. Synthesis and characterization of thiolated heparin and HP based SPIO-NPs

Heparin (300 mg, 50 μM) conjugation to the Fe_3O_4 was prepared by mechanical absorption polymerization at 4 °C for 48 hours. Size and shape of the heparin-Fe_3O_4 nano-shells were evaluated by atomic force microscopy (AFM) and scanning electron microscopy (SEM). For AFM experiments, 100 mL of the solution was deposited onto a clean mica surface and then its image was obtained with a PSIA XE-100 AFM system. Thirty microliters of the solution was equilibrated and dried on a freshly prepared carbon tape at 37 ^oC, and then observed by a Philips 535M SEM.

C. Preparation of self-assembled SPIO-HP-DOX conjugates

SPIO-HP-doxorubicin (DOX) conjugates were synthesized by the conjugation of DOX to Fe_3O_4 and HP complex, by the coupling reaction using EDC. All nanoparticle suspensions were sonicated for 2 min using a probe type sonicator (Ultrasonic Processor GEX-600, Sigma) at 90 W, in which the pulse was turned off for 1 s with an interval of 5 s. The nanoparticle suspensions were passed through a syringe filter (pore size 0.45 mm, Millipore, Billerica, MA, USA) and stored at room temperature.

D. Cell lines and culture conditions

A2780 cells, derived from human ovarian cancer subjects, were cultured in RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator.

E. Cell proliferation assay

An SPIO-HP-DOX solution was diluted with PBS solution to give a final concentration of heparin from 10-200 μM. Human ovarian cancer A2780 cells were seeded in a 24-well plate at a density of 5×10^3 cells/well and grown in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum for 24 h at 37 ^oC. The cells were then incubated with the culture medium alone (control), with SPIO-NP-HP-DOX and SPIO-NP-DOX for 3 days at 37 ^oC. The number of viable cells was determined by the CCK-8 cell viability assay, which depends on the mitochondrial dehydrogenase activity inside the cells.

F. Evaluation of cellular uptake and apoptosis-inducing effect of SPIO-HP-DOX complex

Fluorescein-conjugated heparin was prepared by conjugating fluorescein-5-maleimide to thiolated heparin. In brief, 5 mg of thiolated heparin was reacted with 0.1 mg of fluorescein-5-maleimide in 0.1 M phosphate buffer (pH 7). The solution was dialyzed (Mw cutoff of 6 kDa) and then lyophilized. Using the fluorescent heparin, fluorescein-conjugated SPIO-HP-DOX complex was prepared by the process described above. A2780 cells were plated over a cover slide on a six-well plate at a density of 2×10^5 cells/well and cultivated for 24 h at 37 ^oC. The cells were incubated with SPIO-HP-DOX and SPIO-HPF-DOX in the culture medium for 3 days at 37 ^oC. The cells were washed with PBS solution and fixed with a 1:1 (v/v) mixture of methanol and acetone. After washing with PBS solution, the cell nuclei were stained with propidium iodide (50 mg/mL in PBS solution) for 30 min. Apoptosis-inducing effect of heparin nanogels were evaluated by using a Magic Red caspase detection kit. The cells were examined by using an LSM510 confocal laser scanning microscope (Carl Zeiss, Germany).

G. Western blot analysis

Western blot analysis was done to determine the expression of caspase-3, bax, bcl-2 and survivin protein. Whole cell protein extracts were taken after the incubation of A2780 cells for 72 hours under above-described conditions. Total protein was isolated on ice and subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels using modified radio immuno-precipitation assay buffer, and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Western blotting was performed with a 1:1000–1200 dilutions of monoclonal antibodies against either anti-human caspase-3, bax, bcl-2, survivin or β-actin anti-body in 5% nonfat dry milk, and then with horseradish peroxidase-conjugated goat anti-rabbit (1:5000) as a secondary antibody. The bands were detected using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

H. Statistical analysis

All data were presented as means ± standard deviation in triplicate and analyzed using SPSS software (v. 15.0; SPSS Inc., Chicago, IL). The difference among various groups was analyzed by ANOVA test, and *P* values of less than 0.05 were considered significant.

III. RESULTS AND DISCUSSION

A. Morphological characterizations

TEM image of SPIO-HP were shown in Fig. 1. Nanoparticles appeared mono-dispersed spheres with a solid and consistent structure.

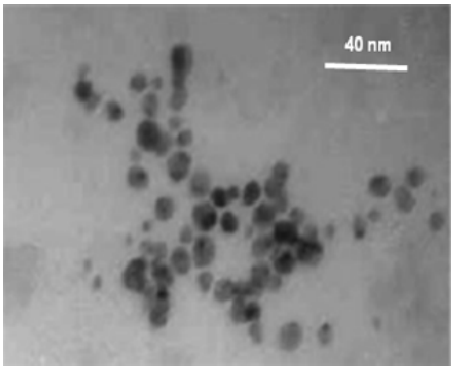

Fig. 1 Transmission electron microscopic image of SCIO-HP at 125,000× magnification.

B. *Synergistic effect of different concentrations HP on the cytotoxicity of A2780 cells*

The HP concentrations used in this experiment were 10, 30, 50, 70, 100, 150 and 200 µM, where 50 µM HP showed 92% inhabitation rate after 72 hours of incubation of the A2780 cells at 37 °C. Furthermore, it was observed that the cancer cell growth inhibition rate was significantly lower in the absence of HP (20%, $p<0.05$) as compared to the A2780 cells containing 50 µM HP, conjugated to the SCIO-DOX (Figure 2).

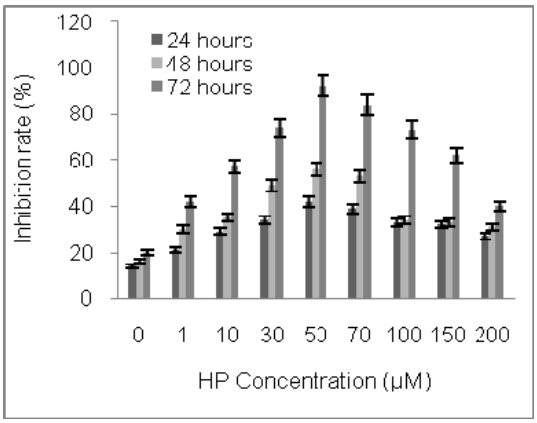

Fig. 2 Synergistic effect of different concentrations HP on the cytotoxicity of A2780 cells. HP (10, 30, 50, 70, 100, 150 and 200 µM) was added to the culture medium containing A2780 cells and incubation was done for 72 hours at 37 °C.

C. *Synergistic effect of HP-SCIO-DOX on the apoptosis of A2780 cells*

According to the results shown in Figure 3, 12% of the A2780 cells underwent apoptosis in the presence of 50 µM HP, which was not considerable as compared to the control group (9%; $p\geq0.05$). The apoptosis of A2780 cells, incubated with 10 mg/L SPIO-DOX for 72 hours, was 15.8% ($p<0.05$). While, the combination of SPIO-DOX-HP increased the apoptosis up to (40%; $p<0.05$).

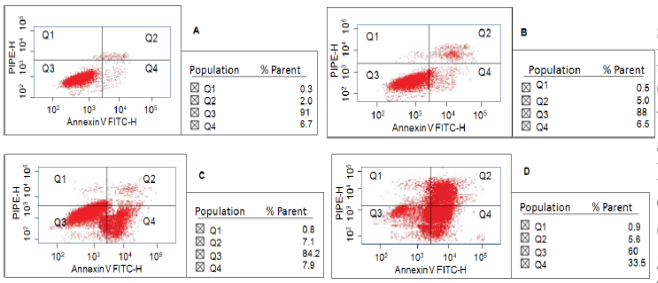

Fig. 3 Effect of SPIO-HP-DOX on the apoptosis of A2780 cells for 72 hours. A) Control; B) Incubated with 50 µM HP; C) Incubated with 10 mg/L SPIO-DOX (Fe_3O_4); D) Incubated with SPIO-HP-DOX.

D. *Morphological changes of A2780 Cells*

The morphological changes of A2780 cells were observed under the optical microscope. As shown in Figure 4, A2780 cells, in control group, displayed normal and healthy shape, demonstrated by the clear skeletons (Figure 4A). While, after treatment with 10 mg/L SPIO-DOX for 72 hours, typical cyto-morphological features of apoptosis in A2780 cells were evident, such as cell shrinkage, chromatin condensation, margination, and the presence of apoptotic bodies (Figure 4B). On the other hand, 50 µM HP caused necrosis in the A2780 cells (Figure 4C). The same, but more significant results were seen when the cells were treated with SPIO-HP-DOX complex. While, the cells displayed the typical phenomena of apoptosis including chromatin condensation, nucleolus pyknosis, and nuclear fragmentation in the presence of our modified "anticancer nanomedicine" (Figure 4D).

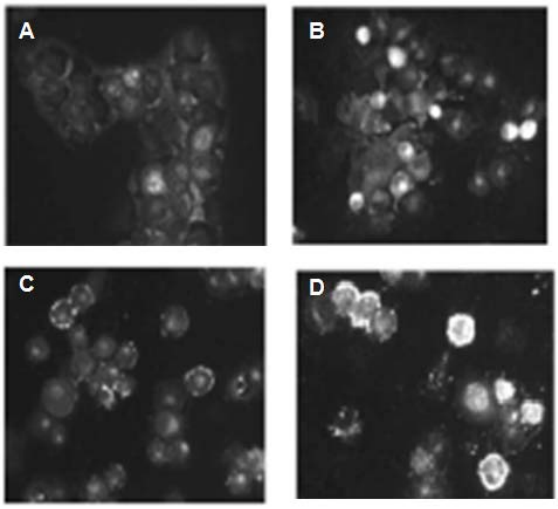

Fig. 4 Demonstration of cellular process destruction in the presence of SPIO-HP-DOX. a) A2780 Cells grown in PBS, b) cells with 50 µM HP only, c) cells with SPIO-DOX only, and d) cells with SPIO-HP-DOX.

E. *Western blot analysis for the determination of caspase-3, bax, bcl-2 and survivin expression*

Our results have shown that the caspase-3, bax, bcl-2 and survivin proteins, in the A2780 cells, treated with 50 µM HP had no significant changes as compared to control group ($p<0.05$). However, the level of caspase-3 and bax proteins was increased in the in presence of SPIO-DOX dramatically as compared to control group ($p<0.05$) (Figure 5). Furthermore, these two kinds of proteins, whose genes were dramatically up-regulated in the presence of SPIO-DOX-HP (Figure 5; $p< 0.05$). Reversely, compared with the control group and the HP-treated group, the level of bcl-2 and caspase was lower, in the presence pf SPIO-DOX-HP, as described previously ($p<0.05$).

Although many chemotherapy drugs are used clinically, the overall survival of cancer patients is still unsatisfactory. The majority of chemotherapy medicines have serious adverse effects in addition to their clinical effects. So, there is a serious need to introduce new methods and compounds that

prove to be successful fighters of cancer as well as show least side effects.

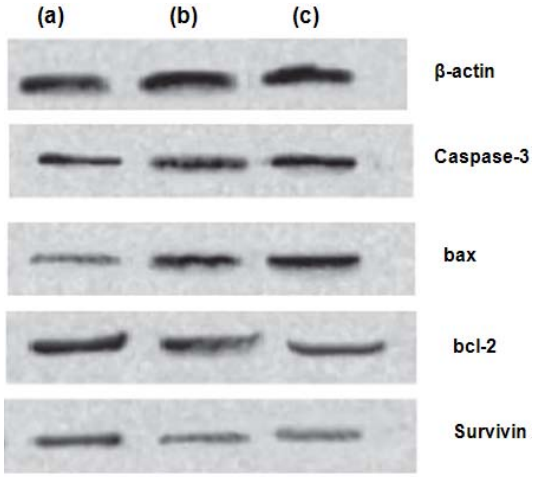

Fig. 5 Expression of caspase-3, bax, bcl-2 and survivin protein in A2780 cells by western blot after separate and combined treatment with SPIO-DOX, HP and SPIO-DOX-HP for 72 hours. β-actin served as a control. A2780 cells were incubated with (a) 10 mg/L SPIO-DOX; (b) with 50 μM HP and (c) with SPIO-HP-DOX complex.

Biodegradable polymers containing entrapped drug can be placed in the body, and are used for localized drug delivery and/or the controlled release of a drug over a period of months. As the polymer slowly degrades, therapeutic levels of the anti-tumour peptide are maintained for up to 3 months, making the therapy very convenient for patient use [8]. Although such polymer-based drug-delivery systems have been important advances, the development of nano-sized vectors enables tumours to be targeted more precisely — the vectors can move around in the body and selectively localize a therapeutic drug payload to metastatic tumours. Conjugation to hydrophilic polymeric carriers can also improve the water solubility of hydrophobic drugs such as doxorubicin and paclitaxel, enabling easier formulation and patient administration.

For example, it is recently suggested that heparin strongly interferes with the activity of growth factors such as basic fibroblast growth factor (bFGF), thereby inhibiting angiogenesis essential for tumor progression [9]. Moreover, heparin is able to attenuate tumor metastasis by blocking selectin-mediated adherence of cancer cells to vascular endothelium or platelets [10]. More recent studies showed strong evidence that free heparin molecules internalized within the cells interacted with transcription factors, playing an important role in inducing apoptotic cell death [11, 12]. These distinctive functional activities of heparin have significant implications in the development of effective tumor targeted delivery systems, which prompted us to develop heparin based nanoparticulate carriers that could be delivered within tumor cells for apoptotic cell death. The present results prove this fact.

Most anticancer agents exert their anticancer effects by inducing apoptosis [13]. Recently, super paramagnetic iron oxide (Fe_3O_4) nanoparticles SPIO-NPs are widely used for targeted drug carriers to enhance the efficiency of anticancer drug delivery based on the ability of target-orientation and sustained-release properties [12]. Previous studies have demonstrated the synergistic effect of SPIO nanoparticles with anticancer drugs on the intracellular accumulation in cancer cells [14-16]. During the present study, the potential synergistic effects of SPIO-HP was demonstrated while using doxorubicin as the shelled anticancer drug. The present cytotoxic analyses have shown that SPIO-HP-DOX caused an increased toxicity in A2780 cells and the presence of SPIO decreased the IC50 of HP in A2780 cells. Effect of present modified nanomedicine was also tested on the apoptosis of A2780 cells. The addition of 10 mg/L SPIO caused a 40% increase in the apoptotic percentage of A2780 cells as compared to those treated with the HP alone (12%) for 72 hours. Our outcomes clearly indicate that a SPIO-HP-based drug delivery system can decrease the IC50 of HP and enhance the effect of DOX by inducing apoptosis in A2780 cells.

In order to check whether the effects of SPIO-DOX, along with 50 μM HP was different from that of HP alone, we demonstrated that the A2780 cells, incubated with 50 μM HP and 10 mg/L SPIO-DOX for 72 hours, showed a typical morphological features of apoptosis under the optical microscope, while 50 μM HP led cells to necrosis. These results suggest that a combination of SPIO-DOX and HP could be a feasible candidate in the development of anticancer drugs. Apoptosis is the consequence of a series of precisely regulated events that are frequently altered in tumor cells. In general, the sequence of events has been broadly categorized into two pathways: the extrinsic pathway, which involves the activation of the tumor necrosis factor (TNF)/Fas death receptor family and the intrinsic pathway, which involves the mitochondria. In both pathways, an apoptotic death stimulus results in the activation of caspases, the major executioners of this process, either directly or via activation of the mitochondrial death program [17, 18].

It is well known that caspase-3 is the most characterized effector caspase, and its activation leads to the final stages of cellular death by proteolytic dismantling of a large variety of cellular components on one hand, and activation of proapoptotic factors on the other hand [17, 18]. Our study showed that HP combined with SPIO-DOX dramatically upregulated the transcription and expression of caspase-3 in A2780 cells. This result supports the promotion of HP-induced apoptosis by SPIO-DOX was related to the level of genes and proteins expression. In tumor cells, apoptosis can be induced either by activation of molecules upstream of apoptosis signaling or by inhibition of antiapoptotic factors.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is overexpressed in virtually every human cancer. In several tumor cell lines, the presence of survivin correlates with resistance to apoptosis and is associated with increased malignancy [19]. Previous *in vitro* studies showed that inhibition of survivin restored or enhanced the cytotoxicity of chemoreagents [20], and animal studies

showed a superb efficacy against xenografts using an adenoviral strategy targeted to surviving [21]. At present, survivin is validated as a cancer therapeutic target [22]. Our data showed that the expression of antiapoptotic genes such as bcl-2, survivin of A2780 cells were significantly down-regulated after co-treatment of HP with SPIO-DOX, whereas the expression of bax was up-regulated. Bax and bcl-2 both belong to the bcl-2 family [23].

Overexpression of Bax has been shown to accelerate cell death [24], while that of antiapoptotic proteins such as bcl-2 represses the death function of bax [25]. Thus, the ratio of bcl-2/bax might be a critical factor of a cell's threshold for undergoing apoptosis [26]. Although bcl-2 and survivin are both apoptosis inhibitors, they work through different pathways in the regulation of cell apoptosis. The antiapoptotic protein bcl-2 mainly inhibits the mitochondrial pathways [27], while survivin directly blocks the processing and activation of effector caspase-3 and caspase-7, which commonly acts downstream of both apoptosis signaling pathways [28], which suggests that SPIO-DOX loaded with HP induced cell apoptosis through various pathways.

IV. CONCLUSION

In conclusion, our study demonstrates for the first time that SPIO-DOX can promote apoptosis induction of HP *in vitro* in A2780 cells, and the synergistic effect of that composite on apoptosis induction may owe to the regulation of various proliferative and antiapoptotic gene products, including caspase-3, bax, bcl-2 and survivin. Thus, it may be possible that a combination of SPIO-DOX and HP may be a sufficient and less toxic method in ovarian cancer therapy.

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