CHEMOTHERAPY AND DETECTION OF CANCER USING HYBRID LIPOSOMES

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ABSTRACT—We have produced hybrid liposomes (HL) which can be prepared simply by sonication of a mixture of vesicular and micellar molecules in a buffer solution. The physical properties of HL such as size, shape, and membrane fluidity can be controlled by changing the constituents and compositional ratio. We have employed HL for chemotherapy and detection of cancer. Interesting results obtained are as follows; (A) The uniform and stable structure of HL composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenedodecyl ether (C12(EO)n) with a diameter of 80 nm was revealed. (B) The remarkable inhibitory effects of HL on the growth of various tumor cells were attained in vitro. (C) Induction of apoptosis by HL was obtained and the pathway of apoptosis induced by HL was clarified. (D) A good correlation between the membrane fluidity of HL and inhibitory effects of HL for tumor cells was obtained. (E) Significantly prolonged survival and remarkable reduction of tumor volume were obtained using mice model of carcinoma after the treatment with HL without any side effects in vivo. (F) In clinical applications, prolonged survival and remarkable reduction of neoplasm were attained in patients with lymphoma treated with HL without any side effects after the approval of the bioethics committee. Furthermore, (G) Specific accumulation and growth inhibitory effects of HL were obtained in human tumor cells without affecting normal cells at all in vivo.

Key Words: Hybrid liposomes, Antitumor effect, Apoptosis, Chemotherapy, Membrane fluidity, Cancer detection

1. INTRODUCTION

Biological membranes provide compartment of defined sizes, shapes, and micro-environments. They organize living matter in cells and create a fluid two dimensional matrix. In general, membranes of animal cells are typically composed of 40-50% lipids and 50-60% proteins. There are wide variations in the types of lipids including phospholipids and proteins as well as in their ratios. Membrane-fluctuations of cancer cells are very different from those of normal cells. In general, the membranes of cancer cells are more fluid as compared with normal cells.

Liposomes are microscopic spheres made from fatty materials, predominantly phospholipids. Because of their similarity to phospholipid domains of cell membranes and an ability to incorporate and/or carry bioactive substances, liposomes can be used for the enzymological (for instance, membrane-bound enzyme models) and medical (for instance, drug delivery systems) applications.
It is well known that liposomes are used as drug carriers. The drugs include antitumor agents, hormones, and immunomodulators [1-2]. In particular, polyoxyethylene-glycol (PEG) – phosphatidylcholine (PC) liposomes have been found to be effective for prolonging blood circulation [3-4]. On the other hand, we have recently produced specific hybrid liposomes (HL) composed of vesicular and micellar molecules; they are free from any contamination from organic solvents and stable for a longer period. Changing the composition of HL can control the physical properties of these liposomes, such as size, membrane fluidity, and phase transition temperature [5-6]. A schematic representation of HL is shown in Figure 1A.

![Figure 1. (A) Schematic representation of HL. (B) An electron micrograph of HL. (C) Time course of d_hy change for HL](image)

In the course of our study on HL, the following interesting results have been obtained. (A) The uniform and stable structure of HL composed of dimyristoylphosphatidyl-choline (DMPC) and polyoxyethylenedodecyl ether (C_{12}(EO)_n : n=21~25) with a diameter of 80 nm was revealed [7-11]. (B) The remarkable inhibitory effects of HL on the growth of various cancer cells were attained in vitro [7-11]. (C) The induction of apoptosis by HL was obtained and the pathway was elucidated [10]. (D) A good correlation between the membrane fluidity of HL and inhibitory effects of HL for cancer cells was obtained [11]. (E) HL distinguished between the cancer and normal cells which had higher and lower membrane fluidities respectively, then fused and accumulated preferentially into the membranes of cancer cells [9, 11]. (F) Significantly prolonged survival and remarkable reduction of tumor volume were obtained using mice model of carcinoma after the treatment with HL without any drug in vivo [12-17]. There were no abnormal findings on normal rats after administering HLs on the basis of acute and chronic toxicity tests [13-14, 17]. (G) In clinical applications, prolonged survival was attained in patients with lymphoma after the intravenous injection of HLs without any side effect after the approval of the bioethics committee [13].

In this study, we report on the uniform and stable HL composed of DMPC and C_{12}(EO)_n, which are effective for inhibiting the growth of tumor cells in vitro, in vivo and in clinical applications, and on detection of cancer.
2. CHEMOTHERAPY WITH HL

2.1 Antitumor Effects in Vitro
We examined the morphology of HL on the basis of electron microscopy and dynamic light scattering measurements. The electron micrograph of HL shows the presence of spherical vesicles (Figure 1B). Interestingly, a clear stock solution of HL having a hydrodynamic diameter of 80 nm with a single and narrow distributions could be kept over 30 days, as shown in Figure 1C. On the other hand, DMPC liposomes were unstable and precipitated after 14 days. These results indicate that the uniform and stable structure of HL could be obtained and suggest that HL could avoid reticular endothelial system (RES) in vivo.

We examined the inhibitory effects of HL on the growth of various tumor (human B lymphoma (RAJI), human promyelocytic leukemia (HL-60), human breast tumor (MDA-MB-453)) and human lung carcinoma (A549) cells on the basis of WST assay using highly water-soluble tetrazolium salt. HL were prepared by dissolving both DMPC and C12(EO)n in 5% glucose solution with sonication at 300 W and 45˚C for 5 min, followed by filtration with a 0.20 μm filter. Fifty percent inhibitory concentration (IC50) values of HL were from one fourth to a half of those of the single-component DMPC liposomes, indicating that the inhibitory effects of HL are large when compared with those of the single-component DMPC liposomes [7-11, 13-20]. These results indicate that inhibitory effects of HL should be advantage as compared with single-component DMPC liposomes.

2.2 Induction of Apoptosis
The induction of apoptosis and apoptotic signaling pathway for HL-60 [10] and MDA-MB-453 cells [14] by HL was examined using flow cytometry, and western blot. Interesting results are as follows: (A) A certain amount of HL accumulated in the cells and then induced apoptosis. It is well known that the apoptosis signal is transduced by sequential activation of caspase family cysteine proteases. (B) Concentration dependence of caspase-8, 9, and 3 inhibitors for apoptotic DNA rate of MDA-MB-453 cells was obtained (Figure 2A). (C) The apoptotic signal induced by HL passed through Fas and FADD. (D) Mitochondrial transmembrane potential decreased by adding HL, and then cytochrome c released from mitochondria (Figure 2BC). We propose the mechanism of apoptosis induced by HL as shown in Figure 3. That is, HL fused and accumulated into tumor cell membranes, and the apoptotic signal passed firstly through mitochondria, caspase-9, and caspase-3, secondly through Fas, caspase-8, caspase-3 and then reached the nucleus [10, 14].
Figure 2. (A) Activation of caspase of MDA-MB-453 cells after the treatment with HL. (○) Caspase-3, (□) Caspase-8, (△) Caspase-9. (B) Decrease of mitochondrial membrane potential for MDA-MB-453 cells after the treatment with HL for 30min. Control (shaded histogram), HL (unshaded histogram). (C) Release of mitochondrial cytochrome c in MDA-MB-453 cells after the treatment with HL.

Figure 3. Schematic representation of the signalling pathway for apoptosis of tumor cells induced by HL.

2.3 Membrane Targeted Antitumor Mechanism

It is well known that the fluidity of cell membranes of disease such as tumor is generally larger than that of normal cells. The fusion and accumulation of HL into tumor cell (liver cancer HuH-7 and Hep-G2 cells, colon cancer HCT116 and WiDr cells, stomach cancer MKN-45 cells, lung cancer A549 cells, and cervical cancer HeLa cells) membranes on the basis of total internal reflection fluorescence (TIRF) microscopy have been investigated using 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBDPC) as a fluorescence probe [18]. The fluorescence of HL/NBDPC was observed around the plasma
membranes of HuH-7, Hep-G2, and HCT116 cells (Figure 4A). Also, the mean-fluorescence intensity of NBDPC in the plasma membranes of cancer cells was plotted with time in Figure 4b. The fluorescence intensity reflects the amount of HL/NBDPC accumulated into the cell membranes. The fluorescence intensities for HuH-7, Hep-G2, and HCT116 cells drastically increased during 8 min after the treatment with HL/NBDPC, though those for A549 and HeLa cells were low and almost constant (Figure 4B). These results indicate that HL/NBDPC could accumulate more in the plasma membranes of HuH-7, Hep-G2, and HCT116 cells as compared with those of A549 and HeLa cells. We focused on the membrane dynamics of cancer cells and attempted to investigate the relationship between membrane fluidity of cancer cells and inhibitory effects of HL on the growth of cancer cells. The fluidity of cancer (A549, MKN-45, HCT116, WiDr, Hep-G2, HuH-7, and HeLa) cell membranes was evaluated from the fluorescence polarization ($P$) of fluorescent probe DPH embedded in the plasma membranes. The fluorescence depolarization is caused by the molecular motion of the fluorescent probe, which reflects the microviscosity of the surrounding region. As the $P$ value of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the cell membranes becomes smaller, the cancer cell membranes have larger fluidity. $P$ values are plotted against the IC$_{50}$ values of HL as shown in Figure 4C. Interestingly, good correlations between the $P$ values of DPH in the cell membranes and the IC$_{50}$ values of HL for the tumor cells were obtained. This result suggests that the tumor cells having larger membrane fluidity could be further inhibited by HL.

Figure 4. Accumulation of HL including NBDPC (HL/NBDPC) in the plasma membranes of cancer cells. (A) TIRF images of cancer (HuH-7, Hep-G2, HCT116, A549, HeLa) cells treated with HL/NBDPC. Scale bar, 10 μm. (B) Time course of mean-fluorescence intensity of NBDPC embedded into cancer cell membranes (HuH-7, red; Hep-G2, green; HCT116, blue; A549, yellow; HeLa, purple). Mean ± S.E. (C) Correlation between the IC$_{50}$ of HL on the growth of cancer cells and the fluorescence polarization ($P$) of DPH in the cancer cell membranes.
2.4 Therapeutic Effects and Toxicity in Vivo

Screening for therapeutic effects is generally carried out in mice or rats bearing tumor cells. The animals were handled during the study in accordance with the guidelines for animal’s experiment of Japanese law.

We examined the therapeutic effects of HL-25 using mice of acute lymphatic leukemia (ALL) with peritoneal dissemination after the intraperitoneal treatment with HL-25. The results are shown in Figure 5 [19]. The photographs show that the mice of control group had distended abdominal regions. Interestingly, massive ascites was obtained in peritoneal cavity of the mice of control group after dissection. The mice treated with DMPC and HL-25 had a significantly lower volume of ascites compared with the mice in control group (Figure 5A). Furthermore, survival rates are shown in Figure 5B. It is noteworthy that a significantly prolonged survival (> 400 %, \( p<0.01 \)) was obtained in the mice treated with HL-25. These results indicate that HL-25 could strongly inhibit the growth of MOLT-4 cells \textit{in vivo}.

![Figure 5. Therapeutic effects of HL-25 for model mice of ALL with peritoneal dissemination \textit{in vivo}. (A) Photographs of ascites-bearing mice of control group or mice treated with HL-25 for 14 days after being inoculated with MOLT-4 cells intraperitoneally, (B) Survival curves for model mice of ALL with peritoneal dissemination treated with HL-25](image)

Next, we examined the therapeutic effects of HL-21 using xenograft mice models after the subcutaneous inoculation of human breast tumor (MDA-MB-453) cells \textit{in vivo} [14]. MDA-MB-453 cells suspended into matrigel were subcutaneously inoculated to dorsal of nude mice. HL-21 were intravenously administered once each day for two weeks. The results are shown in Figure 6A. After the administration of HL-21 for two weeks, the median of tumor volume was 458 ± 32 mm³ and 147 ± 61 mm³ in the control and treatment groups, respectively. It is noteworthy that the remarkable reduction of tumor volume (70%) in mice inoculated MDA-MB-453 cells was obtained after the treatment with HL-21. Moreover, we examined the induction of apoptosis by HL-21 for breast tumor in xenograft mice using the TUNEL method. The results are shown in Figure 6B. Brown color was observed in the tumor cells of xenograft mice after the treatment with HL-21, although the apoptotic cells were slightly observed after adding DMPC liposomes. These results indicate that HL-21 have remarkable reduction effects along with apoptosis on the growth of breast tumor \textit{in vivo} as compared with DMPC liposomes.
Figure 6. Therapeutic effects of HL-21 for xenograft mice models after the subcutaneously inoculated of human breast rumor (MDA-MB-453) cells. (A) The tumor volume of mice treated with HL-21. (B) Induction of apoptosis for tumor section in xenograft mice model using TUNEL method.

Furthermore, we examined the therapeutic effects of 90 mol% DMPC/10 mol% C_{12}(EO)_{25} (HL-25) using xenograft mouse models of hepatic metastasis in human colorectal cancer (WiDr) cells [20]. WiDr cells were intrasplenic inoculated into the SCID (severe combined immunodeficiency) mice. HL-25 were intravenously administered once each day for 14 days after the inoculation of WiDr cells. Remarkably important therapeutic effects of HL-25 were obtained in the xenograft mouse model of colorectal cancer on the basis of autopsy (Figure 7A), hematoxylin-eosin staining (Figure 7B) and CEA immunostaining (Figure 7C) in vivo. Induction of apoptosis was observed in the xenograft mouse model of colorectal cancer after treatment with HL-25 on the basis of the TUNEL method (Figure 7C). Prolonged survival was obtained in our xenograft model of colorectal cancer after treatment with HL-25 (Figure 7D). These results suggest that HL should be effective for inhibiting metastasis of colorectal cancer cells to liver.
Figure 7. (A) Therapeutic effects for a xenograft mouse model of colorectal cancer treated with HL-25 on the basis of autopsy analysis. The red circles indicate tumors. Assessment of the therapeutic effects of a xenograft mouse model of colorectal cancer treated with HL-25 on the basis of histological analysis using (B) HE staining and (C) CEA immunostaining. Photographs of liver and spleen in hepatic metastasis mice model after the treatment with HL-25. (D) Micrographs of tumor in a xenograft mouse model of colorectal cancer treated with HL-25 using the TUNEL method. The arrows and circles indicate apoptotic cells. (E) Survival curves of a xenograft mouse model of colorectal cancer treated with HL-25. There was a significant difference (*: \( p < 0.05 \)) compared with the control group.

In addition, the safety of HL-23 was examined using normal rats [13, 17]. Six rats were assigned to each group by the stratified continuous randomization method and HL-23 (dose for DMPC was 67.8 mg/kg, 136 mg/kg, and 203 mg/kg) were intravenously administered via a vein once a day for six months. The rats were weighed during the experimental period. The numbers of red and white blood cells of rats treated with HL-23 were within normal limits. All of the other biochemical parameters, such as ALP, GOT, and GPT activities, as well as levels of albumin, urea, nitrogen, creatinine, glucose, total protein, calcium, inorganic phosphorus, sodium, potassium, and chloride, were not significantly different from those observed in the controls. Furthermore, no weight loss was observed in the rats. These results indicate that HL should have no side-effects in vivo.

2.5 Clinical Application

Clinical applications of HL without any drug for 10 patients with lymphoma (2 patients), gastric cancer (2 patients), kidney cancer (one patient), mammary cancer (one patient), pharyngeal cancer (one patient), hepatoma (one patient), gallbladder cancer (one patient), and rectal cancer (one patient) were examined after passing the committee of bioethics at the different hospitals.
Informed consents were obtained in accordance with the Declaration of Helsinki. We report the pilot study using HL-23 for one patient with advanced stage B-lymphoma who did not recover by any chemotherapeutics after the approval of the Bioethics Committee at the National Kumamoto Hospital. HL-23 were administered intravenously once every day at a dose of 11.0-16.5 mg/kg for DMPC for 10 months. HL-23 were also locally administered (2 times/week, dose for DMPC was 0.5 mg/kg) to the lymph node neoplasm (solid tumor) for two months and photographs were taken using ultrasound echo [13]. There were no abnormal findings post administration on routine blood test and hematochemistry (data not shown). A prolonged survival, more than one year, was attained in this patient after the intravenous injection of HL-23 without any side-effects. It should be noted that a remarkable reduction of solid tumor was observed after the local administration (2 times/week) of HL-23, as shown in Figure 8A.

Figure 8. A pilot clinical test of HL in a patient with recurrent malignant lymphoma. (A) Reduction of lymph node neoplasm (solid tumor) after the local administration of HL for two months. (B) Induction of apoptosis for solid tumor using the TUNEL method after the treatment with HL.

Moreover, we examined induction of apoptosis in solid tumor using the TUNEL method. Tissue sections were prepared from solid tumor and stained by a TUNEL-based method. A fluorescence micrograph is shown in Figure 8B. The green color was obtained in solid tumor after the injection of HL-23, indicating that HL-23 could induce apoptosis in solid tumor. During the period of treatment, no abnormal findings were obtained on the basis of various hematological and biochemical tests. These results demonstrate that HL should be safe and effective in clinical applications.
3. APPLICATION OF HL FOR DETECTION OF CANCER

3.1 Selective Accumulation for Tumor Cells in Vitro

Membrane fluidity of tumor cells differ from that of normal cells. The membranes of tumor cells are generally more fluid as compared with normal ones. We examined accumulation of HL toward tumor cell membrane and normal cell membrane and normal cell membrane with confocal laser microscopy using HL having NBCPC. The cell membrane accumulation of HL in human lung cancer cells (A549, H460, H23, H520) and human normal lung cells (WI-38, NHLF, HMVEC-L) were observed with the confocal laser microscopy using HL having NBCPC [21]. The results are shown in Figure 9. Interestingly, accumulation of HL after adding to human lung cancer cells (A549, H460, H23, H520) remarkably increased, though that after adding to human normal lung cells (WI-38, NHLF, HMVEC-L) was low. The specific high accumulation of HL should be caused by the high fluidity of cancer cell membranes as compared with normal cell membranes [9, 11, 18, 21].

![Figure 9. Fluorescence micrographs of lung cancer cells (A549, H460, H23, H520) and normal lung cells (WI-38, NHLF, HMVEC-L) after the treatment with HL including NBDPC using confocal laser microscopy. Scale bar: 20 μm.](image)

3.2 Specific Accumulation for Tumors in Vivo

We examined the accumulation of HL-25, including NBDPC as a fluorescence probe into tumor cells, in the liver of mouse models of colorectal cancer (CRC) liver metastases using confocal laser scanning microscope. The results are shown in Figure 10A. 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. 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 [22]. The results are shown in Figure 10B. CEA positive cells in all liver tissue of hepatic metastatic mouse models were confirmed after 7 days of the intrasplenic inoculation of HCT116 cells. Interestingly, remarkably high selective accumulation of HL-25 in the CEA positive area was observed as compared with control and DMPC. These results 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.

It is noteworthy that specific accumulation of HL were obtained in human tumor cells without affecting normal cells for a long hours. These results demonstrate that HL could be effective for detection of cancer cells.
Figure 10. (A) Selective accumulation of HL-25 including NBDPC into liver on mouse models of CRC liver metastases for a long hours after 7 days of the intrasplenic inoculation of HCT116 cells. (B) CEA immunostaining of liver tissue of hepatic metastatic mouse models treated with HL-25 for 6 h after 7 days of the intrasplenic inoculation of HCT116 cells.

4. CONCLUSION

A summary of the noteworthy aspects of this study is as follows: (A) High inhibitory effects of hybrid liposomes (HL) themselves composed DMPC/C12(EO)n on the growth of tumor cells were obtained without any drug. (B) Induction of apoptosis by HL was obtained using flow cytometry, DNA agarose gel electrophoresis, and fluorescence micrograph. The pathway of apoptosis induced by HL was clarified. That is, HL fused and accumulated in tumor cell membranes, and the apoptotic signal passed firstly through mitochondria, caspase-9, and caspase-3, secondly through Fas, caspase-8, caspase-3 and then reached the nucleus. (C) A good correlation between IC50 and membrane fluidity of various tumor (cervical, rectal, colon, lung, liver, brain, and stomach) cells was also observed. This demonstrates that growth inhibition and apoptosis for tumor cells by HL provides the possibility of therapy from a viewpoint of biophysical characteristics of tumor cell membranes. (D) Significantly prolonged survival and remarkable reduction of tumor volume were obtained using mice model of carcinoma after the treatment with HL without any drug in vivo. There were no abnormal findings on blood test, hematochemistry, relative organ weight, and autopsy of normal rats after administering HL on the basis of chronic toxicity tests. (E) After the approval of the bioethics committee, prolonged survival was attained in patients with lymphoma after the intravenous injection of HL with a
diameter of 80 nm, which could avoid RES, without any side effects. Remarkable reduction of neoplasm was obtained after the local administration of HL in clinical applications. (F) For detection of cancer, specific accumulation and growth inhibitory effects of HL were obtained in human tumor cells without affecting normal cells.

In conclusion, the chemotherapy and detection of cancer cells with drug-free HL was established without any side effects for the first time.

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REFERENCES

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