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Original article

Intravenous injection of hybrid liposomes suppresses the liver metastases in xenograft mouse models of colorectal cancer *in vivo*

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ABSTRACT

Therapeutic effects of hybrid liposomes (HL) composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(25) dodecyl ether ($C_{12}(EO)_{25}$) on the metastasis of human colon carcinoma (HCT116) cells were examined *in vivo*. Remarkably high therapeutic effects were obtained in the xenograft mouse models of colorectal cancer (CRC) liver metastases after treatment with HL-25 on the basis of relative liver weight and histological analysis of the liver tissue sections of mouse models with HE staining, and TUNEL staining for detection of apoptotic cells. The survival effects of HL-25 were obtained using xenograft mouse models of CRC liver metastases. Furthermore, with regard to pharmacokinetics, the accumulation of fluorescent labeled HL-25 was observed in the liver tissue of xenograft mouse models of CRC liver metastases for 24 h after the intravenous injection of fluorescent labeled HL-25. Therapeutic effects of HL without any drugs on the liver metastasis of human CRC were revealed for the first time *in vivo*.

1. Introduction

It is well known that colorectal cancer (CRC) has remarkably high metastaticity, leading to cancer-related death with the majority of deaths attributable to distant metastases, and the liver is the most common site of hematogenous metastasis in CRC. Approximately 20% of patients present with synchronous liver metastasis at the time of diagnosis, and the other 30% of patients will develop liver metastasis after resection of the primary CRC. Accordingly, elucidation of the mechanism in liver metastasis is critical for improvement of the survival rate in CRC. Liver resection combined with chemotherapy is the standard of treatment for patients with CRC liver metastasis [1]. However, with enlarged and advanced cancer into the deeper regions, it is difficult to cure cases with surgical treatment. Therefore, an effective chemotherapy for advanced liver metastasis of colon carcinoma has not been established [2,3].

Hybrid liposomes, which are specific nanoparticles, first developed by Ueoka et al. [4,5], can be prepared by simply sonicating a mixture of vesicular and micellar molecules in buffer solutions. Inhibitory effects of HL including flavonoids [6], antitumor drugs [7], sugar surfactants [8–10], or polyunsaturated fatty acids [11,12] on the growth of tumor cells *in vitro* and *in vivo* have been obtained. On the other hand, remarkably high inhibitory effects of HL on the growth of tumor cells *in vitro* [13–16] and *in vivo* [16–19] have been obtained without drugs. Furthermore, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported [20–22]. In addition, it has been demonstrated that HL could induce apoptotic cell death in tumor cells [13–22]. With respect to colon cancer cell lines, HL induced apoptosis in human colon adenocarcinoma WiDr [23] and HCT116 cells [24]. However, therapeutic effects and pharmacokinetics of HL on the mouse models of human CRC liver metastases *in vivo* have not yet been elucidated.

In this study, we investigated the therapeutic effects of HL-25 composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(25) dodecyl ether ($C_{12}(EO)_{25}$) using xenograft mouse models of CRC liver metastases after the inoculation of human colon carcinoma (HCT116) *in vivo*.

2. Results and discussions

2.1. Physical properties of HL-25

We examined the morphology of HL-25 composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ on the basis of dynamic light scattering measurements. As shown in Fig. 1B, the hydrodynamic

Abbreviations: $C_{12}(EO)_{25}$, polyoxyethylene(25) dodecyl ether; CRC, colorectal cancer; DMPC, L-α-dimyristoylphosphatidylcholine; HL-25, hybrid liposomes composed of DMPC and $C_{12}(EO)_{25}$; NBDPC, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine; RES, reticular endothelial system; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

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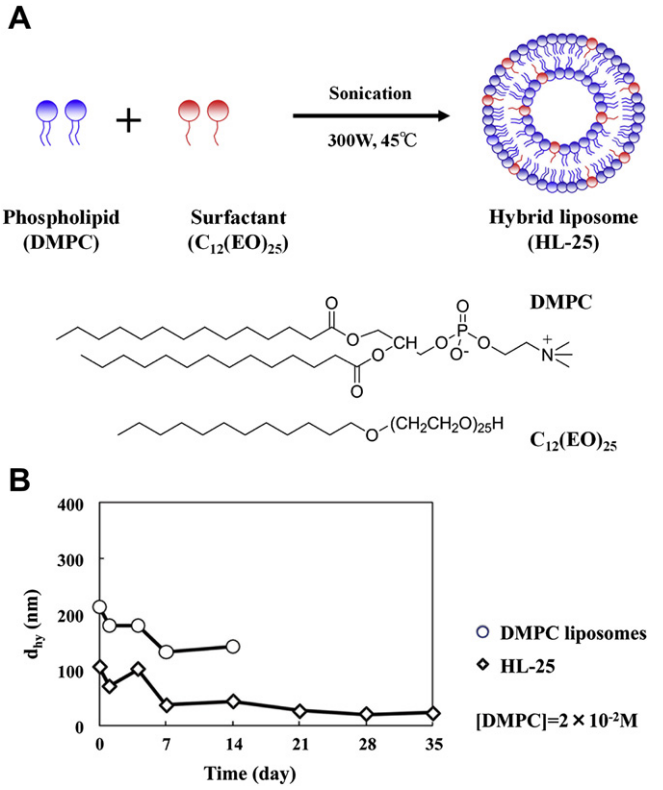

Fig. 1. (A) Schematic representation of HL-25. (B) Time courses of d_{hy} change for hybrid liposomes composed of 95 mol% DMPC and 5 mol% $C_{12}(EO)_{25}$ in 5% glucose solution at 25 °C. [DMPC] = 2.0×10^{-2} M, [$C_{12}(EO)_{25}$] = 1.05×10^{-3} M.

diameter (d_{hy}) of HL-25 was under 100 nm, which was preserved for a period that remained stable for more than one month. On the other hand, DMPC liposomes were unstable and precipitated after 14 days. HL were kept at room temperature (25 °C) due to the convenience of stock for a long term period for clinical application. It is suggested that the diameter of HL gradually decreased, since the membrane fluidity of HL that was kept at room temperature near phase transition temperature (21 °C) [26] gradually stabilized after a preparation at 45 °C. Furthermore, the diameter of HL spontaneously recovered to 100 nm from 50 nm by increasing temperature from room temperature to 37 °C. It is worthy to note that HL-25 having under the 100 nm in diameter could avoid the reticular endothelial system (RES) [27] and should be appropriate for the intravenous administration *in vivo* and clinical applications.

2.2. Prolonged survival effects of HL for xenograft mouse models of CRC liver metastases in vivo

We examined the therapeutic effects of HL-25 using xenograft mouse models of CRC liver metastases after the intravenous treatment with HL-25. HL were intravenously administered *via* a caudal vein once each day for 14 days after the inoculation of HCT116 cells. After dosing period, survival of mice were followed. The results are shown in Fig. 2. The median survival time for the group treated with HL-25 (38 ± 6) was longer than that for the control group (30 ± 0). On the other hand, the median survival time for the group treated with DMPC was 32 ± 5 and the statistically significant difference between control and DMPC alone was not obtained. It is noteworthy that a prolonged survival rate (130%, $p < 0.05$) was obtained in the group treated with HL-25. These

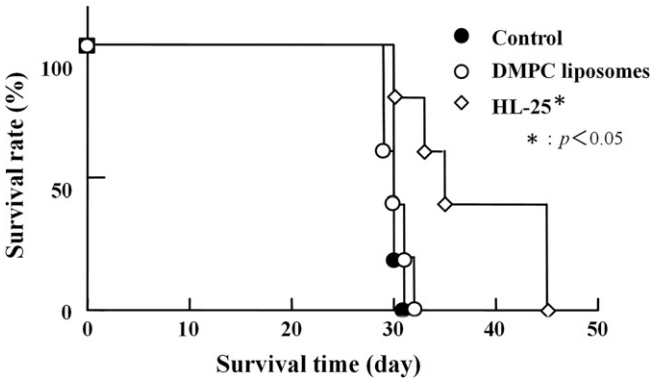

Fig. 2. Survival curves of mouse models of CRC liver metastases after the treatment with HL-25.

results indicate that HL-25 could strongly inhibit the growth of HCT-116 cells *in vivo*.

2.3. Therapeutic effects of HL-25 on CRC liver metastases in vivo

We examined the inhibitory effects of HL-25 on the growth of tumors in xenograft mouse models of CRC liver metastases after intravenous treatment. HL-25 was administered into the caudal vein of mice once each for 14 days after the HCT116 cells were inoculated to mice. Liver was removed from anaesthetized mice immediately after the treatment with HL-25. First, we examined the therapeutic effects of HL-25 on the liver metastasis mouse models in an autopsy. The results are shown in Fig. 3A. The livers of

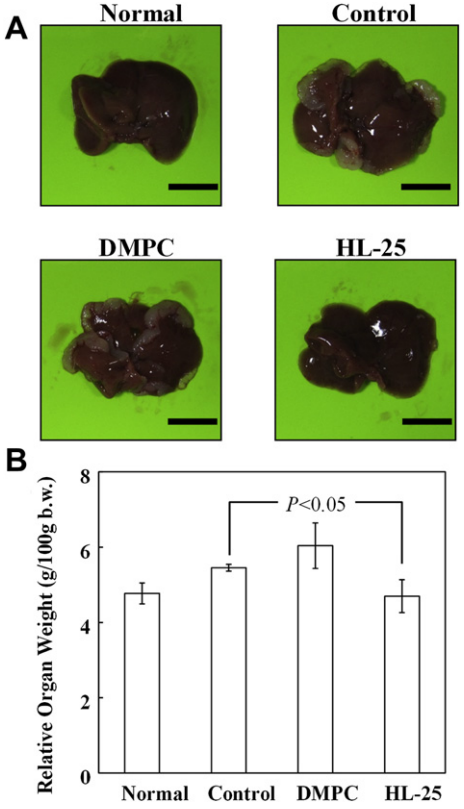

Fig. 3. (A) Liver of mouse models of CRC liver metastases treated with HL-25. Scale bar, 10 mm. (B) Relative liver weight of mouse models of CRC liver metastases treated with HL-25.

the group treated with HL-25 were almost the same as that of the normal group, although the enlargement and the tumor-nodes by metastasis of HCT116 cells in the livers of the untreated control group and those treated with DMPC were confirmed. Next, we examined the relative liver weight of mouse models of CRC liver metastases treated with HL. The results are shown in Fig. 3B. It is worthy to note that the relative liver weight of the group treated with HL-25 was close to that of the normal group, although that of the untreated control group obviously increased. There was a significant difference ($p < 0.05$) in the relative liver weight between the untreated control group and the HL-25 treated group. These results indicate that HL-25 could be effective for the liver metastasis mouse models *in vivo*.

2.4. Histological bioanalysis

We histologically evaluated the therapeutic effects of HL-25 using the liver tissue for the mouse models of CRC liver metastases *in vivo*. HL-25 was administered into the caudal vein of mice once each for 14 days after the HCT116 cells were inoculated to mice. Liver was removed from anaesthetized mice immediately after the treatment with HL-25. We examined hematoxylin and eosin (HE) staining to evaluate the therapeutic effects of HL-25. As shown in Fig. 4, it was confirmed that the large metastatic nodules were obviously observed in the livers of the untreated control group and the group treated with DMPC, indicating a malignant transformation by metastasis of carcinoma cells in the liver. On the other hand, no abnormal findings were observed in the livers of the group treated with HL-25, which were fairly similar to normal livers. These results indicate that HL-25 could have the anti-metastatic effects on the liver metastasis mouse models *in vivo*.

2.5. Induction of apoptosis by HL-25

We examined the mechanism of the therapeutic effects of HL-25 on the liver metastasis of HCT116 cells in *vivo* using the TUNEL method. HL-25 was administered into the caudal vein of mice once each for 14 days after the HCT116 cells were inoculated to mice. Liver was removed from anaesthetized mice immediately after the treatment with HL-25. As shown in Fig. 5, many apoptotic cells were observed in the tumor cells in the liver tissue of the group treated with HL-25. On the other hand, no apoptotic cells were observed in the normal and control groups. With regard to

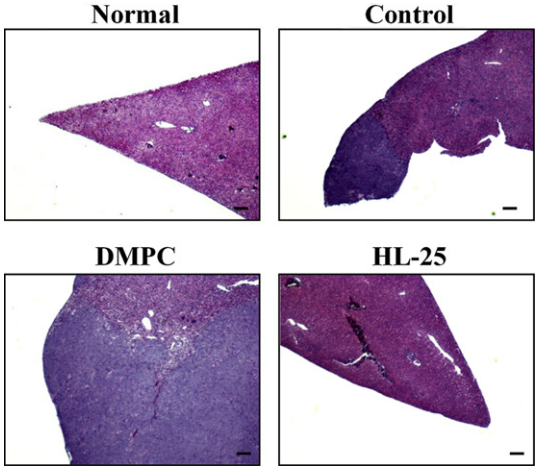

Fig. 4. HE staining of liver tissue of mouse models of colorectal cancer liver metastases treated with HL-25. Scale bar, 100 μm.

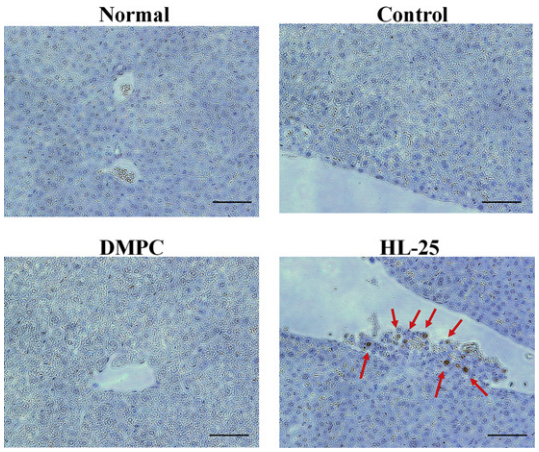

Fig. 5. TUNEL staining of liver tissue of mouse models of CRC liver metastases treated with HL-25. Arrow indicates apoptotic cells. Scale bar, 100 μm.

colon cancer cell lines, it has been reported that HL induced apoptosis in human colon adenocarcinoma *in vitro* [23]. Furthermore, HL have distinguished between normal colon (CCD33Co) cells and colon carcinoma (WiDr) cells, then fused and accumulated into the plasma membranes of tumor cells, leading to apoptosis *in vitro* [23]. We have reported that HL fused and accumulated into tumor cell membranes, and the apoptosis signal first passed through mitochondria, caspase-9, and caspase-3, second through Fas, caspase-8, caspase-3 and then reached the nucleus [15]. Same apoptotic signal-transduction could be possible in this study. These results suggest that HL-25 have remarkable inhibitory effects on the mouse models of CRC liver metastases along with apoptosis *in vivo*.

2.6. Selective accumulation of HL-25 into tumors

We examined the accumulation of HL-25, including NBDPC as a fluorescence probe into tumor cells, in the liver of mouse models of CRC liver metastases using a confocal laser scanning microscope. The results are shown in Fig. 6A. A decrease of green fluorescence within 3 h in the liver of normal healthy SCID mice (Control) treated with HL-25 including NBDPC was obtained. It has been reported that HL composed of DMPC and polyoxyethylene(23) dodecyl ether were metabolized in the liver after the intravenous administration to normal BALB/c mice [28]. On the other hand, accumulation of HL-25 including NBDPC into tumor cells in the liver were observed for 24 h. Next, we carried out immunostaining using carcinoembryonic antigen (CEA) as a histochemical marker of metastatic colon carcinoma to establish the therapeutic effects of HL-25. The results are shown in Fig. 6B. CEA positive cells in all liver tissue of hepatic metastatic mouse models were confirmed after 7days of the intrasplenic inoculation of HCT116 cells. It is noteworthy that remarkably high selective accumulation of HL-25 in the CEA positive area was observed as compared with control and DMPC. Significantly, we have reported that a good correlation between the membrane fluidity of HL and their growth-inhibitions for colorectal cancer cells *in vitro* [23]. HL having larger membrane fluidity showed higher inhibitory effects on the growth of colorectal cancer cells. Furthermore, we also reported that HL distinguished between normal colon CCD33Co cells and tumor colon WiDr cells, then fused and accumulated into the plasma membranes of tumor cells, leading to apoptosis [23]. In addition, we have already reported that HL have no side-effects *in vivo* in the safety tests intravenously administered *via* a vein once a day for 14 days (mice) [18] and six months (rats) [21,22]. These results

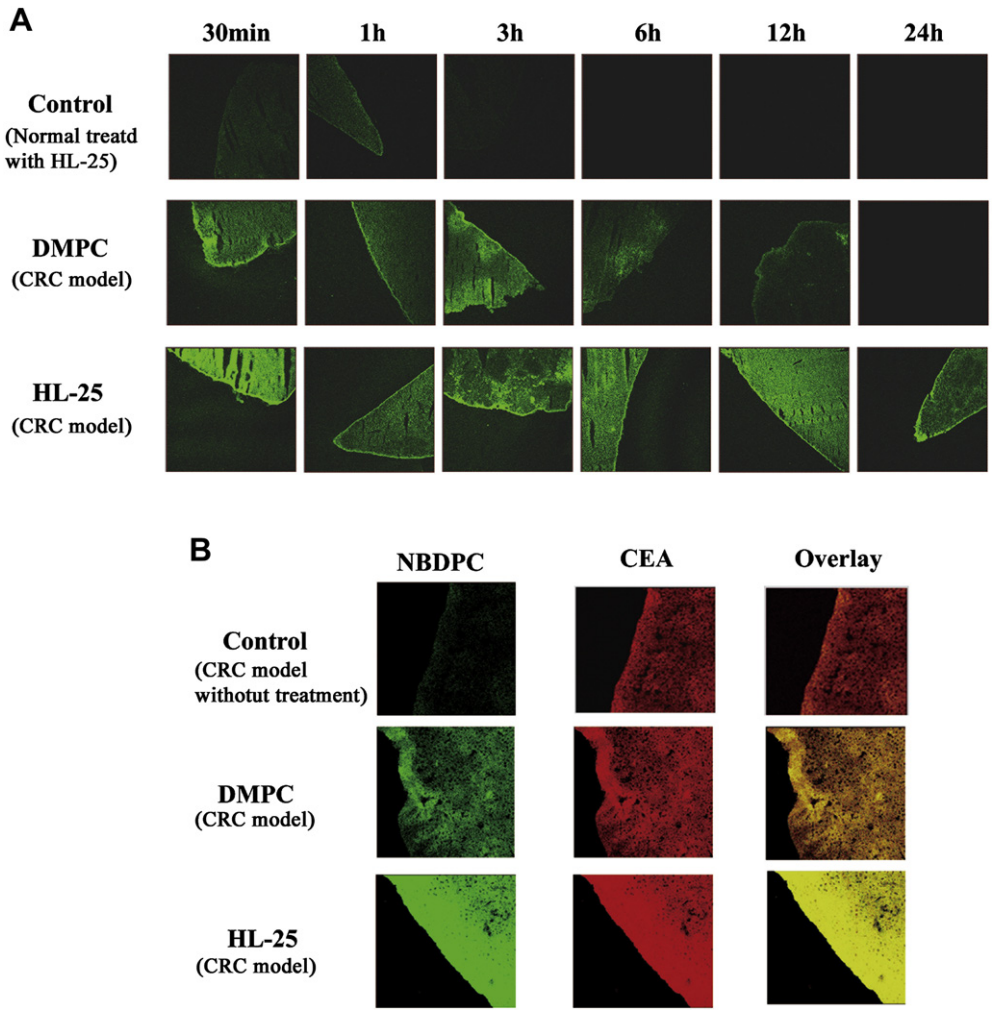

Fig. 6. (A) Selective accumulation of HL-25 including NBDPC into liver on mouse models of CRC liver metastases after 7days of the intrasplenic inoculation of HCT116 cells. Control: Liver section of normal healthy mice after the intravenous administration with HL-25. DMPC: Liver section of mouse models of CRC liver metastases treated with DMPC liposomes. HL-25: Liver section of mouse models of CRC liver metastases treated with HL-25. (B) CEA immunostaining of liver tissue of hepatic metastatic mouse models treated with HL-25 for 6 h after 7days of the intrasplenic inoculation of HCT116 cells. Control: Liver section of mouse models of CRC liver metastases without treatment. DMPC: Liver section of mouse models of CRC liver metastases treated with DMPC liposomes. HL-25: Liver section of mouse models of CRC liver metastases treated with HL-25.

suggest that HL-25 could selectively accumulate into tumor cells in the liver of mouse models of CRC liver metastases and inhibit the growth of HCT116 cells.

3. Conclusion

We clearly demonstrated that the therapeutic effects of HL-25 along with apoptosis were obtained for xenograft mouse models having human CRC liver metastases. The noteworthy aspects in this study are as follows: (a) Prolonged survival was obtained in mouse models of CRC liver metastases after the treatment with HL-25. (b) Remarkably high therapeutic effects of HL were obtained in mouse models of CRC liver metastases on the basis of an autopsy, relative liver weight, and hematoxylin–eosin staining. (c) Induction of apoptosis was observed in mouse models of CRC liver metastases after the treatment with HL-25 on the basis of the TUNEL method. (d) Accumulation of fluorescent labeled HL-25 was observed in the liver tissue of xenograft mouse models of CRC liver metastases for 24 h after the intravenous injection. The results in this study should be advantageous in the chemotherapy for patients with CRC liver metastases in clinical applications.

4. Experimental section

4.1. Preparation of hybrid liposomes

Hybrid liposomes (HL) were prepared by sonication of a mixture containing 95 mol% L-α-dimyristoylphosphatidylcholine (DMPC, NOF, Japan) and 5 mol% polyoxyethylene(25) dodecy ether ($C_{12}(EO)_{23}$, Nikko Chemicals, Tokyo, Japan) in 5% glucose solution using bath type sonicater (VS-N300, VELVO-CLEAR, Japan) at 45 °C with 300 W as shown in Fig. 1A, and filtered with a 0.20 μm cellulose acetate filter (Advantec, Japan).

4.2. 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 (Equation (1)), where κ is the Boltzmann constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient:

$$d_{hy} = kT/3\pi\eta D \quad (1)$$

4.3. Cell culture

Human colon carcinoma (HCT116) cell lines were purchased from the American Type Culture Collection (Manassas, VA). HCT116 cells were maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD) supplemented with penicillin 100 U/mL, streptomycin 50 μg/mL, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT). The cells were cultured in a 5% CO_2 humidified incubator at 37 °C.

4.4. Assessment of antitumor effects in vivo

The mice were handled in accordance with the guidelines for animal experimentation set out in Japanese law. The animal studies were approved by the Committee on Animal Research of Sojo University. Female SCID mice (C.B-17/Icr-scid) were obtained from CLEA (Tokyo, Japan). The mice were randomly grouped on the basis of body weight by the stratified randomization method. The number of mice was five in each group. The HCT116 cells (5.0×10^6 cells) were intrasplenically transplanted into the SCID mice [25]. HL (Dose: 136 mg/kg for DMPC) were intravenously administered once each day for 14 days after the inoculation of HCT116 cells. The livers were weighed after anatomizing the mice after 14 days of inoculation of HCT116 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 observed by optical microscope (Nikon TS-100, Tokyo, Japan).

4.5. TUNEL method

Detection of apoptotic cells was performed on the basis of the TUNEL method using an in situ apoptosis detection kit (ApopTag Plus Peroxidase, Intergen, U.S.A) according to manufacturer's directions. HCT116 cells (5.0×10^6 cells) suspended were intrasplenically inoculated into SCID mice. HL-25 was administered into the caudal vein of mice once each for 14 days after the HCT116 cells were inoculated to mice. Liver was removed from anaesthetized mice after the treatment with HL-25 and fixed in 10% formalin solution. Paraffin-embedded sections were made, and the detection of apoptosis of a tumor cell in liver was performed on the basis of the TUNEL assay according to the conventional method.

4.6. Assessment of survival rate in vivo

For assessment of survival rate, female SCID mice (C.B-17/Icr-scid) were obtained from CLEA (Tokyo, Japan). The mice were randomly grouped on the basis of body weight by the stratified randomization method. The number of mice was five in each group. The HCT116 cells (5.0×10^6 cells) were intrasplenically transplanted into the mice. HL (Dose: 136 mg/kg for DMPC) were intravenously administered once each day for 14 days after the inoculation of HCT116 cells. After dosing period, survival of mice were followed. The median lifespan was calculated using following the equation, median lifespan = (median survival days after the treatment)/(median survival days of control group) × 100.

4.7. Pharmacokinetics and immunostaining with CEA

The fusion and accumulation into the membrane of OS-RC-2 cells of HL including a fluorescence probe (1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine) (NBDPC; Avanti Polar Lipids, Alabama, U.S.A.) was performed. Female SCID mice (C.B-17/Icr-scid) were obtained from CLEA (Tokyo, Japan). The mice were randomly grouped on the basis of body weight by the stratified randomization method. The number of mice was three in each group. The HCT116 cells (5.0×10^6 cells) were intrasplenically transplanted into the mice. HL including NBDPC were intravenously administered for mice after 7days of the inoculation of HCT116 cells. The livers were resected from the mouse models, and embedded directly in optimum cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) at room temperature. The OCT-embedded livers were rapidly frozen in hexane/dry ice at −20 °C, and then the frozen tissue blocks were cut into 5 μm sections using a freezing microtome (Leica CM1850, Wetzlar, Germany). Furthermore, the sections were incubated with anti human carcinoembryonic antigen (CEA) rabbit polyclonal antibody (NBP1-4003, Novus, U.S.A.) at 4 °C for 1 h and with Alexa Fluor 594 donkey anti-rabbit IgG(H + L) (Molecular Probes, U.S.A.) in humidified box at 4 °C for 1 h. The cryosections of the liver tissue were observed by confocal microscope (Leica TCS-SP, Heidelberg, Germany) using a 488 nm Ar laser (detection, NBD:515–555 nm, Alexa Fluor 594:560–620 nm).

4.8. Statistical analysis

Results are presented as mean ± S.D. Data were statistically analyzed using Student's *t*-test. A p value of less than 0.05 was considered to represent a statistically significant difference.

Acknowledgments

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References

[1] G. Portier, D. Elias, O. Bouche, P. Rougier, J.F. Bosset, J. Saric, J. Belghiti, P. Piedbois, R. Guimbaud, B. Nordlinger, R. Bugat, F. Lazorthes, L. Bedenne, Multicenter randomized trial of adjuvant fluorouracil and folinic acid compared with surgery alone after resection of colorectal liver metastases: FFCD ACHBTH AURC 9002 trial, J. Clin. Oncol. 24 (2006) 4976–4982.
[2] E.K. Abdalla, J.N. Vauthey, L.M. Ellis, V. Ellis, R. Pollock, K.R. Broglio, K. Hess, S.A. Curley, Recurrence and outcomes following hepatic resection, radiofrequency ablation, and combined resection/ablation for colorectal liver metastases, Ann. Surg. 239 (2004) 818–825.
[3] S.K. Maithel, M. Gönen, H. Ito, R.P. Dematteo, P.J. Allen, Y. Fong, L.H. Blumgart, W.R. Jarnagin, M.I. D'Angelica, Improving the clinical risk score: an analysis of molecular biomarkers in the era of modern chemotherapy for resectable hepatic colorectal cancer metastases, Surgery 151 (2012) 162–170.
[4] R. Ueoka, R.A. Moss, S. Swarup, Y. Matsumoto, G. Strauss, Y. Murakami, Extraordinary micellar enantioselectivity coupled to altered aggregate structure, J. Am. Chem. Soc. 107 (1985) 2185–2186.
[5] R. Ueoka, Y. Matsumoto, R.A. Moss, S. Swarup, A. Sugii, K. Harada, J. Kikuchi, Y. Murakami, Membrane matrix for hydrolysis of amino acid esters with marked enantioselectivity, J. Am. Chem. Soc. 110 (1988) 1588–1595.
[6] R. Ueoka, Y. Matsumoto, H. Oyama, H. Takekuma, M. Iio, Effects of flavonoids in hybrid membranes on the activity of tripeptide as an enzyme model and their inhibition of hybridoma growth *in vitro*, Chem. Pharm. Bull. 36 (1988) 4640–4643.
[7] I. Kitamura, M. Kochi, Y. Matsumoto, R. Ueoka, J. Kuratsu, Y. Ushio, Intrathecal chemotherapy with 1,3-bis(2-chloroethyl)-1-nitorsourea encapsulated into hybrid liposomes for meningeal gliomatosis: an experimental study, Cancer Res. 56 (1996) 3986–3992.
[8] Y. Matsumoto, T. Kato, H. Suzuki, S. Hirose, Y. Naiki, M. Hirashima, R. Ueoka, Highly specific inhibitory effects of three-component hybrid liposomes including sugar surfactants on the growth of glioma cells, Bioorg. Med. Chem. Lett. 10 (2000) 2617–2619.
[9] R. Ueoka, Y. Matsumoto, S. Hirose, K. Goto, M. Goto, S. Furusaki, Remarkably enhanced inhibitory effects of three-component hybrid liposomes including sugar surfactants on the growth of lung carcinoma cells, Chem. Pharm. Bull. 50 (2002) 563–565.
[10] Y. Matsumoto, Y. Tanaka, K. Goto, R. Ueoka, Specific fixed aqueous layer of three-component hybrid liposomes related to inhibition of hepatoma cells growth, Chem. Lett. 37 (2008) 118–119.

[11] K. Goto, Y. Tanaka, Y. Matsumoto, R. Ueoka, Induction of apoptosis of human tumor cells by hybird liposomes including docosahexaenoic acid, Bioorg. Med. Chem. Lett. 18 (2008) 1880–1883.
[12] Y. Tanaka, K. Goto, Y. Matsumoto, R. Ueoka, Remarkably high inhibitory effects of docosahexaenoic acid incorporated into hybrid liposomes on the growth of tumor cells along with apoptosis, Int. J. Pharm. 35 (2008) 264–271.
[13] K. Nakano, Y. Iwamoto, W. Takata, Y. Matsumoto, R. Ueoka, Specific accumulation and growth inhibitory effects of hybrid liposomes to hepatoma cells *in vitro*, Bioorg. Med. Chem. Lett. 12 (2002) 3251–3254.
[14] H. Nagami, Y. Matsumoto, R. Ueoka, Induction of apoptosis by hybrid liposomes for human breast tumor cells along with activation of caspases, Biol. Pharm. Bull. 29 (2006) 380–381.
[15] Y. Matsumoto, Y. Iwamoto, T. Matsushita, R. Ueoka, Novel mechanism of hybrid liposomes-induced apoptosis in human tumor cells, Int. J. Cancer 115 (2005) 377–382.
[16] T. Towata, Y. Komizu, S. Suzu, Y. Matsumoto, R. Ueoka, S. Okada, Hybrid liposomes inhibit the growth of primary effusion lymphoma *in vitro* and *in vivo*, Leuk. Res. 34 (2010) 906–911.
[17] H. Nagami, Y. Matsumoto, R. Ueoka, Chemotherapy with hybrid liposomes for lymphoma without drugs *in vivo*, Int. J. Pharm. 315 (2006) 167–172.
[18] S. Shimoda, H. Ichihara, Y. Matsumoto, R. Ueoka, Chemotherapy with hybrid liposomes for human breast tumors along with apoptosis *in vivo*, Int. J. Pharm. 372 (2009) 162–168.
[19] H. Ichihara, J. Ueno, M. Umebayashi, Y. Matsumoto, R. Ueoka, Chemotherapy with hybrid liposomes for acute lymphatic leukemia leading to apoptosis *in vivo*, Int. J. Pharm. 406 (2011) 173–178.
[20] R. Ueoka, Y. Matsumoto, H. Ichihara, T. Kiyokawa, Chemotherapy with hybrid liposomes composed of dimyristoylphosphatidylcholine and polyoxyethylenealkyl ether without drugs, in: M.R. Marten, T.H. Park, T. Nagamune (Eds.), Biological Systems Engineering, ACS Symposium Series, American Chemical Society, Washington, DC, 2002, pp. 177–189.
[21] H. Ichihara, H. Nagami, T. Kiyokawa, Y. Matsumoto, R. Ueoka, Chemotherapy using hybrid liposomes along with induction of apoptosis, Anticancer Res. 28 (2008) 1187–1196.
[22] R. Ueoka, Y. Matsumoto, K. Goto, H. Ichihara, Y. Komizu, Membrane targeted chemotherapy with hybrid liposomes for tumor cells leading to apoptosis, Curr. Pharm. Des. 17 (2011) 1709–1719.
[23] Y. Komizu, Y. Matsumoto, R. Ueoka, Membrane targeted chemotherapy with hybrid liposomes for colon tumor cells leading to apoptosis, Bioorg. Med. Chem. Lett. 16 (2006) 6131–6134.
[24] Y. Komizu, H. Ueoka, K. Goto, R. Ueoka, Remarkable inhibitory effects of hybrid liposomes on growth of human colon cancer cells through induction of cell cycle arrest along with apoptosis, Int. J. Nanomed. 6 (2011) 1913–1920.
[25] J.S. Jeong, S.W. Lee, S.H. Hong, Y.J. Lee, H.I. Jung, K.S. Cho, H.H. Seo, S.J. Lee, S. Park, M.S. Song, C.M. Kim, I.H. Kim, Antitumor effects of systemically delivered adenovirus harboring trans-splicing ribozyme in intrahepatic colon cancer mouse model, Clin. Cancer Res. 14 (2008) 281–290.
[26] Y. Matsumoto, T. Kato, S. Iseki, H. Suzuki, K. Nakano, M. Iwahara, R. Ueoka, Remarkably enhanced inhibitory effects of hybrid liposomes on the growth of specific tumor cells, Bioorg. Med. Chem. Lett. 9 (1999) 1937–1940.
[27] S.K. Huang, K.D. Lee, K. Hong, D.S. Friend, D. Papahadjopoulos, Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice, Cancer Res. 52 (1992) 5135–5143.
[28] H. Ichihara, H. Nagami, K. Yamamoto, Y. Matsumoto, R. Ueoka, Chemotherapy with hybrid liposomes without any drug *in vivo*, Yakugaku Zasshi 123 (2003) 25–34 (Japanese).