In vitro and in vivo antitumor effects of doxorubicin loaded with bacterial magnetosomes (DBMs) on H22 cells: The magnetic bio-nanoparticles as drug carriers

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Abstract

Hepatocellular carcinoma (HCC) is the most common form of cancer although effective therapeutic strategy especially targeted therapy is lacking. We recently employed bacterial magnetosomes (BM) as the magnetic-targeted drug carrier and found an antitumor effect of doxorubicin (DOX)-loaded BMs (DBMs) in EMT-6 and HL60 cell lines. The aim of this study was to evaluate the in vitro and in vivo anti-neoplastic effects of DBMs on hepatic cancer. DBMs, DOX and BMs displayed tumor suppression rates of 86.8%, 78.6% and 4.3%, respectively, in H22 cell-bearing mice. The mortality rates following administration of DBMs, DOX and BMs were 20%, 80% and 0%, respectively. Pathological examination of hearts and tumors revealed that both DBMs and DOX effectively inhibited tumor growth although DBMs displayed a much lower cardiac toxicity compared with DOX. The DBMs were cytotoxic to H22 cells manifested as inhibition of cell proliferation and c-myc expression, consistent with DOX. The IC_{50} of DOX, DBMs and BMs in target cells were 5.309 ± 0.010, 4.652 ± 0.256 and 22.106 ± 3.330 μg/ml, respectively. Our data revealed both in vitro and in vivo antitumor property of DBMs similar to that of DOX. More importantly, the adverse cardiac toxicity was significantly reduced in DBMs compared with DOX. Collectively, our study suggests the therapeutic potential of DBMs in target-therapy against liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers with a high mortality. Although surgical resection represents the mainstream of HCC treatment, most HCC patients are
already in a diagnostic stage where surgery is no longer possible. Furthermore, HCC has a very poor prognosis with a dismal 8.9% five-year survival rate in US and recurrence rates remain high following tumor resection [1,2]. General chemotherapy and radiotherapy offer somewhat unsatisfactory responsiveness, making new therapeutic strategies an immediate need to combat HCC. Targeted cancer therapy is promising to minimize the non-specific toxicity and to improve therapeutic efficiency compared to conventional chemotherapy [3].

Magnetic-targeted drug carriers, one of main research focuses on drug targeting, are prepared using Fe$_3$O$_4$ or Fe$_2$O$_3$ as cores coated with biocompatible polymers for drug delivery [4–8]. This technology has evolved rapidly since the 1970s [9] although its application has been hampered by certain technical problems such as low drug loading, propensity of congregating, poor dispersion and control of microspheric shape or size [10]. Recently, bacterial magnetosomes (BMs) synthesized by magnetotactic bacteria [11] have drawn great interest [12,13] as replacements for targeted drug carriers due to their unique features such as paramagnetism, nano-scale, narrow size distribution and membrane-bounded [14–18].

BMs have been used as carriers for enzymes [19], nucleic acids [20,21] and antibodies [22,23]. However, their role as drug carriers has not been explored. Our group recently tested if BMs may be used as drug carrier for the anti-neoplastic agent doxorubicin (DOX). DOX was chosen as the targeting drug given it represents one of the most commonly used antitumor drugs with a broad spectrum of applications including hematopoietic malignancies and solid tumors [24,25]. In addition, DOX may produce free radicals leading to serious side effects especially cardiomyopathy en route to congestive heart failure [25–27]. Therefore, DOX is a preferred candidate for drug targeting research to evaluate both effectiveness and toxicity.

2. Materials and methods

2.1. Materials

*Magnetospirillum gryphiswaldense* MSR-1 (DSM 6361) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Brunswick, Germany). Doxorubicin hydrochloride was purchased from Junning Technology Company (Shenzhen, China). MTT assay kit was obtained from Sigma (St. Louis, MO). All other chemicals were from Sinopharm Chemical Reagent Co., Ltd (Beijing, China) unless otherwise specified. The H22 mouse liver cancer cells were kindly provided by Director Xiaohua Tan (Department of Blood, Beijing General Hospital of Beijing Military Command, Beijing, China). The H22 cells were isolated from the ascites of BALB/c mice following abdominal injection of the H22 cells (0.5 ml 1 × 10⁶ cells/ml) for 7 days. Cell culture medium was composed of RPMI1640 supplemented with 10% fetal bovine serum, 100 U/ml streptomycin and penicillin solution (from Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China). All cells were incubated at 37 °C in humidified air with 5% CO$_2$. Female BALB/c mice (18–22 g) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). Mice were housed 5/cage under standard 12/12 light–dark circadian cycle condition at room temperature with free access to food and water.

2.2. Preparation of the DBMs

Cells of *M. gryphiswaldense* MSR-1 were suspended in 0.1 M PBS (pH 7.4) and disrupted by an ultrasonic cell crusher (300 W for 15 min). BMs were absorbed to the bottom of the beaker using a magnet with cell debris removed. The BMs sediments were reabsorbed after being resuspended in 0.1 M PBS (pH 7.4) and dispersed by low level ultrasonication (50 W, repeated for 20–30 times). The BMs suspensions were treated with 5 μg/ml of DNase I for 2 h at 37 °C. The BMs were purined with rinsing and were conserved at −20 °C following freeze-drying. Twenty milliliters of purified BMs was sterilized by Co$_{60}$ irradiation (15 kGy) and were resuspended in 70 ml PBS. Thirty milliliters of DOX solution (1 mg/ml, dissolved in distilled water) was added into the BMs suspension and was treated with ultrasonic bathing for 5 min to distribute the BMs evenly. Following the addition of 1 ml glutaraldehyde (50%), the mixture was treated with ultrasonic bathing. The DBMs were then collected from blending and were washed with magnet absorption in conjunction with ultrasonic bathing to eliminate redundant glutaraldehyde and DOX that was non-covalently, yet relatively tightly, attached, until the supernatants displayed no reddish color. Five ml DOX solution (1 mg/ml) was added into the DBMs suspension and was treated with ultrasonic bathing for 14 times to eliminate the residual glutaraldehyde. The resultant DBMs were collected and washed as mention above.

The amount of doxorubicin loaded with 1 mg of BMs was 0.87 ± 0.08 mg. It was convenient for analysis to assume the concentration of DOX carried by DBMs to be that of DBMs and to assume 1 mg/ml BMs actually contain 1 mg/ml of DOX and 1 mg/ml BMs actually contain 1/0.87 mg/ml of BMs. DOX, DBMs and BMs
used in vivo were prepared with 0.01 M sterilized PBS and the concentrations of them were adjusted to 1 mg/ml based on the assumption.

2.3. Examination of the DBMs

The DBMs and BMs were examined by TEM (JEM-1230, JEOL, Tokyo, Japan) and AFM (NanoScopeIIIa SPM, Digital Instruments, Santa Barbara, CA). Zeta potential was measured by Zetasizer 3000HS (Malvern Instruments Ltd., Malvern, UK).

2.4. DOX release studies

The DOX release test was performed in 24-well plate. DBMs (75 μg) were suspended in 1.5 ml PBS (0.01 M) buffer which contained 50% FBS in each well. The DOX content in the supernate of each well was evaluated by UV–VIS spectrophotometry after incubation for 2, 24 and 48 h. The solution was then removed by centrifugation (2000g for 5 min) following a 48-h incubation. The pellets were washed by distilled water and dissolved in 1.5 ml 50% ethanol solution supplemented with 10% hydrochloric acid for measurement of DOX content. All assays were performed in quadruplicates.

2.5. MTT assays

The MTT assay was performed to determine the cytotoxicity based on previously described method with minor modifications [28]. In brief, 200 μl of H22 cell suspensions (1×10^4 cells) were added to 96-well round-bottomed plates (Corning Incorporated Costar® 3799, Corning, NY), and each plate was incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere. Following incubation, 20 μl of reagent per well with different dose for experiment or complete medium for control were distributed in the 96-well plates, and each plate was incubated for 24 h at 37 °C in a humidified environment with 5% CO₂. Following the incubation, 20 μl of 5 mg/ml MTT solution (dissolved in PBS and sterilized by filtration) was added to each well and the cultures were incubated in dark for 4 h at 37 °C in a humidified environment with 5% CO₂. The culture medium was removed from the wells and replaced with 150 μl of DMSO after centrifuged at 2000g for 5 min with a centrifuge (Sorvall Legend RT, Newtown, CT). The absorbance of each well was measured at 570 nm with Bio-Rad Automated EIA Analyzer (Bio-Rad Laboratories, Hercules, CA) after the plates were incubated for 30 min at 37 °C. The cell inhibition rate was calculated using the following formula: cell inhibition rate = [1 – (absorbance of experimental wells)/ (absorbance of control wells)] × 100. All assays were performed at least three times in quadruplicates to determine the IC₅₀. The IC₅₀ was calculated from the dose responsibility using the method of Bliss [29].

2.6. Cell growth

One milliliter of the H22 cell (2×10⁵ cells) was added to each well of 24-well plates and was incubated with 8 μg of reagents (in quadruplicates). The number of the intact cells was counted every 24 h by a hemacytometer. Four wells were selected to determine the cell activity for each reagent every 24 h.

2.7. RT-PCR assays

For gene expression studies to elucidate the antitumor mechanism of DBMs, 1 ml of target cell suspensions (2×10⁵ cells) was seeded to each well of 24-well plates for 24 h and was then incubated with 8 μg of reagents for 24 h. Cells were harvested by centrifuging at 2000g for 5 min. Total cellular RNA was isolated using the Qiagen RNaseasy Mini Kit (Cat# 74104) based on silica-gel–membrane purification. RNA quality was checked by agarose gel electrophoresis and quantification was performed by densitometric analysis at 260 nm. Purified RNA was stored at −80 °C until RT-PCR.

The mRNA levels of c-myc and β-actin were determined by using a RT-PCR assay kit (Cat. No. A3500, Promega Corp., Madison, WI). A Perkin-Elmer 9600 thermal cycler was used for the amplification. For these studies, approximately 2.0 μg of total RNA was preincubated for 10 min at 70 °C and then mixed in a 40 μl final volume of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 5 mM MgCl₂, 1 mM of each deoxyribonucleotides (dNTPs), 1 U of Recombinant Rnasin Ribonuclease Inhibitor, 30 U of AMV Reverse Transcriptase and 1.0 μg of Oligo(dT)₁₅ primers. The mixture was incubated for 60 min at 42 °C followed by 5 min at 94 °C. The RT-Products obtained were stored at −20 °C. PCR amplification was carried out in a 50 μl final volume of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.8 mM MgCl₂, 0.16 mM of each deoxynucleotides (dNTPs), 1.25 U of Taq DNA polymerase, 1 μl of RT-Products and 0.4 μM of specific primer pairs as given in the following paragraph. The PCR amplification consisted of an initial incubation at 94 °C for 5 min followed by 35 amplification cycles (94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s). The PCR products were separated on an agarose gel containing ethidium bromide, and the intensity of each product was determined by Gene tools from SynGene.

All the primers for PCR were obtained from AuGCT Biotech Co., Ltd. (Beijing, China). Primers for β-actin were designed with Primer PREMIER 5.0 software. Primers for c-myc were got from reference [30] and the length was different between the PCR product of DNA and RNA. β-actin sense-strand was 5‘-CTTTGATGTGCAGCAGATTT-3’ and β-actin antisense-strand was 5‘-TGTCCTGTAGCCTCGTGC-3’, which yields 222 bp product. c-myc sense-strand was 5‘-AGTGCAATTGACCGATTTT-3’.
CCTCAGTGGTCTTTCCCCTA-3' and c-myc antisense-strand was 5'-CAGCTCGTTCCCTCCTGACGTTC CAAGACGTT-3', which yields 548 bp product.

2.8. Determination of in vivo antitumor effect

Antitumor activity against a solid tumor mass was evaluated in BABL/c mice. Ten days after receiving tergal s.c. inoculation of \(1 \times 10^6\) H22 cells prepared as described in Section 2.1, 40 BABL/c mice were randomly selected and divided into four groups (n = 10) for drug injection. Drugs were administered s.c. adjacent to (not injected into) tumors, with a schedule of q4d\(\times\)3 (on days 10, 14 and 18) in a volume of 0.1 ml (10 mg/kg drugs). The control group received sterile PBS injection. All mice were sacrificed on day 34 and tumors were taken out and weighed. The tumor and heart of mice were collected for pathological sectioning with hematoxylin–eosin (HE) stain. Tumor volume and body weight were measured prior to drug injection and sacrifice of mice. Tumor volume size at the initiation of therapy in these 40 animals was 265.4 ± 153.3 mm\(^3\). Relative tumor growth rate was expressed as the change in the tumor volume. \(R_t\) is the relative tumor growth rate in each drug-treated mouse on day 34 and \(R_c\) is the one in each mouse of control group. \(R_t\) and \(R_c\) were arranged and numbered, respectively, in a sort ascending order among their groups. And the antitumor activity of the treatments was evaluated in terms of inhibition rate (IR), which was calculated as \(IR\) (%) = \((1 - R_t/R_c)\times100\), where the sequence number of \(R_t\) was same as that of \(R_c\).

2.9. Statistical analysis

SPSS 12.0 for windows (SPSS Inc., Chicago, IL) was used for statistical analysis. All of the values are expressed as means ± SE. The IC\(_{50}\) was calculated by Bliss method [29]. Comparisons within groups were made using one-way ANOVA, and differences between groups were determined by Scheffe’s test. \(P < 0.05\) was considered to be significant.

3. Results

3.1. Characterization of DBMs

Electron microscopic image revealed a clearly identified contour of DBMs with a distinct membrane outside of BMs. The size and morphology of DBMs were uniform similar to BMs. However, DBMs were surrounded by thick nebulous materials which were presumed to be DOX-loaded (Fig. 1). Atomic force microscopic examination displayed consistent results in that the diameter of DBMs was 71.02 ± 6.73 nm, larger than that of BMs (34.93 ± 8.24 nm). The zeta potential of DBMs and BMs in PBS were −8.5 and −24.4 mV, respectively.

3.2. DOX release from DBMs

Table 1 displayed that DOX content in the supernate containing serum was significantly higher than that of PBS after incubation for 24 or 48 h, with a further increase following prolonged incubation. These data showed that DOX was slowly released from DBMs in serum containing solution. Furthermore, all DBMs retained at least 80% of the original DOX load following a 48-h incubation, indicating that DBMs were relatively stable in circulatory system and DOX may not be completely released before reaching the target sites.

3.3. Influence of DBMs on tumor growth

Both DBMs and DOX significantly suppressed tumor growth. The tumor mass of the DBMs (n = 8) and DOX group (only two mice survived) were both significantly reduced compared with control (n = 10) and BMs groups (n = 10) following a 24-day treatment. The tumor inhibition rate (IR) of the DBMs group (86.8%) was comparable to that of the DOX group (78.6%) and was much higher than that of the BMs group (4.3%) (Fig. 2). The photographs of tumors indicated that tumors in the DBMs and DOX treatment groups were effectively confined with few surrounding angiogenesis (Fig. 3a). Abundant particles of DBMs were found around the tumor of
the DBMs-treated group from which DOX could be extracted despite the fact that the latest injection of DBMs was performed 16 days earlier. To the contrary, tumors of both BMs and control groups grew well with abundant blood vessels around. Pathological examinations of tumors displayed similar results (Fig. 3b). There were a mass of necrotic cells in tumors of the DOX and DBMs groups with only a few normal residuals. On the other

Table 1
The results of the DOX release test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DOX content (μg)</th>
<th>DBMs</th>
<th>Supernate</th>
<th>Deposit</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>PBS</td>
<td>75</td>
<td></td>
<td>3.48 ± 0.20</td>
<td>2.00 ± 0.20</td>
</tr>
<tr>
<td>50% FBS</td>
<td>75</td>
<td></td>
<td>3.45 ± 0.30</td>
<td>4.59 ± 0.17</td>
</tr>
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*P < 0.05 compared with that of PBS (Scheffe’s test).

Fig. 2. Effect of DBMs on tumor growth. IR value was calculated with relative tumor growth rate as per Section 2. Means ± SE, #P < 0.01 vs. DBMs group (Sheffe’s test).

Fig. 3. Photographs of the tumors (a; scale, 1 mm) and photomicrograph (original magnification, 400×) of pathological sections (HE stains) from tumors (b) and hearts (c). The arrows showed the DBMs.
hand, tumor cells of the BMs and control groups grew relatively well with some nuclei in pathological fissile phase and abundant vessels in the tumors.

3.4. Influence of DBMs on mortality and cardiotoxicity

DOX-induced toxic side effect was significantly higher than that of DBMs. Following drug injection, the DOX-treated mice displayed lethargy and were mostly dead with only two survivors. Interestingly, however, only two mice were dead in the DBMs-treated group and none was dead in other groups. The myocardium of DOX-treated mice was partially necrotic with phagocyte infiltration. Numerous neonatal capillary vessels which were haematose were found in myocardium (Fig. 3c). Cytoplasmic vacuolization due to dilation of the sarcotubules and loss of myofibrils was observed in DOX group, which was the main cardiomyopathy features induced by DOX [25–27]. To the contrary, cardiac muscle cells of the DBMs, the BMs and the control groups were largely found normal.

3.5. Influence of DBMs on cytotoxicity in response to drug concentration

The cytotoxicity of DOX, DBMs and BMs in H22 cells were all strengthened with increase in drug concentration (Fig. 4a). The IC₅₀ value (µg/ml) of DOX, DBMs and BMs in the target cells were 5.309 ± 0.010, 4.652 ± 0.256 and 22.106 ± 3.330, respectively. The IC₅₀ value of DBMs was comparable to that of DOX and significantly smaller than that of BMs (Fig. 4b).

3.6. Influence of DBMs on cytotoxicity in response to incubated time

Experiments on cytotoxicity in response to incubated time indicated that rapid proliferation and little inhibition in cells treated with BMs or control groups. On the other hand, the target cell growth was significantly inhibited by DBMs and DOX (Fig. 4c).

3.7. Influence of DBMs on c-myc gene expression

The main antitumor mechanism of DOX is that DOX represses the duplication, especially the transcription of DNA. Therefore, the influence of DBMs on the expression of c-myc was evaluated in an attempt to elucidate the antitumor mechanism of DBMs. β-actin was also monitored as an internal control for the RT-PCR assays. RT-PCR results indicated that DBMs effectively suppressed the c-myc mRNA levels in the H22 cells, in a manner reminiscent of DOX. BMs itself elicited less effect on c-myc expression (Fig. 5).
4. Discussion

BMs have drawn much attention since their ultrastructural details were first described in 1980 [11]. The unique features of BMs make them excellent candidates for magnetic-targeted drug carrier [12,13]. Our preliminary study showed efficient in vitro antitumor effects of DBMs in EMT-6 and HL60 cells (Sun JB et al., unpublished data). In this study, the tumor suppressant and cardiac morphological studies demonstrated that DBMs were effective for HCC with lesser cardiac toxicity compared with DOX. The tumor inhibition rates of DBMs, DOX and BMs were 86.8%, 78.6% and 4.3%, while the drug-induced mortality was 20%, 80%, 0%, respectively. Pathological examination of heart and tumor revealed that both DBMs and DOX inhibited tumor growth with a much lower cardiac toxicity from DBMs.

The DBMs were certainly cytotoxic to H22 cells with inhibition of cell proliferation and suppression in c-myc expression, comparable to DOX. The IC_{50} value (µg/ml) of DOX, DBMs and BMs were 5.309 ± 0.010, 4.652 ± 0.256 and 22.106 ± 3.330, respectively. Compared with DOX, the main advantages of DBMs are magnetic targeting (the magnetic property of BMs in DBMs has long been known) and slow releasing, which should reduce the amount of drugs used and adverse effects [3,6], which is supported by our current data.

Target delivery of DBMs to the tissues of interest is an important issue needing in-depth investigation [31,32] although it is beyond the scope of our current study. For solid tumors, local delivery of drugs is preferred as oppose to vascular injection since the drug will directly interact with the tumor mass. In this study, DBMs were administered subcutaneously adjacent to (not injected into) the tumor which should make it harder to be diffused or transported elsewhere. This maneuver seemed to mimic drug targeting as the result indicated that the DBMs injected appeared to target to the tumor by a magnet. DBMs may concentrate and retain the DOX in the focus when they are administrated via local injection. Although the latest injection of the DBMs was performed 16 days prior to animal sacrifice, abundant particles of DBMs were found around tumors of the DBMs group from which DOX could be extracted. These findings suggested that the DOX coupled with BMs may be slowly released in vivo partly courtesy of the aggregation of DBMs. Our additional data on drug release elucidated that DOX was released slowly from DBMs in solutions containing 50% of serum and all DBMs remained at least 80% of the original DOX following incubation for 48 h. This may allow DOX to interact with the tumor for a long time with minimal side effect on normal tissues.

DOX has severe tissue toxicity and is only administered clinically by i.v. injection or in the case of ovarian carcinomas, i.p. injection [33,34]. The tissue toxicity was observed in this study when the DOX was injected by s.c. which presumably caused the death of the mice since three injections at 10 mg/kg by i.v. in most mice is not a lethal dose of DOX. It may be too small for statistical analysis that only two mice remained alive in the DOX group but it would not weaken the power of statistical significance that the DBMs inhibited the tumor growth. The reasons for the injection of DOX by s.c. were as follows: (1) As a control of the DBMs, DOX had to be injected as same as the DBMs; (2) If the DOX was released slowly from the DBMs in vivo, the tissue toxicity of DBMs should be lower than DOX, which would be easily observed if they were

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Fig. 5. Effect of DBMs on c-myc expression. A representative gel picture from three independent experiments is shown in (a) and (b), and the expected product sizes are depicted in parenthesis. The intensity of each product was determined using Gene tools from SynGene and the values of the c-myc mRNA levels showed in (c) were normalized to the respective β-actin samples. The error bars represent ±SE of values. *P < 0.05; **P < 0.01 compared with the CK group (Sheffe’s test).
their high drug loading capacity and lower size. Approximately 870 μg DOX was loaded onto 1 mg carriers while the other known carriers may only load up to 140 μg DOX. The size of DBMs was <100 nm while the others are usually bigger than 100 nm [35,36].

It should be stated that a thorough understanding of the pharmacokinetics of DBMs especially how DBMs are targeted and metabolized in the body following i.v. administration is equally important as the determination of their antitumor effect given that it is another research area. It is unknown whether BMs can be readily eliminated from the body after complete release of DOX from the DBMs and whether a long time retention of BMs in body is safety. Obviously, it warrants a thorough study before DBMs can be considered clinically.

Last but not the least, it is essential to determine the biocompatibility of BMs since BMs were isolated from bacterial cells surrounded with membrane that consists of lipids and proteins. Our earlier study regarding evaluation of the purity and safety of BMs indicated that the purified and sterilized magnetosomes are not toxic to mouse fibroblasts in vitro [37]. Recently, we have developed novel bacterial magnetic particles (BMPs)-polyethylene-limenine (PEI) gene delivery system with a high transfection efficiency and low toxicity to BHK-21 cells, HeLa cells and CHO cells [38]. Assessment of the acute toxicity and immune toxicity of BMs showed a good biocompatibility (data not shown). Despite of the promising results from our current investigation, there are still a plethora of practical issues which may be difficult to reconcile for the ultimate use of DBMs for the novel target-therapy in cancer management.

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