Antitumor Activity of Doxorubicin Encapsulated in Poly(ethylene glycol)-Coated Liposomes

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Received March 15, 1995; accepted June 6, 1995

The antitumor activity of doxorubicin (DXR) which had been encapsulated in poly(ethylene glycol) (PEG)-coated long-circulating liposomes was examined in mice inoculated with colon 26 carcinoma cells. Six mol% of the distearoylphosphatidylethanolamine derivative of PEGs with different molecular weights was incorporated in liposomes (90—110 nm, mean diameter) composed of distearoylphosphatidylcholine/cholesterol (1/1, molar ratio), and the encapsulating efficiency of DXR in liposomes was more than 98% by the pH gradient method. Each concentration of DXR in blood and tumor tissue was significantly greater after administration of the drug encapsulated in PEG-coated liposomes (DXR-PEG-liposome) compared to the non-coated control liposomes or non-encapsulated free drug. DXR-PEG-liposome prepared with PEG1000 (DXR-PEG1000-liposome) more effectively increased the level of DXR in blood and tumor than did the preparations with PEG5000 or PEG12000. A single treatment with DXR-PEG1000-liposome (10 mg DXR/kg) resulted in increased survival time. Further therapeutic improvement in terms of tumor growth retardation and prolongation of survival time were observed following multiple treatments with DXR-PEG1000-liposome (3 x 5 mg DXR/kg). Long-circulating liposome coating optimized PEGs should be useful for the delivery of chemotherapeutic agents for the treatment of solid tumors.

Key words liposome; long-circulating liposome; drug delivery system; doxorubicin; poly(ethylene glycol)

Many reports have demonstrated that the encapsulation of doxorubicin (DXR) in liposomes reduces its toxicity and enhances its therapeutic efficacy in animal systems.1—4 However, conventional liposomes are rapidly removed from the blood circulation by phagocytic cells of the reticuloendothelial system (RES).2,5 therefore, efforts have been made to formulate liposomes with a reduced affinity for the RES. It has been reported that liposomes containing ganglioside GM1 (GM1),5—7) hydrogenated phosphatidylinositol8) or polyethylene glycol (PEG)-derivatized phospholipids9—13) exhibit prolonged circulation in blood. More importantly, small liposomes of these types with a mean diameter of around 100 nm showed greater accumulation in solid tumors.7) We previously reported14) that the i.v. administration of DXR encapsulated in GM1-containing liposomes to colon 26-bearing mice produced increased DXR levels in tumors compared with conventional drug-containing liposomes or free drug. Papahadjopoulos and co-workers15) demonstrated that liposome formulations coated with amphiphilic PEG (PEG-liposomes) exhibited improved pharmacokinetics and greater antitumor therapeutic efficacy of encapsulated DXR. They also found that the extent of tumor uptake appears to be critically dependent on the vesicle size and circulation half-life of PEG-liposomes. We have shown13) that PEG-liposomes with rigid lipid composition and a controlled size of less than 300 nm exhibit high retention in the blood circulation. However, there is little evidence concerning the effects of PEGs of different molecular weight on the distribution and antitumor efficacy of encapsulated DXR.

In this report, we describe the antitumor effect of DXR-encapsulated PEG-liposomes (DXR-PEG-liposome) prepared with different molecular weights of PEGs, in terms

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MATERIALS AND METHODS

Materials Distearoylphosphatidylcholine (DSPC, COATSCONE MC-8080), distearoylphosphatidylethanolamine (DSPE) and monomethoxy polyethylene glycol succinimidyI succinates (PEG-OSu), with average molecular weights of 1000, 5000 and 12000 daltons, were kindly donated by Nippon Oil & Fats (Tokyo). A series of DSPE—PEG conjugates was synthesized according to the method reported by Klivanov et al.9) An aliquot of PEG-OSu in CHCl3/methanol (3:1, v/v) was added to a solution of DSPE in CHCl3, followed by the addition of triethylamine (3:1:3.5, mol ratio). The mixture was stirred overnight at room temperature and evaporated.

Full conversion of the primary amine group in DSPE was confirmed by the negative ninhydrin reaction after separation by TLC. A small amount of water was added to the evaporated reaction residues to form the micelles. DSPE—PEG micelles were dialyzed (Spectra-Por CE 300000 MWCO, Spectrum Medical) against water for 5 d and lyophilized. DXR was kindly provided by Kyowa Hakko Kogyo (Tokyo). Cholesterol (CH) was purchased from Wako Pure Chemicals Co. (Tokyo).

Preparation of DXR-Encapsulating PEG-Coated Liposomes Liposomes were prepared from DSPC/CH (1/1, molar ratio) and 6 mol% of DSPE—PEG derivatives with various molecular weights by means of the reverse-phase evaporation method.16) Briefly, the lipid mixture was dissolved in isopropyl ether/CHCl3 (1:1, v/v) and 300 mm citric acid (pH 4.0) was added. The mixture was sonicated with a bath-type sonicator (VS-150, Iuchi) to give a
w/o-type emulsion, and then organic solvents in the emulsion were evaporated gradually at 60°C to form a large unilamellar vesicle (LUV) suspension. The resulting LUV were extruded (EXTRUDER, Lipex, Vancouver, Canada) ten times at 60°C through Nuclepore polycarbonate filters (0.2 and 0.1 μm) to make small unilamellar vesicles (SUV). Liposome size was measured by a Nicomp 370 submicron particle analyzer (HIAC Pacific Scientific).

DXR was encapsulated into liposomes by the pH gradient method reported by Mayer et al.17 The liposome suspension was diluted with water to make a total lipid concentration of 10 mg/ml. The exterior pH of the vesicles was then titrated to 7.8 with 1.0 n NaOH to form a pH gradient across the lipid membrane. This liposomal suspension was allowed to stand at 60°C for 3 min and then combined with a pre-heated DXR aqueous solution at a DXR-to-lipid ratio of 0.2 (w/w). The mixture was heated at 60°C for 10 min with intermittent vortexing and then chromatographed on a Sephadex G-50 column pre-equilibrated with saline to remove the unencapsulated DXR and citric acid. The amount of entrapped DXR was determined with a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm, Hitachi F-3000) after dilution with 0.3 n HCl-50% ethanol.

Blood Level and Tissue Distribution Mouse colon carcinoma (colon 26) cells (1 × 10⁵) were inoculated into the hind feet of male BALB/c mice (8 weeks, 22—25 g), and the tumors were allowed to grow for approximately 8 d, at which time the mean tumor volume was around 0.25 cm³. Free or liposomal DXR was injected intravenously via the tail vein at a dose of 5 mg DXR/kg. At fixed times after the injection, blood was collected from the retro orbital sinus under light anesthesia for 24 h, and the tumor was isolated after 6 h after injection. DXR was extracted from blood and tissues as described before14 and was measured by an HPLC method.19

Tumoricidal Effect and Toxicity A single dose of DXR (10 mg/kg body weight) was injected into the tail vein at 8 days after tumor cell inoculation (ten mice per group). In the case of multiple doses, 5 mg DXR/kg in each DXR formulation was injected i.v. three times at 3-d intervals (total dose: 15 mg DXR/kg). Tumor volumes were measured as before19 and the survival time was recorded for 80 d after tumor implantation.

The lethal toxicity of DXR formulations was examined in 8-week-old healthy BALB/c male mice after a single i.v. injection of various doses, and the number of surviving mice was recorded up to 120 d after injection.

RESULTS

Characterization of PEG-liposomes Encapsulating DXR DXR was entrapped into PEG-liposomes in more than 98% of the used drug, regardless of the molecular weight of PEGs. The mean diameters of all liposome preparations were controlled to within the range of 90—110 nm. No change in liposome size was observed after encapsulating the DXR. When PEG-liposomes were stored at 4°C for 30 d, the liposome size was unchanged and the encapsulated drug had not leaked out (data not shown).

Plasma Clearance and Uptake in Tumor Tissue The elimination profile of DXR in different formulations from blood circulation was estimated in tumor-bearing mice until 24 h post-injection. As shown in Fig. 1, rapid elimination of free DXR from the blood circulation was observed within 1 h after injection. Conventional liposomes without PEG also showed low blood levels. In contrast, three different types of PEG-liposomes gave significantly increased blood levels of DXR. The blood DXR levels obtained with DXR-PEG1000-liposome appeared to be higher than those in the cases of DXR-
PEG5000-liposome and DXR-PEG12000-liposome.

Figure 2 shows the DXR levels in the solid tumor at 6 h after the administration of DXR by various formulations. Relatively high DXR levels in the tumor were obtained with PEG-liposomes, and the highest drug level was obtained after the administration of DXR-PEG1000-liposome. Thus, the DXR level in the tumor after administration of PEG-liposomes is markedly affected by the molecular weight of PEG. From Figs. 1 and 2, the retardation of plasma clearance seems to be reflected in the tumor accumulation of DXR.

**Antitumor Efficacy**  Figure 3 shows the survival curves of tumor-bearing mice after a single i.v. administration of various DXR formulations. There was no improvement in survival time after the administration of free DXR or control DXR-liposome. In contrast, the administration of DXR as a formulation of PEG-liposomes significantly increased the survival time. DXR-PEG1000-liposome, DXR-PEG5000-liposome and DXR-PEG12000-liposome increased the survival time 1.83-, 1.57- and 1.49-fold, respectively, compared with the control mice receiving saline.

The antitumor efficacy of DXR-PEG1000-liposome after multiple i.v. administration was examined. As shown in Fig. 4A, the most obvious inhibition effect on tumor growth was observed by multiple administration of the DXR-PEG1000-liposome. Free DXR, DXR-liposome and DXR-PEG1000-liposome increased the survival time 1.51-, 1.92- and 2.35-fold, respectively (Fig. 4B). These results show clearly that encapsulation of DXR in PEG-liposomes causes a marked improvement in therapeutic efficacy.

Table 1 compares the survival of non-tumor-bearing mice after the injection of various doses of free DXR or DXR-PEG1000-liposome. Eight of ten mice given 20 mg/kg of DXR-PEG1000-liposome survived, but none of the mice survived after the same dose of free DXR. It is apparent that the toxicity of DXR is reduced by its encapsulation in PEG-liposomes. Thus, PEG-liposomes offer a broadened therapeutic dosage window for DXR.

**DISCUSSION**

In recent years, novel formulations of long-circulating liposomes with reduced uptake by the RES and enhanced accumulation in tumors have been developed, expanding the range of potential applications in anti-cancer drug delivery. Encapsulation of anthracyclines in long-circulating liposomes has been demonstrated to result in a superior therapeutic index in various tumor-bearing experimental animals.\(^{15,19,21-23}\) We recently reported that GM1- or PEG-containing long-circulating thermosensitive liposomes exhibit a marked enhancement of the antitumor activity of encapsulated DXR in combination with hyperthermia.\(^{24,25}\)

The mechanism by which PEG-liposomes favor pro-
longed circulation has not been fully clarified. Increased hydrophilicity\textsuperscript{26} and a steric hindrance against recognition by the RES\textsuperscript{11,27} promote the long circulation of the liposomes. It has been reported by Mori \textit{et al.}\textsuperscript{27} that the activity of PEG in prolonging the circulation time of fluid liposomes composed of egg phosphatidylcholine (PC)/CH is directly proportional to the chain length of the PEGs. On the other hand, Maruyama \textit{et al.}\textsuperscript{13} reported that the long-circulating activity of PEG-coated DSPC/CH liposomes was not dependent on the molecular weight of PEGs. They also showed that DSPC/CH liposomes containing PEG of a higher molecular weight, such as PEG5000 or 12000, showed relatively lower stability in the circulation than did liposomes containing PEG1000. For rigid liposomes, essentially stable in the circulation, short-chain-length PEG might be more effective than long-chain-length PEG in prolonging the circulation time.

Thus, it is important to optimize the molecular weight of PEG in the PEG-liposome to enhance the antitumor activity of entrapped drugs. We prepared DXR encapsulated in PEG-liposome with different molecular weights of PEGs, and examined their tumor accumulation and antitumor efficacy.

As shown in Figs. 1 and 2, the highest DXR concentrations in blood and tumor were obtained by liposomes coated with low-molecular-weight PEG. These results indicate that liposomes coated with high-molecular-weight PEG may be less stable in blood circulation than those coated with low-molecular-weight PEG.

In the treatment study, the DXR-PEG1000-liposome significantly increased the therapeutic effectiveness of the drug in comparison with other formulations (Fig. 3). These results indicate that the improved antitumor activity of PEG-liposomes seems to be correlated to the prolonged circulation time and to the enhanced tumor accumulation of DXR. Internalization of the drug-containing liposomes by tumor cells is not always required to explain the antitumor response. We speculate that intact extravasated liposomes within the tumor area gradually break down and release the entrapped drug. Amphiphatic molecules such as DXR, once released from the liposomes, would quickly penetrate deep into the tumor.

The present results indicated that the molecular weight of PEGs available for liposome coating is one of the important variables for achieving good therapeutic efficacy.

In conclusion, the use of long-circulating liposomes as drug carriers has many potential applications in cancer chemotherapy, offering reduced toxicity in normal tissues and selective drug delivery to tumor tissue. In particular, long-circulating liposomes containing optimized PEGs appear useful for delivering chemotherapeutic agents for the treatment of solid tumors.

REFERENCES