Modulation of tumor immunity is a known factor in the antitumor activity of many chemotherapeutic agents. Exosomes are extracellular nanometric vesicles that are released by almost all types of cells, which includes cancer cells. These vesicles play a crucial role in tumor immunity. Many in vitro studies have reproduced the aggressive secretion of exosomes following treatment with conventional anticancer drugs. Nevertheless, how chemotherapeutic agents including nanomedicines such as Doxil® affect the in vivo secretion of exosomes is yet to be elucidated. In this study, the effect of intravenous injection of either free doxorubicin (DXR) or liposomal DXR formulation (Doxil®) on exosome secretion was evaluated in BALB/c mice. Exosomes were isolated from serum by using an ExoQuick™ kit. Free DXR treatment markedly increased serum exosome levels in a post-injection time-dependent manner, while Doxil® treatment did not. Exosomal size distribution and marker protein expressions (CD9, CD63, and TSG101) were studied. The physical/biological characteristics of treatment-induced exosomes were comparable to those of control mice. Interestingly, splenectomy significantly suppressed the copious exosomal secretions induced by free DXR. Collectively, our results indicate that conventional anticancer agents induce the secretion of circulating exosomes, presumably via stimulating immune cells of the spleen. As far as we know, this study represents the first report indicating that conventional chemotherapeutics may induce exosome secretion which might, in turn, contribute partly to the antitumor effect of chemotherapeutic agents.

Key words chemotherapeutic agent; doxorubicin; liposomal doxorubicin; exosome; splenectomy

Conventional thinking maintains that chemotherapeutic agents directly kill tumor cells. This concept arises from the fact that chemotherapeutic agents interfere with DNA synthesis or produce chemical damage to DNA, which leads to tumor cell death. Nevertheless, a mounting body of evidence indicates that the antitumor activities of chemotherapeutic agents also rely on several other mechanisms.1–4) Alteration of the tumor microenvironment accounts for the enhanced tumor immunity of some anticancer agents. Inhibitors of phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling, such as rapamycin and nelfinavir, alter angiogenesis and oxygenation in tumors, and thereby improve the response to combined radiation and chemotherapy.5) Inhibitors for human epidermal growth factor receptor 2 (HER2), such as trastuzumab, exert potent antitumor activity against HER2 positive breast cancer presumably via normalization of the breast tumor vessels.2) Interestingly, the modulation of tumor immunity has recently been verified as a contributor to the antitumor efficacy of several chemotherapeutic agents. Cyclophosphamide activates natural killer (NK) cells and depletes regulatory T cells (Treg), which promotes immunogenic cell death (ICD).6) Gemcitabine depletes circulating myeloid-derived suppressor cells (MDSC)7) and reprograms tumor-associated macrophages (TAM)8) that play key roles in tumor progression. These findings indicate that, besides their direct cytotoxic effect against tumor cells, conventional chemotherapeutics could synergistically exert an antitumor effect by regulating tumor immunity and/or the tumor microenvironment.

Exosomes are nano-sized extracellular vesicles (40–100 nm in diameter) that mediate cell-to-cell communication by delivering bioactive agents such as proteins, mRNA, microRNA and lipids.7,8) Exosomes are secreted from various types of cells consisting mainly of immune cells such as T cells, B cells, dendritic cells, NK cells, and macrophages.9–12) Immune cell-derived exosomes are known to play a crucial role as a potent stimulator in antitumor immunity. For example, B cell-derived exosomes directly stimulate CD4+ T cells and then elicit humoral immune responses.13) Dendritic cell-derived exosomes (dexosomes) eradicate tumors in a T cell-dependent and major histocompatibility complex (MHC)-restricted manner.14,15)

Exosome release is known to be triggered by various stimuli. Recently, many in vitro studies have demonstrated that exosomes are aggressively secreted by treatment with conventional anticancer drugs. Lv et al.16) reported that microvesicles, including exosomes, were actively released by treatment with anticancer drugs such as paclitaxel, carboplatin, etoposide, and irinotecan from hepatocellular carcinomas. In the same context, Pessina and coworkers17) found that exosomes are secreted from mesenchymal stromal cells (MSCs) following treatment with paclitaxel. In that study, paclitaxel-induced exosomes contributed to the in vitro tumor growth inhibition of a murine SR4987 cell line. However, no studies have explained how conventional chemotherapy and/or chemotherapeutic agents containing nanomedicine, such as Doxil®, affects in vivo exosomal secretion at animal level.

In the present study, free doxorubicin (DXR) and a liposomal DXR formulation (Doxil®) were adopted as model anticancer drugs, and their effect on exosome secretion was...
investigated in conventional BALB/c mice. DXR is a widely used anthracycline anticancer drug adopted for the treatment of several types of tumors. Doxil® is a potent anticancer drug active against a wide range of human tumors including leukemia, lymphomas and solid tumors. Treatment with free DXR increased in vivo exosomal secretion presumably by stimulating the immune cells of the spleen, but the effect was not achieved with a liposomal formulation. Furthermore, the induced exosomes had the same physical/biological characteristics as endogenously non-induced exosomes.

MATERIALS AND METHODS

**Materials** Doxorubicin (Adriacin®, DXR) was purchased from Kyowa Hakko Kirin (Tokyo, Japan). Doxil® was purchased from Janssen Pharmaceutical K.K. (Tokyo, Japan). ExoQuick™ Exosome Precipitation Solution was purchased from System Biosciences (CA, U.S.A.). DC™ Protein Assay Kit was purchased from Bio-Rad Laboratories (CA, U.S.A.). Anti-CD9 (ab92726), anti-TSG101 (ab30871), and horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin G (IgG) H&L (ab6721) were purchased from Abcam (Cambridge, U.K.). Anti-CD63 (sc-15363) antibody was purchased from Santa Cruz Biotechnology (CA, U.S.A.). All other reagents were of analytical grade.

**Animals** BALB/c mice (male, 5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were housed under controlled environmental conditions (constant temperature, humidity, and a 12-h dark/light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University.

**Surgery for Splenectomy** Splenectomy was performed as previously described. BALB/c mice were anesthetized and a 2-cm incision was made in the skin at the left flank. The peritoneal membrane was opened, and the entire spleen was removed intact after ligating the splenic vein and artery at the hilum. The peritoneal membrane and skin were closed separately with surgical silk thread. This procedure ensured that the spleen would be removed in total and that no splenic fragments would be left behind, as confirmed by examining the mice at the time of death. The splenectomized mice were used for further experimentation on day 6 post-surgery.

**Treatment with DXR or Doxil®** BALB/c mice or splenectomized mice were intravenously injected with a single dose of either DXR (5, 10 or 15 mg/kg) or Doxil® (5, 10 or 15 mg DXR/kg). At selected time points (4, 12, 24 or 48 h) after the injection, whole blood was collected and the serum exosomes were isolated.

**Isolation of Serum Exosomes** Blood samples were collected from the postcaval vein of the mice under anesthesia, and serum was obtained by centrifugation of the blood (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature. The serum exosomes were precipitated using ExoQuick (3000 rpm, 4°C, 30 min) after incubation for 30 min at room temperature.

Effect of Treatment with DXR or Doxil® on in Vivo Exosomal Secretion First, we assessed the effect of free DXR or liposomal DXR formulation (Doxil®), as a model anticancer drug, on exosome secretion in vivo. Free DXR or Doxil® was intravenously injected into BALB/c mice. At 24 h post injection, exosomes were isolated from serum using an ExoQuick™ isolation kit. Under the current experimental condition, minor changes in body weight were observed among mice treated with either free DXR or Doxil®. Serum exosome levels were determined as exosomal protein concentration per unit of serum. By following the instructions for using an ExoQuick™ kit for exosome isolation, the serum exosomal protein concentration in the control group was established at 6505 ± 1020 µg/mL (Fig. 1), which was comparable to that determined by other research groups, and indicated the reliability of the isolation technique. Treatment with free DXR at different concentrations significantly increased the serum exosome secretion as manifested by the remarkable increase in exosomal protein concentration (8439 ± 1194 µg/mL at 5 mg/kg
DXR and 8856±110 µg/mL at 15 mg/kg DXR), compared with the control (non-treated) group. On the other hand, Doxil® treatment did not change the serum exosomal protein levels (6765±511 µg/mL at 5 mg/kg Doxil® and 7029±444 µg/mL at 15 mg/kg Doxil®), compared with that of the control group. These results indicated that treatment with free DXR increased the in vivo secretion of serum exosomes, but treatment with Doxil® did not.

Characterization of the Serum Exosomes after Treatment with DXR or Doxil®

Based on their intracellular origin and biochemical composition, the most common populations of extracellular vesicles are classified into exosomes (size 50–200 nm) or shedding microvesicles (with a size range of up to several µm).21 Accordingly, to characterize the features of the purified serum exosome pellet obtained after the treatment with/without free DXR or Doxil®, we performed size distribution analysis and Western blotting analysis. Figure 2A depicts the size distribution of obtained serum exosomes. Serum exosomes derived from non-treated control mice showed 2 main peaks of size distribution: a small peak (about 30 nm) and a large peak (about 150 nm) with an average particle size of 105.4±11.2 nm. This particle size range is characteristic for extracellular vesicles including exosomes.22 Treatment (free DXR or Doxil®)-induced exosomes showed a particle size distribution pattern that was similar to that of control exosomes. The average particle size was 91.9±4.9 nm in the DXR-treated group and 125.8±18.8 nm in the Doxil®-treated group. In addition to size, we used Western blotting to confirm the presence of exosomes via specific markers (CD9, CD63 and TSG101) (Fig. 2B). The density of bands was calculated using ImageJ software, and the values are represented in upper side of each band. As shown in Fig. 2B, serum exosomes from DXR- or Doxil®-treated mice expressed comparable levels of CD9, CD63 and TSG101 proteins as the control mice. These results indicate a successful isolation of exosomes from serum. In addition, treatment-induced serum exosomes showed characteristics comparable to serum exosomes from control mice.

Time-Dependency on Serum Exosome Levels after Treatment with DXR or Doxil® in Vivo

To gain insight into the impact of the different characteristics of either free DXR or liposomal DXR (Doxil®) on exosome secretion, changes in the serum exosomal protein levels with time following treatment with either free DXR or Doxil® were evaluated (Fig. 3). In the control group, serum exosomal protein concentration was 5361±641 µg/mL. Treatment with free DXR did not change the serum exosomal protein level until 12 h post-injection (5069±1061 µg/mL at 4 h and 5305±557 µg/mL at 12 h), which was significantly increased at 24 h (7836±374 µg/mL) and remained at that level for as long as 48 h post-injection (7044±875 µg/mL at 48 h). Treatment with Doxil® did not change the serum exosomes at any of the time points examined (4854±489 µg/mL at 24 h and 4959±320 µg/mL at 48 h).
Chemotherapy plays an important role in cancer treatment. Conventional wisdom has historically held that anticancer agents exert their tumor growth inhibition via direct cytotoxic effects against tumor cells. Nonetheless, accumulating evidence now emphasizes the possible contribution of other mechanisms such as manipulation of the tumor microenvironment and/or tumor immunity in overall anticancer efficacy. Exosomes are nanometric vesicles that are released by almost all types of cells, including cancer cells, and they are considered to be among the most important physiological endogenous carriers for the delivery of molecular information among cells. Exosome release is triggered by various stimuli. Nonetheless, the effect of anticancer agents on \textit{in vivo} exosome secretion remains to be elucidated. In the present study, treatment with DXR significantly elevated serum exosome levels, expressed as exosomal protein concentration per unit of serum, in mice at 24 h post injection (Figs. 1, 3). Furthermore, splenectomy substantially suppressed the secretion of DXR-induced serum exosomes (Fig. 4). These results indicate that treatment with DXR triggers exosome secretion, presumably, mainly from immune cells of the spleen.

Exosome release can be both constitutive and inducible (i.e., stimulus-triggered). Induction of exosomal release can be mediated \textit{via} either cell activation or stress. Recently, Albertin et al. have reported that treatment of human prostate cancer cells with the cytotoxic agent DXR triggered a massive production of extracellular vesicles, including exosomes, both \textit{in vitro} and \textit{in vivo} in immunodeficient mice. In this study, we investigated the effect of DXR on serum exosomal protein level in conventional mice which hold whole immune system. In the current study, we showed that treatment with free DXR significantly increased serum exosomal protein levels, but treatment with Doxil did not (Figs. 1, 3). Pharmacokinetic profiles and biodistribution of either free anticancer drugs or those encapsulated within a nanocarrier system may be anticipated to affect the \textit{in vivo} secretion of exosomes. Despite the fact that intravenous Doxil preferentially accumulates in the spleen due to its long circulating properties, the rigidity nature of liposomal membrane which is composed of saturated phospholipids and cholesterol is expected to hinder the release of encapsulated DXR in both serum and the spleen following intravenous injection and consequently might attenuate its potential to stimulate splenic immune cells for exosomal secretion (Figs. 1, 3). Furthermore, the preferential hepatic accumulation of Doxil following intravenous administration might trigger the rapid systemic clearance of a large fraction of DXR in the Doxil and thereby alleviate its stimulatory effect on

discussion

These results show that free DXR treatment affects serum exosome secretion in a time-dependent manner.

\textbf{Impact of Splenectomy on the Secretion of Serum Exosomes after Treatment with Either DXR or Doxil}  

Splenectomized BALB/c mice were intravenously injected with a single dose of either free DXR or Doxil (10 mg DXR/kg). At different time points post-injection, the serum exosomes were isolated using ExoQuick. The protein concentration of serum exosomes was determined by DC protein assay. The data are presented as the mean±S.D. (n=3–4). (***p<0.01 vs. control).

These results show that free DXR treatment affects serum exosome secretion in a time-dependent manner.

\textbf{Impact of Splenectomy on the Secretion of Serum Exosomes after Treatment with Either DXR or Doxil \textit{in Vivo}}  

Several reports have emphasized the contribution of various types of immune cells, such as B cells, T cells, macrophages and dendritic cells, to the secretion of exosomes. Accordingly, to verify the possibility that DXR-induced exosomes are immune cell-derived, splenectomy was performed in mice prior to DXR treatment, and the serum exosome levels were then determined. The spleen is well known to play a crucial role in the systemic immune response. As shown in Fig. 4, treatment with Doxil in splenectomized mice did not change serum exosomal protein levels, compared with that of the control group (5006±610 µg/mL in Doxil\textsuperscript{®} vs. 4078±1325 µg/mL in control group). As we expected, splenectomy attenuated exosome secretion in splenectomized mice treated with free DXR and restored it to the control levels (4767±848 µg/mL in the DXR treatment group vs. 4078±1325 µg/mL in the control group). Notably, serum exosomal protein levels in splenectomized mice (4078±1325 µg/mL) were almost the same as in the control mice that had no surgical procedure performed (4883±932 µg/mL). These results suggest that free DXR induces the secretion of circulating exosomes, presumably, \textit{via} stimulating immune cells of spleen.

\textbf{Fig. 3. Time-Dependency on Serum Exosome Levels after Treatment with DXR or Doxil}  

BALB/c mice were intravenously injected with a single dose of either free DXR or Doxil (10 mg DXR/kg). At different time points post-injection, the serum exosomes were isolated using ExoQuick. The protein concentration of serum exosomes was determined by DC protein assay. The data are presented as the mean±S.D. (n=3–4). (**p<0.01 vs. control).

\textbf{Fig. 4. Impact of Splenectomy on the Induction of Serum Exosome Secretion after Treatment with Either DXR or Doxil}  

Splenectomized BALB/c mice were intravenously injected with a single dose of either free DXR or Doxil (10 mg DXR/kg). At 24 h post injection, the serum exosomes were isolated using ExoQuick. The protein concentration of serum exosomes was determined by DC protein assay. The data are presented as the mean±S.D. (n=5). (***p<0.01).
exosomal secretion. On the other hand, it is worth noting that intravenous DXR enhanced serum exosome levels particularly at 24h post injection (Figs. 1, 3). Intravenous DXR showed a short circulation half-life (0.5±0.1h) and a prolonged residence time in the spleen (91.8±45.6h). This indicates that DXR accumulates in the spleen soon after intravenous injection and is retained there for an extended period of time where it exerts its stimulatory effect on exosomal secretion.

Depending on their cellular origin, exosomes play many distinct roles that include intercellular communication, modulation of immune function, and promotion of tumor cell invasion. Many reports emphasize the contribution that immune cell-derived exosomes exert in tumor immunity and consequently on the outcome of tumor progression. Romagnoli et al. revealed how dendritic cell-derived exosomes may contribute to the conversion of poorly immunogenic tumor cells into highly immunogenic targets, and consequently, the control of tumor progression. In the same context, Lu et al. demonstrated how dendritic cell-derived exosomes elicit tumor regression in hepatocellular carcinoma mouse models via the activation of functional T cells, particularly CD8+ cytotoxic T lymphocyte (CTL). In the present study, treatment with free DXR enhanced the secretion of exosomes, presumably immune cell-derived exosomes, in non splenectomized mice (Figs. 1, 3), while, splenectomy substantially suppressed the secretion of DXR-induced serum exosomes to the normal control level (Fig. 4). These findings suggest that DXR triggered the release of immune-cell derived exosomes. However, a more thorough examination on the origin of the secreted vesicles with immune cell markers is under investigation to verify our assumption.

Cancer cells may secrete abundant levels of exosomes under stress conditions such as surgery, chemotherapy and radiotherapy. The impact of secreted exosomes on the immune system and on tumor progression has been a controversial issue. Exosomes can mediate either immunosuppression or immune system activation depending on their cellular origin. In the present study, we reported that free chemotherapeutic agents could potentially trigger the secretion of immune cell-derived exosomes (Figs. 1, 4), which are expected to participate in the antitumor potential of chemotherapeutic agents. Such promising findings along with a deep understanding of the immune-related functions of exosomes could form a curious hypothesis wherein treatment-induced immune cell-derived exosomes could efficiently participate in the overall antitumor efficacy of conventional anticancer agents. To follow this hypothesis, a study to demonstrate the contribution of DXR-induced exosomes on antitumor activity against tumor-bearing mice model is in progress in our laboratory.

CONCLUSION

In the present study, free DXR markedly enhanced the secretion of circulating exosomes, presumably, from spleen in mice, but the administration of Doxil® did not. The current study indicates that chemotherapy-induced exosomes could play a role in mediating the overall antitumor effect of free DXR to help create a synergistic endogenous antitumor immune response.

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Conflict of Interest The authors declare no conflict of interest.

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