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Nano-chemotherapy using cationic liposome that strategically targets the cell membrane potential of pancreatic cancer cells with negative charge

Muneaki Motomura, Hideaki Ichihara, Yoko Matsumoto *

Division of Applied Life Science, Graduate School of Engineering, Sojo University, 4-22-1, Ikeda, Nishi-ku, Kumamoto 860-0082, Japan

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ABSTRACT

Negatively charged phosphatidylserine (PS) and sialic acid-containing glycosphingolipids (GM1) were observed to be over represented on the cell membranes of pancreatic cancer cells (BxPC-3) as opposed to normal pancreatic cells. Cationic liposomes (CL) were also found to selectively accumulate into the negatively charged cell membranes of BxPC-3 cells and inhibited their growth but have no effect on the viability of normal pancreatic cells. CL induced apoptosis in BxPC-3 cells via activation of caspase-3, -8, and -9 and mitochondrial events and inhibited tumor enlargement in xenograft mouse models of pancreatic cancer.

The early stages of pancreatic cancer are often difficult to detect and hence most cases are detected at the advanced stages of the disease. Surgical removal of pancreatic cancer is often carried out at the early stages of the disease. In cases where surgery is not possible for advanced and metastatic pancreatic cancer, radiation therapy, chemotherapy, or both are often carried out to limit tumor growth. FOLFIRINOX (5-fluorouracil/Folinic acid/irinotecan/Oxaliplatin) is one of the main drugs used in the combination chemotherapy of advanced pancreatic cancer alongside[1,2] gemcitabine which may also be given on its own.[3] However, severe side effects may be caused by anticancer chemotherapy.[4,5]

Hybrid liposomes (HL) can be prepared by just the sonication of vesicular and micellar molecules in a buffer solution.[6] High inhibitory effects of HL without any anti-cancer drug on the growth of tumor cells *in vitro*,[7,8] *in vivo*[9,10] and for clinical application have been reported.[11] HL are fused and accumulate in membranes of tumor cells, and then induced apoptosis *via* activation of caspases and mitochondrial event.[7]

We have produced novel hybrid cationic liposomes (CL) composed of phospholipid, cationic lipid, and PEG surfactants.[12] CL (containing cationic lipid) exerted a marked inhibitory effect on the growth of human renal cell carcinoma and colorectal cancer and induced both *in vitro* and *in vivo* apoptosis in the absence of drugs.[12,13]

The tumor marker commonly used for pancreatic cancer is CA19-9 which contains anionic sialic acid. However, CL therapy which targets negatively charged cell membranes of pancreatic cancer cells has not yet been investigated.

In this study, we investigated the inhibitory effects of CL composed of 87 mol% dimyristoylphosphatidylcholine (DMPC), 8 mol % *O,O′*-ditetradecanoyl-*N*-(α-trimethyl-ammonioacetyl) diethanolamine chloride ($2C_{14}ECl$), and 5 mol% polyoxyethylene(21)dodecyl ether ($C_{12}(EO)_{21}$) on the growth of pancreatic cancer (BxPC-3) cells as well as its ability to induce apoptosis *in vitro* and *in vivo*. We also analyzed the difference between the membrane potential of pancreatic cancer cells and normal pancreatic cells.[14,15] Furthermore, the nano-chemotherapeutic effect of CL was examined *in vivo* using subcutaneous mouse models of pancreatic cancer.

CL were prepared by using sonication (VS-N300; VELVO, Tokyo, Japan) of a mixture containing DMPC (purity > 99%; NOF Co. Ltd., Tokyo, Japan), micellar molecules: $C_{12}(EO)_{21}$ (purity > 99%; Sogo Pharmaceutical Co. Ltd. Tokyo, Japan) and $2C_{14}ECl$ (purity > 99%; Sogo Pharmaceutical Co. Ltd. Tokyo, Japan) in 5% glucose solution at 45 °C with 300 W, followed by filtration with a 0.20 μm filter.

We evaluated the inhibitory effects of CL on the growth of human pancreatic cancer (BxPC-3) cells on the basis of the WST-1 method.[16] The IC_{50} values of CL were determined from a plot of concentration of CL versus cell viability recorded at each test concentration (Fig. 1A). The IC_{50} value of CL obtained for BxPC-3 cells were lower than those of the DMPC and HL, while the IC_{50} values of CL for normal pancreatic cells was over 1000 μM. IC_{50} values

* Corresponding author.
E-mail address: matumoto@life.sojo-u.ac.jp (Y. Matsumoto).

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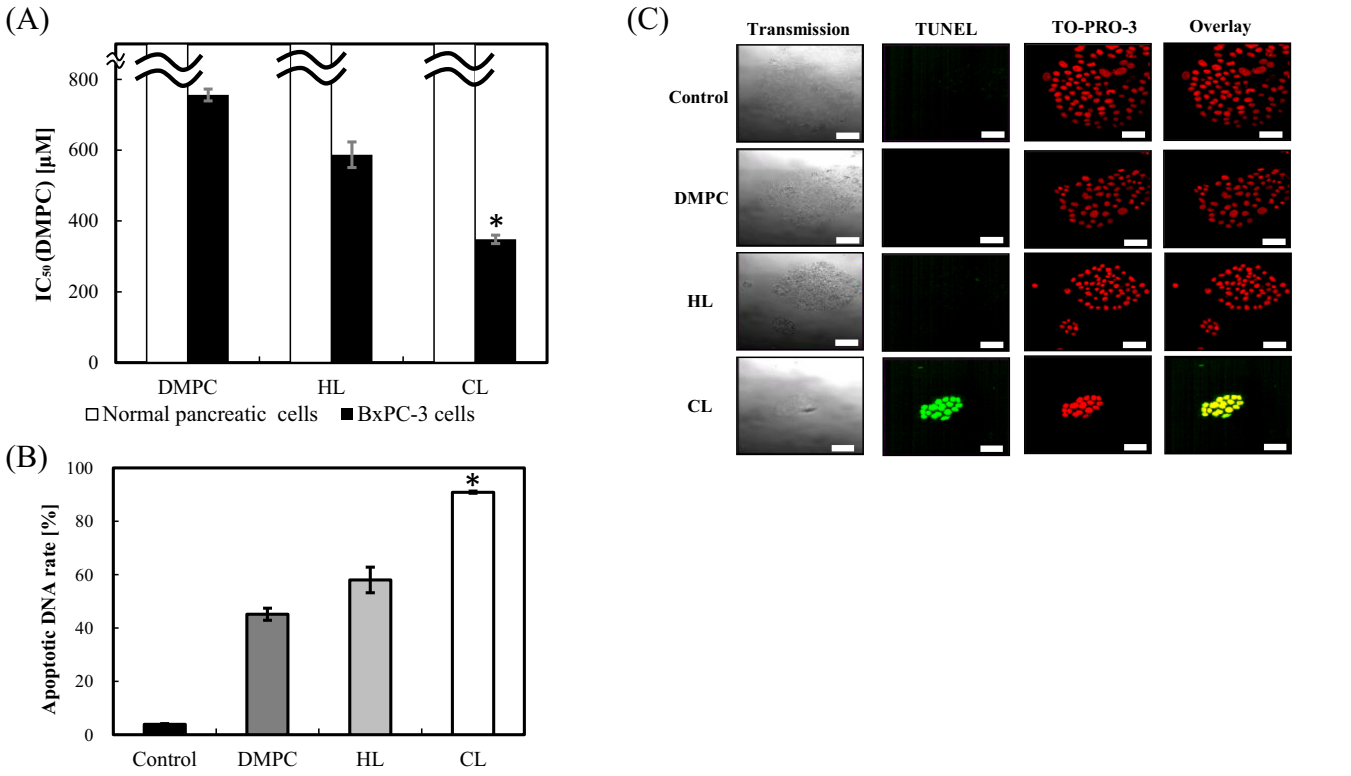

Fig. 1. (A) Inhibitory effects of CL on the growth of human pancreas cancer (BxPC-3) cells. The values here are represented as mean ± S.E. of three independent experiments. Values of $p < 0.05$ were considered statistically significant. The p value was evaluated by Student's t-test. $^{*}p < 0.01$; versus DMPC and HL. (B) Apoptotic DNA rate for BxPC-3 cells treated with CL for 48 h. The values here are represented as mean ± S.E. of three independent experiments. Values of $p < 0.05$ were considered statistically significant. The p value was evaluated by Student's t-test. $^{*}p < 0.01$; versus control, DMPC and HL. (C) Fluorescence micrographs of BxPC-3 cells using TUNEL method treated with CL for 24 h. CL:[DMPC] = 0.70 mM, [$C_{12(EO)21}$] = 0.04 mM, [$2C_{14}ECl$] = 0.064 mM. TUNEL (green) indicates apoptotic cells. TO-PRO-3 (red) indicates nucleus.

of CL for BxPC-3 cells was one-third or less that of normal pancreatic cells. DMPC, HL, and CL did not affects on the viability of normal pancreatic cells (IC_{50} > 1000 μM), which indicates non-toxicity of those liposomes for normal pancreatic cells. These results indicate that CL exerted a high level of selective inhibition on BxPC-3 cells without affecting the growth of normal pancreatic cells. The induction of apoptosis in BxPC-3 cells by CL was examined using flow cytometric analysis and the propidium iodide (PI) staining method (Fig. 1B).[17] Apoptotic DNA rates of CL for BxPC-3 cells were higher than those of the DMPC and HL reaching a high apoptotic rate of 90%. We observed DNA fragmentation of BxPC-3 cells caused by CL using the TUNEL method and confocal laser microscopy (Fig. 1C).[18] Green color indicating apoptotic BxPC-3 cells treated with CL was observed. No green coloring was observed with the use of HL and DMPC. These results indicate that CL induced apoptosis in BxPC-3 cells.

It is known that the membrane potential of cancer cells is remarkably different from that of normal cells. We examined electrostatic interactions between the cell membrane of BxPC-3 cells and CL. We also tested for the presence of negatively charged phosphatidylserine (PS) in the outer membrane of BxPC-3 cells and normal pancreatic cells using the Annexin V-FITC staining method by flow cytometry (Fig. 2A).[19] Relative fluorescence intensity of Annexin V-FITC in the cell membrane of BxPC-3 cells was three times higher than that in normal pancreatic cells. Subsequently, flow cytometry was used to measure the content of gangliosides (GM1) containing sialic acid residues in the cell membrane of BxPC-3 cells and normal pancreatic cells using the subunit B cholera toxin (CTB) staining method (Fig. 2B).[20] Relative fluorescence intensity of GM1 for BxPC-3 cells was four times that of normal pancreatic cells. The zeta potential of BxPC-3 cells and normal pancreatic cells was also analyzed (Fig. 2C).[21] Zeta potential in BxPC-3 cells (−50 mV) was observed to be much lower than that in normal pancreatic cells (−18 mV). We also examined the zeta potential changes of BxPC-3 cells treated with CL (Fig. 2D).[21] Time-dependent increase of the zeta potential of BxPC-3 cells treated with CL from −50 mV to −20 mV was observed. These results indicate that CL selectively targets BxPC-3 cells with negatively charged cell membranes.

In order to understand how CL induces apoptosis in BxPC-3 cells, we examined the fusion of CL containing NBDPC ((1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl]-*sn*-glycero-3-phosphocholine (NBDPC)) as a fluorescence probe with the cell membrane of BxPC-3 cells using confocal laser microscopy (Fig. 3A).[22] Fusion of CL containing NBDPC with the membrane of BxPC-3 cells was accelerated, occurring within 3 h, although less accumulation of HL was obtained. On the contrary, there was no membrane fusion observed for BxPC-3 cells treated with DMPC containing NBDPC. We investigated the involvement of mitochondria in the induction of apoptosis of BxPC-3 cells by CL (Fig. 3B).[23] A decrease in mitochondrial transmembrane potential of BxPC-3 cells treated with CL was observed suggesting that. CL induced apoptosis of BxPC-3 cells through the mitochondrial pathway. We further examined the activation of caspase-3, caspase-8, and caspase-9 to investigate the mechanism of induction of apoptosis in BxPC-3 cells by CL using the fluorescence substrate assay (Fig. 3C).[24] Green coloring indicating activated caspase-8, caspase-9, and caspase-3 substrate was observed in BxPC-3 cells treated with CL suggesting that CL induced apoptosis of BxPC-3 cells through the activation of