Bovine Serum Albumin-Doxorubicin Conjugate Overcomes Multidrug Resistance in a Rat Hepatoma

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ABSTRACT

A bovine serum albumin-conjugated doxorubicin via the glutaraldehyde bridge (BSA-DXR conjugate) showed potent dose-dependent inhibition of cell growth against daunorubicin-resistant AH66 (AH66DR) cells as well as parental AH66 (AH66P) cells in vitro as compared to treatment with DXR or BSA-glutaraldehyde conjugate without DXR (BSA-GA). In the culture of AH66DR with BSA-DXR conjugate, drug accumulation in the AH66DR cells increased as a function of time up to 24 h reaching approximately the same drug level as AH66P cells treated with DXR. The intracellular accumulation of the BSA-DXR conjugate was inhibited by the addition of ammonium chloride, while that of DXR alone was not inhibited. Intracellular DXR was effluxed rapidly from AH66DR cells, but BSA-DXR conjugate or pharmacologically active DXR adduct remained in the cells at a relatively high concentration over a 36-h time period. The life-prolonging effect of the conjugate was assessed using rats inoculated i.p. with AH66P or AH66DR. The rats were treated with the BSA-DXR conjugate, DXR, a mixture of DXR with BSA, or BSA-GA by either the i.p. or i.v. route. Treatment with DXR had no significant surviving effect as compared to that with saline in AH66P-bearing rats. By contrast, BSA-DXR conjugate showed a significant life-prolonging effect as compared with DXR alone in the same degree both in AH66P- and AH66DR-bearing rats. BSA-GA did not show any toxicity in vivo as well as in vitro. These results indicate that the BSA-DXR conjugate allows DXR to escape from the multidrug resistance mechanism.

INTRODUCTION

Resistance of tumors to a variety of chemotherapeutic agents is a major obstacle to successive cancer chemotherapy. Increased drug excretion out of tumor cells is often noted when tumors acquire resistance to anticancer drugs. The mechanism underlying chemoresistance has been associated with overproduction of the drug-efflux pump called Pgp (1–3). Various attempts to overcome multidrug resistance have been performed. These include cotreatment with a calcium antagonist to block the efflux pump of Pgp (4–8) and the use of i.v. route. Treatment with DXR had no significant surviving effect as compared to that with saline in AH66P-bearing rats. By contrast, BSA-DXR conjugate showed a significant life-prolonging effect as compared with DXR alone in the same degree both in AH66P- and AH66DR-bearing rats. BSA-GA did not show any toxicity in vivo as well as in vitro. These results indicate that the BSA-DXR conjugate allows DXR to escape from the multidrug resistance mechanism.

MATERIALS AND METHODS

Cell Line. The azo dye-induced rat ascites hepatoma cell line, AH66P, and the daunorubicin-resistant mutant subline (AH66DR) were maintained in RPMI 1640 as described previously (15) and this subline showed cross-resistance to DXR (14).

Preparation of the Conjugate of BSA and DXR. Conjugation of DXR with BSA were carried out according to the method of Hurwitz et al. (16). The degree of substitution was estimated by the drug absorbance at 495 nm by radioactivity. Protein concentration was measured by a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). The conjugate of glutaraldehyde to BSA without DXR was also prepared.

Drug Sensitivity Assay. To assess drug resistance both types of AH66 viable cells (2 × 10⁶) were cultured continuously for 96 h in 24-well culture plates (Corning, Corning, NY) with 1 ml of growth media containing a graded concentration of DXR. To determine the effects of the conjugate and other controls, viable cells (2 × 10⁶) were also cultured with 1 ml of growth media containing various concentrations of DXR or conjugates at equivalent concentrations of DXR or BSA-GA conjugate at an equivalent BSA concentration. To investigate the mechanism responsible for the cytotoxicity of the drug conjugate, ammonium chloride (10 mM final concentration) known as lysosomotropic amine, was added to culture medium containing AH66DR cells 30 min before addition of either DXR or the BSA-DXR conjugate. After 96 h incubation, the viable cell number in drug-exposed cells was counted and the results were expressed as the increase in cell numbers of drug-exposed cells as a percentage of the control cells.

Uptake of DXR or BSA-DXR Conjugate. Viable AH66DR or AH66P cells (1 × 10⁶) were incubated with 1 μM [14C]DXR (specific activity, 27,500 dpm/μg) or BSA-[14C]DXR conjugate (specific activity 44,700 dpm/μg) in growth medium (2 ml) in culture tubes (Corning No. 25200) with without ammonium chloride (10 mM final concentration) added 30 min before treatment. The incubation was terminated by thorough washing with ice-cold 0.15 M saline by centrifugation. After the viable cell numbers were counted, the cells were lysed by NCS (Amersham Japan, Tokyo, Japan) and the amount of intracellular [14C]DXR radioactivity was measured using a liquid scintillation counter. The viability of cells was assessed using a Trypan blue dye exclusion method.

Efflux of DXR or BSA-DXR Conjugate. For loading studies of DXR in AH66DR or AH66P cells, both cells were cultured with 1 μM [14C]DXR in Hanks' balanced salt solution for 1 h. Sodium azide (5 mM final concentration) was added to the culture before addition of DXR and not removed during drug treatment. For loading studies of the BSA-DXR conjugate in the cells, AH66P and AH66DR cells were cultured with 1 μM BSA-[14C]DXR conjugate in culture media for 24 h. The exposure of the cells for 24 h was necessary because of slow accumulation of the conjugate. The drug-loaded cells were detached by pipetting and were recovered by centrifugation, resuspended in 1 ml of growth media (1 × 10⁶ cells), and incubated for various periods of time. After the cells were washed with ice-cold saline, their radioactivity was measured by the method described above.

Determination of Intracellular Degradation Products of the Conjugate in Cultured Cells. AH66DR cells (1 × 10⁶) were cultured with 1 μM [14C]DXR conjugate in growth medium for 18 or 36 h. The cells were dissolved in 1 ml of 10 mM sodium phosphate buffer, pH 7.0-1% SDS. A small amount of unlabeled DXR (50 μg) was added as the carrier for [14C]DXR. After the cell lysis, the extract was then loaded on a Sephadex G-25 column (0.8 x 25 cm, Pharmacia, Uppsala, Sweden) preequilibrated with the same growth medium.
buffer. An aliquot was collected and radioactivity was counted by a liquid scintillation counter. The unlabeled DXR was detected by the measurement of absorbance at 495 nm. The spent culture medium was also fractionated by the same Sephadex G-25 column equilibrated with the growth medium instead of the extraction buffer and radioactivity was counted.

In Vivo Therapy. Either AH66P or AH66DR cells (1 × 10^6 cells/rat) were inoculated i.p. into male Donryu rats (mean body weight, 110 g at Day 1; Clea Japan, Tokyo, Japan). The rats were treated with an i.p. (for Experiment A) and an i.v. (for Experiment B) injection of test materials every alternate day starting from Day 3 for a total of 3 and 4 doses, respectively. Test materials were as follows: Experiment A, saline 0.5 ml, DXR 17 µg/rat, or BSA-DXR conjugate at equivalent doses of DXR; and Experiment B, saline 0.5 ml, DXR 26 µg/rat, BSA-GA 740 µg/rat, mixture of BSA and DXR (BSA + DXR) 740 + 26 µg/rat, or conjugate at equivalent doses of DXR. The 50% lethal doses of i.p. and i.v. administration of DXR are 16.0 and 13.1 mg/kg, respectively. The effect of the drug was evaluated by the survival time. Body weights of each group of rats were checked weekly for toxic side effects.

SDS-PAGE and Western Blotting Analysis. Cell extracts diluted in SDS-PAGE sample buffer were separated by SDS-PAGE followed by electrophoresis on nitrocellulose paper as reported previously (17). The paper was incubated with mouse anti-Pgp monoclonal antibody (C219; Centocor, Malvern, PA) followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin (Bio-Rad). Bands were visualized by enhanced chemiluminescence (Amersham).

Drugs and Chemicals. DXR was kindly provided from Farmitalia-Carlo Erba (Tokyo, Japan). BSA and glutaraldehyde were purchased from Sigma (St. Louis, MO). [3H]DXR (specific activity, 55 mCi/mmol) was purchased from Amersham. All other chemicals were of analytical grade.

Statistical Analysis. Fisher's exact test was used.

RESULTS

DXR Sensitivity of AH66DR Cells. In the culture with continuous exposure to DXR for 96 h, the AH66DR cell line was approximately 200 times more resistant to DXR than the AH66P line (AH66DR IC50 of 16 µM versus AH66P IC50 of 0.08 µM) and exhibited overproduction of Pgp in the cell membranes (Fig. 1).

DXR Conjugate to BSA. The extent and substitution varied in different preparations and the conjugates with the molar ratio of 3.28 or 4.10 mol DXR/mol BSA were used in the present study.

![Fig. 1. SDS-PAGE Western blotting analysis of Pgp in the AH66P (P) and AH66DR (DR) cell extracts. kDa, molecular weight markers in thousands.](image)

Cytotoxic Effect of BSA-DXR Conjugate on AH66DR Cells. The cytotoxic activity of BSA-DXR conjugates to AH66DR cells is shown in Fig. 2. The BSA-DXR conjugate showed potent and a dose-dependent growth-inhibitory effect against AH66DR cells as compared with that of DXR or BSA-GA. Phase-contrast microscopic examination showed that dead cells gradually increased and were easily detectable after 48 h of incubation with the conjugate. The IC50 for BSA-DXR conjugate in the AH66DR cell line was 0.05 µM at an equivalent DXR concentration. The growth inhibition of the conjugate was almost equivalent to that of the IC50 (0.08 µM) for DXR in the AH66P cell line. The BSA-DXR conjugate also showed potent cytotoxic activity against AH66P cells (Fig. 2; IC50 0.004 µM). Pretreatment with ammonium chloride did not increase or decrease the effects of free DXR but moderately blocked the growth-inhibitory effects of the conjugate (Fig. 2).

Cellular Uptake and Accumulation of the Drugs. Drug uptake in AH66P and AH66DR cells was evaluated as shown in "Materials and Methods." Within 1 h of treatment with DXR, the drug was accumulated to an approximately 2-fold higher level (P < 0.05) in sensitive cells than in resistant cells and these differences were maintained over 36 h. By contrast, when the resistant AH66DR cells were treated with BSA-DXR conjugate at doses that were equivalent to DXR, a relatively lower accumulation of the drug was observed within 1 h of treatment. The uptake of the BSA-DXR conjugate in AH66DR cells then increased significantly (P < 0.05) over a 24-h time period and approximately the same level of DXR in AH66P cells (equivalent concentration) was reached. Moderate to slight inhibition of the uptake of the conjugate (67.2–92.3% over a series of experiments) was observed when AH66DR cells were cocultured with ammonium chloride-containing medium (Fig. 3). By contrast, there was no inhibition of DXR uptake between AH66DR or AH66P cells which were cocultured with ammonium chloride.

Efflux of the Drugs. Efflux of the conjugate from AH66DR cells (initial radioactivity, 38,000 dpm/10^6 cells) was very slow with >90% of the initial concentration of the drug remaining in the cells. By
BSA-DXR CONJUGATE OVERCOMES MDR

Fig. 3. Uptake of DXR or conjugate (equivalent concentration of DXR) in AH66P or AH66DR cells at different periods of time. Columns, mean of triplicate determinations of 2 independent examinations; bars, SD. Both cells treated with DXR (□) or with BSA-DXR conjugate (□), both cells treated with DXR plus ammonium chloride (□) or BSA-DXR conjugate plus ammonium chloride (□).

contrast, very rapid transport of DXR outside of the AH66DR cells (initial radioactivity, 24,000 dpm/10⁵ cells) to the extent of approximately 70–75% was observed. The velocity of the outward transport of DXR as well as BSA-DXR from AH66P cells (initial radioactivity, 23,000 dpm/10⁵ cells for DXR and 31,000 dpm/10⁵ cells for conjugate) was slow, but the efflux of the conjugate was slightly less than that of free DXR (Fig. 4).

Degradation of the Conjugate in the Cells. The elution profiles are shown in Fig. 5. The extract from the cells treated for 18 h with the conjugate showed only one radioactive peak at void volume. By contrast, the extract from the cells treated for 36 h showed two additional peaks of radioactivity representing about 12 and 14% of the total, which eluted at post-void volume and close to but somewhat before the peak of DXR, respectively. These intermediate and small molecular weight materials have not yet been identified but are believed to be breakdown products of the conjugate. The elution profiles of the culture media obtained from the treatment of the cells for 36 h as well as for 18 h with BSA-DXR conjugate showed only one peak at void volume (data not shown).

In Vivo Efficacy of BSA-DXR Conjugate. As shown in Fig. 6 and Table 1, treatment of AH66P tumor-bearing rats with DXR prolonged their MST as compared with the control group (MST, DXR group: Experiment A, 36.3 ± 13.8 (SD) versus 19.7 ± 3.7 days for controls; Experiment B, 33.5 ± 8.9 versus 19.4 ± 1.6 days for controls). DXR treatment did not show any significant life-prolonging effect in AH66DR-bearing rats (MST: Experiment A, 29.3 ± 2.8 days; Experiment B, 25.5 ± 3.0 days) as compared to that in the control group of rats which received saline (MST: Experiment A, 23.7 ± 4.4 days; Experiment B, 23.7 ± 3.9 days). The BSA-DXR conjugate was the most efficacious in the groups of rats inoculated not only with the drug-resistant AH66DR cells but also with the parent AH66P cells in both experiments. In Experiment A, 5 of 10 rats bearing AH66DR cells and 4 of 10 rats bearing AH66P cells were 70-day survivors with the MST of the dead rats being 50.4 ± 13.7 and 54.3 ± 15.3 days, respectively. In Experiment B, 3 of 10 rats bearing AH66DR cells and 4 of 10 rats bearing AH66P cells were 70-day survivors with MST of the dead rats being 49.4 ± 12.1 days and 49.5 ± 10.9 days, respectively.

Fig. 4. Efflux of DXR (△, □) or BSA-DXR conjugate (equivalent concentration of DXR, △, □) in AH66P (▲, ▼) or AH66DR (△, □) cells at different periods of time. Results were expressed as a percentage of the intracellular radioactivity at 0 time as compared to the radioactivity at various time intervals of reincubation. Points, mean of duplicate determinations of 2 independent examinations; bars, SD.

Fig. 5. Intracellular degradation of BSA-DXR conjugate by AH66DR cells exposed for 18 (A) or 36 h (B) to 1 μM BSA-14C-DXR conjugate. Cell lysates were chromatographed on a Sephadex G-25 column as described in “Materials and Methods.” Void and DXR indicate the void volume and elution of unlabeled carrier-DXR, respectively.

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were inoculated i.p. with 1 × 10⁴ AH66P (○, △, ▽) or AH66DR (□, □, ●) ascites hepatoma cells on Day 1 (∆). In Experiment A, on Days 3, 5, and 7 (▲) rats received an i.p. injection of one of the following: ○ or ◼, 0.15 M NaCl solution; □ or □, 17 μg/rat of DXR; △ or ●, 17 μg (equivalent doses of DXR)/rat of BSA-DXR conjugate. In Experiment B, on Days 3, 5, 7, and 9 (▲) rats received an i.v. injection of one of the following: ○ or ◼, 0.15 M NaCl solution; small ○ or small ◼, 740 μg/rat of BSA; △ or ●, 740 μg + 26 μg/rat of BSA + DXR mixture; ▲ or ▲, 26 μg (equivalent doses of DXR)/rat of BSA-DXR conjugate.

Survival when compared to all other groups. In Experiment B, BSA + DXR prolonged the lives of AH66P tumor-bearing rats as well as those of AH66DR-bearing rats, whereas BSA-GA showed essentially no life-prolonging activity against both groups of rats bearing AH66P or AH66DR cells. BSA-GA also showed no cytotoxic effect against age- and sex-matched normal rats (without tumor inoculation, data not shown). No remarkable loss of body weight was found in any of the treated groups of rats.

DISCUSSION

In the present study, we have demonstrated that chemical modification of DXR conjugated with BSA effectively increased its cytotoxic activity against DXR-resistant AH66DR cells. Additional studies showed that BSA-DXR conjugate-treated cells had higher intracellular DXR concentrations over a long period of time and that there was also a slow diminution of intracellular drug levels in the drug-resistant AH66DR cells. It is somewhat difficult to compare the velocity of the BSA-DXR conjugate efflux with that of DXR efflux. This is because long-term incubation of the AH66DR cells with conjugate is required to load enough drug into the cells to cause cell killing. These experiments illustrate an important pharmacokinetic principle for drug conjugates, which is that a longer time is needed for higher accumulation and slow efflux of the conjugate to occur in multidrug-resistant cells. This conjugate was also cytotoxic to AH66P cells. The therapeutic efficacy of the BSA-DXR conjugate was assessed in vivo in rats inoculated i.p. with AH66 tumor cells. The conjugate treatment, which was given by the same route, i.p., or systemic route, i.v., prolonged the life of rats bearing the multidrug-resistant subline, AH66DR cells, as well as the parent line, AH66P. In fact DXR treatment had no effect on rats inoculated with the drug-resistant tumor cell (AH66DR). The BSA-DXR conjugate undoubtedly exhibited the cytotoxicity based on the anticancer drug, DXR, but the immune response derived from BSA as a foreign antigen in rat, because the BSA-GA without DXR did not show any toxic activity either in vitro or in vivo. It was of interest that the drug conjugate also exhibited the excellent therapeutic activity against AH66P cells when compared to DXR. This result is due to the fact that the Pgp was expressed frequently on the cell membranes of regenerating active liver cells or hepatocellular carcinoma cells (18–22).

The addition of ammonium chloride to the medium moderately reduced the cytotoxic effect of the conjugate as well as the intracellular accumulation of the conjugate. This may have been a secondary effect in relation to the inhibition of drug uptake. However, it undoubtedly suggests that the growth-inhibitory effects of the conjugate involve endocytosis of the conjugate and this may be critically dependent on the low pH of the postendocytotic compartment. Furthermore, on the basis of gel filtration of the cell extract, data have been obtained suggesting that the digestion of the uptaken BSA-DXR conjugate and the liberation of the pharmacologically active adduct of DXR “without any interaction to Pgp” may take place inside the cell and not in the culture medium. Confirmation of subcellular distribution of the uptaken conjugate and its adduct is necessary to clarify that underlying mechanism(s), but our results indicate that the intracellular pharmacokinetics of the chemically modified DXR is different from that of free DXR, which is actively excreted from the cytoplasm by an energy-dependent pump mechanism (1–3, 7). The need for a longer time to reach higher accumulation of the conjugate inside the cell compared with that of free DXR supports this evidence. It has been reported that the conjugates were taken up by endocytosis of the plasma membrane into the cytoplasm and metabolized in the lysosomes (23–29). We assume that the in vitro and in vivo effectiveness of the BSA-DXR conjugate shown in AH66DR cells is via a similar mechanism.

The improved drug sensitivity of multidrug-resistant cells to DXR conjugated with specific antibody against the cells or to DXR-loaded

Table 1 In vivo effect of the drugs on tumor-bearing rats

<table>
<thead>
<tr>
<th>Agent*</th>
<th>Route</th>
<th>Tumor</th>
<th>MST (days)b</th>
<th>Cured rats</th>
</tr>
</thead>
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<tr>
<td>Saline</td>
<td>i.p.</td>
<td>AH66P</td>
<td>19.7 ± 3.7</td>
<td>0/10</td>
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<td></td>
<td></td>
<td>AH66DR</td>
<td>23.7 ± 4.4</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>AH66P</td>
<td>19.4 ± 1.6</td>
<td>0/10</td>
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<tr>
<td></td>
<td></td>
<td>AH66DR</td>
<td>23.7 ± 3.9</td>
<td>0/10</td>
</tr>
<tr>
<td>DXR</td>
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<td>AH66P</td>
<td>36.3 ± 13.8</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH66DR</td>
<td>29.3 ± 2.8</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>AH66P</td>
<td>33.5 ± 8.9</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH66DR</td>
<td>25.5 ± 3.0</td>
<td>0/10</td>
</tr>
<tr>
<td>BSA-DXR</td>
<td>i.p.</td>
<td>AH66P</td>
<td>54.3 ± 15.3</td>
<td>4/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH66DR</td>
<td>50.4 ± 13.7</td>
<td>5/10</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>AH66P</td>
<td>49.5 ± 10.9</td>
<td>4/10</td>
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<td></td>
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<td>AH66DR</td>
<td>49.4 ± 12.1</td>
<td>3/10</td>
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<td>BSA + DXR</td>
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<td>27.3 ± 3.0</td>
<td>0/10</td>
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<tr>
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<td>19.2 ± 1.7</td>
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<td>AH66DR</td>
<td>23.4 ± 3.9</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Dose of each test material is indicated in Fig. 6 and in "Materials and Methods."

a Mean survival times (± SD) were calculated as total surviving days prior to death in rats before 70 days of experiment/number of dead rats.

b i.p. administration for Experiment A.

c i.v. administration for Experiment B.
nanospheres has been reported recently (30, 31). The present report was, however, the first in vitro and in vivo demonstration that a chemically modified chemotherapeutic agent, such as a conjugate of the drug with protein, can be used successfully as a cytotoxic agent in animals bearing multidrug-resistant tumor cells. The method used to prepare the conjugate is simple and should be applicable for human use with substitution of BSA with human serum albumin or other proteins of human origin. Improved methods may make it possible to conjugate 20–50 molecules of the drugs with one molecule of protein (32–34), so that more effective tumor cell killing activity will result. Moreover, our results indicate that drug conjugates may be useful for cancer chemotherapy not only of acquired multidrug-resistant cancer cells but also of intrinsic multidrug resistant cancer cells that may be derived from cells which express the Pgp on their cell membranes, such as the biliary canalicular surface of hepatocytes, brush border of renal proximal tubules, and cell surfaces of adrenal glandular cells, etc. (35). Our results are encouraging and offer an innovative way to overcome multidrug resistance. Further investigations are planned to elucidate the mechanism(s) via which this drug-protein conjugate overcomes the multidrug resistance.

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