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Histological bioanalysis for therapeutic effects of hybrid liposomes on the hepatic metastasis of colon carcinoma *in vivo*

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ABSTRACT

Therapeutic effects of hybrid liposomes (HL) composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene (23) dodecylether ($C_{12}(EO)_{23}$) on the metastasis of colon carcinoma (Colon26) cells were examined *in vivo*. Fluorescent labeled Colon26 cells were observed in the liver tissue of hepatic metastasis mouse models after the intrasplenic inoculation of the cells. Remarkably high therapeutic effects were obtained in the hepatic metastasis mouse models after the treatment with HL on the basis of relative liver weight and histological analysis of the liver tissue sections of mouse models with hematoxylin–eosin staining, Masson trichrome staining, and CEA immunostaining as a histochemical marker of metastatic colon carcinoma. Furthermore, no toxicity was observed in the hepatic metastasis mouse models after the intravenous injection of HL. Therapeutic effects of HL without any drugs on the hepatic metastasis were revealed on the basis of histological analysis for the first time *in vivo*.

1. Introduction

The control of cancer metastasis is one of the most important strategies for cancer therapy. During the process of metastasis, it is well known that tumor cells could interact with various host cells (platelets, lymphocytes and endothelial cells), extracellular matrix and basement membranes, leading to the development of metastases. The liver is the most common organ for the metastasis of cancers in the digestive system, especially for the hematogenous metastasis of colon carcinoma, and the prognosis for cases with liver metastasis is extremely poor (Nicolson, 1987; Terranova et al., 1986; Fidler, 1990, 2003). On hepatic metastasis of colon carcinoma, the effectiveness of anti-cancer drugs as adjuvant chemotherapy after the curative resection has been reported (Moertel et al., 1990; Koraira et al., 1998; Ito et al., 2001). Elucidation of the mechanism in hepatic metastasis is critical for improvement of the survival rate in colon carcinoma. Although chemotherapy has been introduced to improve the postoperative prognosis, an effective chemotherapy for liver metastasis of colon carcinoma has not been established. So, establishing experimental animal models *in vivo* is essential for the analysis of the mechanisms of cancer metastasis (Ishizu et al., 2007).

On the other hand, we have produced hybrid liposomes (HL) which can be prepared by just the sonication of vesicular and micellar molecules in a buffer solution (Ueoka et al., 1985, 1988). HL are free from any contamination with organic solvents and remain stable for longer periods. The physical properties of these liposomes such as size, membrane fluidity, phase transition temperature, and hydrophobicity can be controlled by changing the constituents and compositional ratios of the HL. In the course of our study for HL, the following interesting results have been obtained. (a) Stereochemical control of the enantioselective hydrolysis of amino acid esters could be established by temperature regulation and changing the composition of the HL (Ueoka et al., 1985, 1988). (b) Inhibitory effects of HL including antitumor drugs (Kitamura et al., 1996), sugar surfactants (Matsumoto et al., 2000) or polyunsaturated fatty acids (Tanaka et al., 2008) have been observed on the growth of tumor cells *in vitro* and *in vivo*. (c) High inhibitory effects of HL on the growth of leukemia (Matsumoto et al., 2005), lung carcinoma (Iwamoto et al., 2005) and human breast tumor (Nagami et al., 2006) cells *in vitro* along with the induction of apoptosis have been obtained without using drugs. (d) A good correlation between membrane fluidity of HL and antitumor effects on the growth of human colon tumor has been observed (Komizu et al., 2006).

In this study, we examined the therapeutic effects of HL composed of 90 mol% L-α-dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene (23) dodecylether ($C_{12}(EO)_{23}$) on the hepatic metastasis mouse models *in vivo*. Furthermore, the therapeutic effects of HL on the hepatic metastasis mouse models were revealed on the basis of histological analysis of liver tissue sections.

Abbreviations: TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; CEA, carcinoembryonic antigen.

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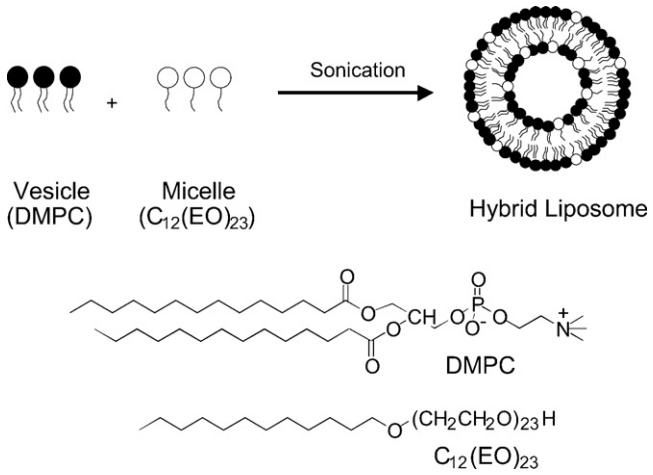

Scheme 1. Schematic representation of hybrid liposomes.

2. Materials and methods

2.1. Preparation of hybrid liposomes

Hybrid liposomes (HL) were prepared by sonication of a mixture containing 90 mol% L-α-dimyristoylphosphatidylcholine (DMPC, Nippon oil and Fats Co. Ltd., Japan) and 10 mol% polyoxyethylene (23) dodecylether ($C_{12}(EO)_{23}$, Sigma–Aldrich Co., USA) in 5% glucose solution using bath type sonicater (VS-N300, VELVO-CLEAR, Japan) at 45 °C with 300 W as shown in Scheme 1, and filtered with a 0.20 μm cellulose acetate filter (Advantec, Japan).

2.2. Dynamic light-scattering measurements

The diameter of HL was measured with a light-scattering spectrometer (ELS-8000, Otsuka Electronics, Japan) using a He–Ne laser (633 nm) at a 90° scattering angle. The diameter (d_{hy}) was calculated using the Stokes–Einstein formula (Eq. (1)), where κ is the Boltzmann constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient:

$$d_{hy} = \frac{\kappa T}{3\pi\eta D} \quad (1)$$

2.3. Cell culture

Mouse colon carcinoma (Colon26) cell lines were obtained from Tokyo Medical University. Cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM: GIBCO, USA) supplemented with penicillin (100 unit/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS, HyClone Laboratories Inc., USA) in humidified atmosphere at 37 °C.

2.4. Observation of Colon26 cells entrapped in liver sinusoids

Colon26 cells were labeled with CellTracker Red probe (CMPTX; Molecular Probes, Eugene, OR) (Acuff et al., 2006) at a final concentration of 25 μM in the culture medium for 45 min at 37 °C. The labeled cells were observed by a confocal laser microscope (Leica TCS-SP, Heidelberg, Germany) using 543 nm He/Ne laser line (detection; 600–724 nm) as shown in Fig. 1A (inset). The cells were inoculated intrasplenically into the mice (5.0×10^4 cells/body). After 5 min and 24 h of inoculation, the livers were resected from the mouse models, and randomly dissected liver tissues were embedded in OCT compound and rapidly frozen. The cryosections of the liver tissue were stained with YO-PRO-1 dye (Molecular Probes, USA) solution including antifade reagent (5% DABCO (1,4-diazobicyclo-(2,2,2)-octane)) for detecting cell nucleus and observed by confocal microscope.

2.5. Therapeutic effects of HL for hepatic metastasis mouse models

The mice were handled in accordance with the guidelines for animal experimentation in Japanese law. Female BALB/c mice were obtained from CLEA (Japan). The mice were randomly grouped on the basis of body weight by the stratified randomization method. The number of mice was six in each group. The Colon26 cells (5.0×10^4 cells) were intrasplenically transplanted into the mice (Jeong et al., 2008). HL (dose: 203 mg/kg for DMPC) were intravenously administered once each day for 14 days after the inoculation of Colon26 cells. The livers were weighed after anatomizing the mice after 14 days of inoculation of Colon26 cells, and held an autopsy, and fixed in 10% formalin solution. The livers were embedded in paraffin and sectioned at 5 μm of thickness. The liver sections were stained with hematoxylin and eosin (HE) and Masson trichrome and observed by optical microscope (Nikon TS-100, Tokyo, Japan).

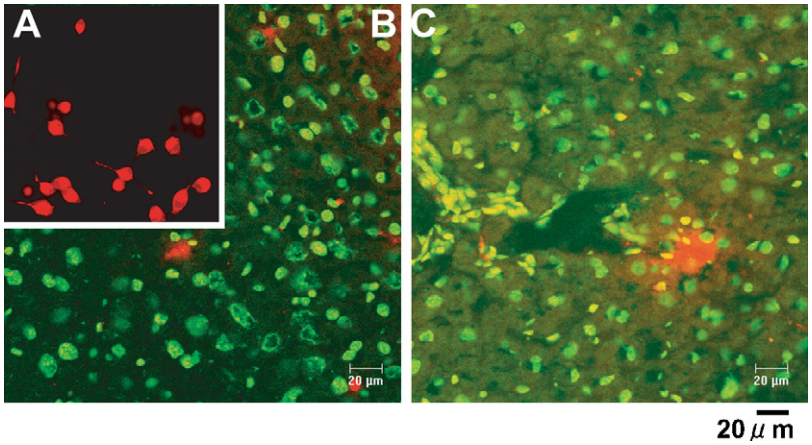

Fig. 1. CellTracker-labeled Colon26 cells observed using a confocal laser microscope. Colon26 cells were labeled with 25 μM of CellTracker Red probe (CMPTX) in the culture medium for 45 min. (A, inset) Labeled Colon26 cells (red) in the culture medium. (B) Labeled Colon26 cells (red) which indicate early stage of metastasis step in liver sinusoids in the mice after 5 min of the intrasplenic inoculation of the cells. (C) Labeled Colon26 cells (red) in the section after 24 h of the intrasplenic inoculation of the cells. It was confirmed that Colon26 cells were entrapped in liver sinusoids in the mice, which indicated the early stage of metastasis step.

2.6. Immunostaining with anti-CEA antibody

Deparaffinized and hydrated liver sections were immersed in 10 mM citric acid buffer at pH 6.0 and were heated by microwave oven (600 W) for antigen activation. The sections were blocked with Super Block TM Blocking Buffer in PBS(−) for 30 min, and incubated with anti-human carcinoembryonic antigen (CEA)/CD66e Ab-2 rabbit polyclonal antibody (Thermo Scientific, USA) at room temperature for 30 min and with FITC labeled anti-rabbit polyclonal donkey antibody in humidified box at 4 °C for 1 h. It was confirmed by the production data sheet that the species reactivity of the antibody, CEA/CD66e Ab-2, was human and mouse. Finally, the sections were counterstained with TO-PRO-3 (Molecular Probes, USA) solution including antifade reagent for detecting cell nucleus and observed by confocal microscope.

2.7. Assessment of toxicity for hepatic metastasis mouse models

Female mice (BALB/cA Jcl) were obtained from CLEA Japan, Inc. The mice were randomly grouped on the basis of the body weight using the stratified randomization method. Number of mice was six in each group. HL were intravenously administered into the caudal vein of mice once each day for 14 days. The mice were weighed during the experimental period. The blood was collected from the heart in mice under anesthesia with ether after fasting for 24 h as previously reported (Kurata et al., 2003; Senoh et al., 2004; Lee et al., 2004; Aiso et al., 2005). White blood cells (WBC), and red blood cells (RBC) were counted using multiple automatic blood cell counting device (F-500, Sysmex Co., Japan).

2.8. Statistical analysis

Results are presented as mean ± S.D. Data were statistically analyzed using Student's *t*-test. A p value of less than 0.01 was considered to represent a statistically significant difference.

3. Results and discussion

3.1. Physical properties of HL

A clear solution of HL having a hydrodynamic diameter of 100 nm with narrow range of size distribution could be kept over one month on the basis of dynamic light-scattering measurements.

3.2. Bioimaging of Colon26 cells entrapped in liver sinusoids

To confirm the hepatic metastasis of mouse colon carcinoma cells (Colon26) after the intrasplenic inoculation of Colon26 cells, we have carried out imaging analysis. As shown in Fig. 1, the CellTracker-labeled Colon26 cells (Fig. 1A) were clearly observed in a liver tissue after 5 min (Fig. 1B) of the intrasplenic inoculation into the mice and retained in the tissue near the blood vessel at least until 24 h (Fig. 1C). Therefore, it was confirmed that Colon26 cells were entrapped in liver sinusoids in the mice, which indicated the early stage of metastasis step.

Table 1
Relative liver weight of hepatic metastasis mouse models treated with hybrid liposomes after 14 days of the intrasplenic inoculation of Colon26 cells.

Group	Relative liver weight (g/100 g B.W.)
Normal	4.17 ± 0.02
Control (without treatment)	5.31 ± 0.10
Treatment with HL	4.39 ± 0.09*

Values are indicated as mean ± S.D. ($n = 6$).
*Significant difference ($p < 0.01$) compared with control group (Student's *t*-test).

3.3. Therapeutic effects of HL on the hepatic metastasis

We examined the therapeutic effects of the HL by using hepatic metastasis mouse models. The relative liver weight of hepatic metastasis mouse models treated with HL is summarized in Table 1. It is worthy to note that the relative liver weight of the group treated with HL was almost the same as that of the normal group, although that of the control group without treatment obviously increased. Furthermore, there was a significant difference ($p < 0.01$) in relative liver weight between control group and treated group with HL. Furthermore, we examined the therapeutic effects of HL in autopsy. As shown in Fig. 2, the liver of group treated with HL (DMPC dose, 203 mg/kg, Fig. 2C) was the same as that of normal group (Fig. 2A), although enlargement and tumor-node by metastasis of colon carcinoma in liver of control group (Fig. 2B) were confirmed.

These results indicate that the therapeutic effects of HL should be obtained on the hepatic metastasis mouse models.

3.4. Histological bioanalysis

We histologically evaluated the therapeutic effects of HL using the liver tissue for the hepatic metastasis mouse models *in vivo*. First, we examined hematoxylin and eosin (HE) staining to evaluate the therapeutic effects of HL. As shown in Fig. 3, it was confirmed that the large metastatic nodules were obviously observed in the liver of the control group (Fig. 3B), which indicated a malignant transformation by metastasis of carcinoma cells to liver. On the other hand, no obvious nodule was observed in liver of the group treated with HL (DMPC dose, 203 mg/kg, Fig. 3C), which was very similar to normal liver (Fig. 3A). On the other hand, no abnormal

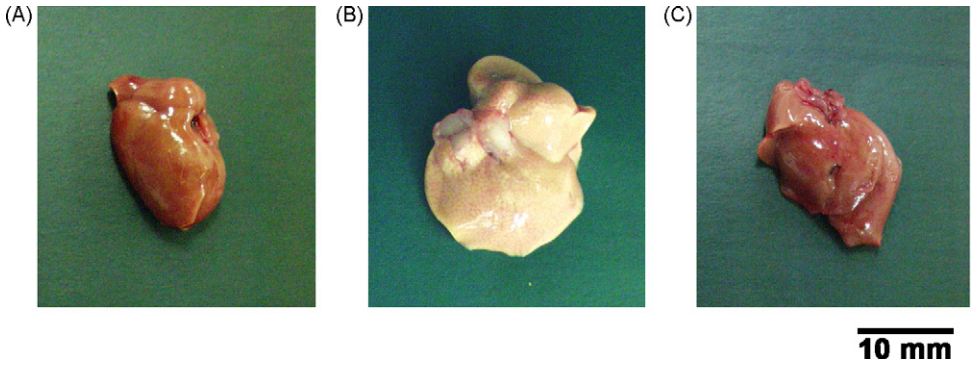

Fig. 2. Photographs of liver in hepatic metastatic mouse models treated with hybrid liposomes after 14 days of the intrasplenic inoculation of Colon26 cells. The Colon26 cells (5.0×10^4 cells) were intrasplenically transplanted into the mice. HL (dose: 203 mg/kg for DMPC) were intravenously administered once each day for 14 days after the inoculation of Colon26 cells. (A) Normal, (B) control (without treatment) and (C) treatment with HL. The liver of group treated with HL (C) was the same as that of normal group (A).

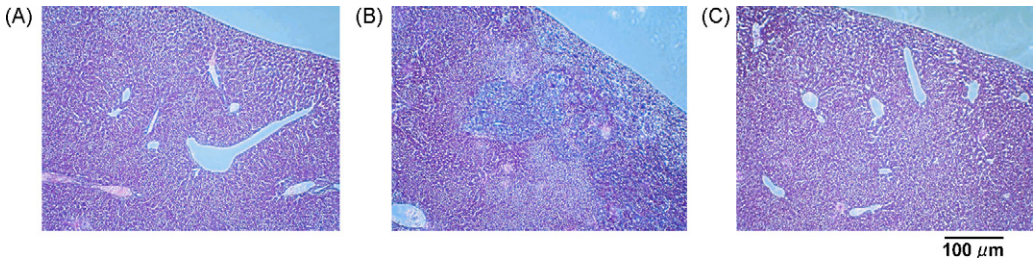

Fig. 3. HE staining of liver tissue of hepatic metastatic mouse models treated with hybrid liposomes after 14 days of the intrasplenic inoculation of Colon26 cells. (A) Normal, (B) control (without treatment) and (C) treatment with HL. No obvious nodule was observed in liver of the group treated with HL (DMPC dose, 203 mg/kg), which was very similar to normal liver.

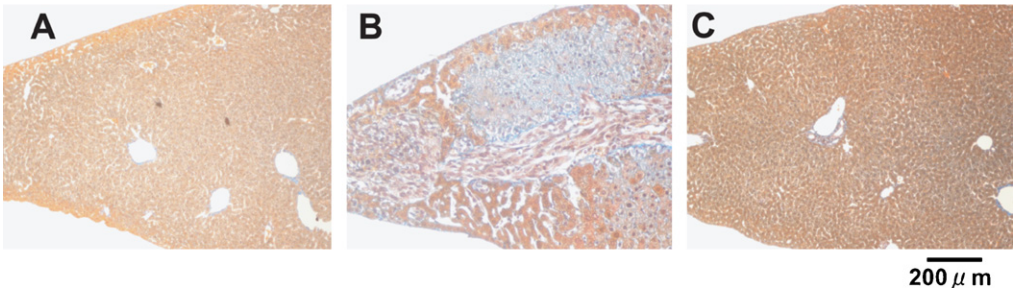

Fig. 4. Masson trichrome staining of liver tissue of hepatic metastatic mouse models treated with hybrid liposomes after 14 days of the intrasplenic inoculation of Colon26 cells. (A) Normal, (B) control (without treatment) and (C) treatment with HL. There was no abnormal finding in the group treated with HL.

findings were observed in liver of the group treated with HL (DMPC dose, 203 mg/kg, Fig. 3C), which was very similar to normal liver (Fig. 3A). Next, we examined Masson trichrome staining to evaluate the therapeutic effects of HL. As shown in Fig. 4B, remarkably abnormal findings with increase of fibrillization were observed near the blood vessel of liver tissue in the control group, which was often observed in hepatic metastasis of colon carcinoma (Caruso et al., 1993). On the other hand, no abnormal finding was obtained in the group treated with HL (Fig. 4C), and was the same as the normal group (Fig. 4A).

Next, we carried out immunostaining using carcinoembryonic antigen (CEA) as a histochemical marker of metastatic colon carcinoma (Blumenthal et al., 2007) to establish the therapeutic effects of HL. It is of interest that the CEA positive cells were not observed in the liver tissue sections of the group treated with HL (Fig. 5C), and was the same as the normal group (Fig. 5A), although numerous CEA positive cells were detected in the tissue near and inside the blood vessel in the control group (Fig. 5B).

These results indicate that HL could be quite effective for inhibiting the hepatic metastasis of Colon26 cells.

3.5. Toxicity of HL in vivo

Safety tests of the HL were carried out using hepatic metastasis mouse models. The mice were weighed during the experiment period of 14 days. The body weights were 19.62 ± 1.3 g in the normal group, 20.48 ± 0.66 g in the control one, 20.31 ± 1.24 g in the treatment with HL, respectively. It is noteworthy that no weight loss and no abnormal finding in any group were observed. Blood was collected from the heart under anesthesia with ether after 14 days. Increase in white blood cells (WBC; $90 \pm 32 \times 10^2/\mu l$) and decrease in red blood cells (RBC; $450 \pm 100 \times 10^4/\mu l$) in the control group were confirmed in comparison with those (WBC; $25 \pm 10 \times 10^2/\mu l$, RBC; $550 \pm 100 \times 10^4/\mu l$) in the normal group. However, the numbers of WBC ($35 \pm 10 \times 10^2/\mu l$) and RBC ($550 \pm 70 \times 10^4/\mu l$) in the group treated with HL were the same as those in the normal group. The biochemical parameters in AST and ALT activities in group treated HL (AST; 115 ± 36 IU/l, ALT; 35 ± 0.6 IU/l) were not significantly different from those obtained in the normal mice (AST; 124 ± 20 IU/l, ALT; 32 ± 2.6 IU/l). On the other hand, an increase of AST and ALT were obtained in control group

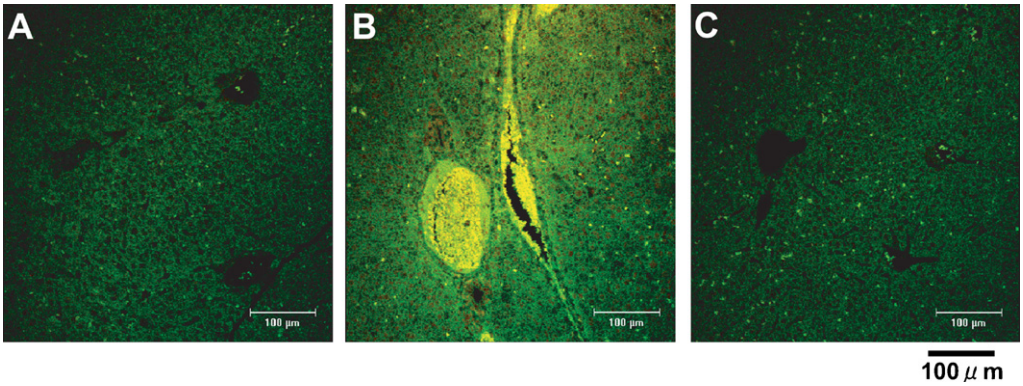

Fig. 5. CEA immunostaining of liver tissue of hepatic metastatic mouse models treated with hybrid liposomes after 14 days of the intrasplenic inoculation of Colon26 cells. Green: CEA, red: nucleus. (A) Normal, (B) control (without treatment) and (C) treatment with HL. The CEA positive cells were not observed in the liver tissue sections of the group treated with HL.

(AST; 220 ± 23 IU/l, ALT; 68 ± 15.3 IU/l). Furthermore, no abnormal findings of HL in biochemical examination of blood as AST, ALT, etc. have been observed in safety test using rats (Nagami et al., 2006; Ichihara et al., 2008) and mice (Shimoda et al., 2009). These results indicate that HL should have no hematotoxicity for the hepatic metastasis mouse models *in vivo*.

4. Conclusion

In conclusion, we examined the therapeutic effects of HL on the hepatic metastasis of colon carcinoma *in vivo*. The noteworthy aspects are as follows. (a) Hepatic metastasis of colon carcinoma cells was confirmed using histological analysis. (b) Remarkably high therapeutic effects of HL were obtained in hepatic metastasis mouse models having various abnormal findings observed in hepatic metastasis of colon carcinoma on the basis of relative liver weight, hematoxylin–eosin staining, Masson trichrome staining, and CEA immunostaining as a histochemical marker of metastatic colon carcinoma. (c) No toxicity was observed in the hepatic metastasis mouse models after the intravenous injection of HL. It is noteworthy that remarkably high therapeutic effects of drug-free HL without any side effects on the hepatic metastasis were evaluated on the basis of histological bioanalysis for the first time *in vivo*.

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