Inhibitory Effects of Hybrid Liposomes on the Overgrowth Of Human Synovial Sarcoma Cells By Induction Of Apoptosis

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Abstract

Hybrid liposomes (HL) composed of L- α dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(23)dodecyl ethers having a diameter under 100 nm were produced. It is noteworthy to note that HL inhibited the overgrowth of human synovial sarcoma (SW982) cells (model cells of rheumatoid arthritis) and induced apoptotic death of SW982 cells through activation of caspase-3, -8 and -9. A significant accumulation of HL including fluorescence probe (ICG) into SW982 cells was observed.

Keywords: Hybrid Liposome; Rheumatoid arthritis; Synovial sarcoma; Apoptosis, Caspase

Abbreviations

$C_{12}(EO)_{23}$: polyoxyethylene(23)dodecyl ethers; DMPC: L- α -dimyristoylphosphatidylcholine; HL: hybrid liposomes

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the overgrowth of synoviocytes. Synoviocytic overgrowth after the supersecretion of inflammatory cytokine forms pannus in a chronically inflamed microenvironment, which induces the destruction of articular cartilage and subchondral bone. Therapeutic effects of anti-rheumatic drugs against RA have been reported [1,2]. However, anti-rheumatic drugs have severe side effects [2,3]. Therefore, a novel anti-rheumatic drug that would be effective for inhibiting the growth of synoviocyte in RA without any side-effects is highly desirable to improve the quality of life.

Hybrid liposomes (HL) can be prepared by simply ultrasonication a mixture of vesicular and micellar molecules in buffer solutions, and contain no organic solvent unlike conventional liposomes [4]. The physiological properties of these liposomes such as size, shape, and the membrane fluidity can be controlled by changing the constituents and compositional ratios of phospholipids and micellar molecules. Therapeutic effects of HL composed of L- α -dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene(20) sorbitan monolaurate (Tween 20) including antitumor drugs have been reported for glioma model rats *in vivo* [5]. On the other hand, HL composed of DMPC and polyoxyethylene(n) dodecyl ethers ($C_{12}(EO)_n$) without any drugs have remarkable inhibitory effects on the growth of various tumor cells such as lymphoma, leukemia and colorectal cancer

along with apoptosis *in vitro*, *in vivo* and for clinical applications [6-13]. Furthermore, with regard to RA, inhibitory effects of HL on the overgrowth of human primary RA fibroblast-like synoviocytes (HFLS-RA) cells *in vitro* have been reported [14]. Primary HFLS-RA cells established from RA patients have been used to study the effects of medicine for RA. However, The Primary HFLS-RA cells have weaknesses such as the ethical problem to collect from the RA patients and the difficulty of the establishment of the cell line. In recent years, the validity of synovial sarcoma (SW982) cell line for RA study has been reported [15-17].

In this study, we examined the inhibitory effects of HL composed of DMPC and $C_{12}(EO)_{23}$ on the overgrowth of human SW982 cells, which are model cells of RA *in vitro*.

HL, nanoparticles, can be prepared by sonication of a mixture containing 95 mol% DMPC (NOF, Japan) and 5 mol % $C_{12}(EO)_{23}$ (Nikko Chemicals, Japan) in 5% glucose solution. The sample solutions were sterilized by filtration with a 0.20 μ m filter. The time course of the hydrodynamic diameter (d_{hy}) change for HL using an electrophoretic light scattering spectrophotometer (ELS-ZO, Otsuka Electronics, Osaka, Japan) were examined. The mean d_{hy} of HL was under 100 nm, which were preserved for a period remaining stable for more than 4 weeks, although DMPC liposomes were unstable and precipitated after 2 weeks. It is noteworthy that HL having under the 100 nm in diameter could avoid the reticular endothelial system *in vivo* and thus should be appropriate for the intravenous administration *in vivo* and clinical applications [18].

First, we examined the 50% inhibitory concentration (IC_{50}) of HL on the overgrowth of human synovial sarcoma (SW982) cells with WST-8 assay [19]. The cells (5.0×10^4 viable cells/ml) were seeded into 96 well plates and incubated for 48 h in humidified 5% CO_2 at 37°C in the presence or absence of HL. Subsequently, the WST-8 solution (Dojindo Laboratories, Japan) was added to each well. After 3 h, the absorption at 450 nm was measured with VersaMax Microplate Reader (Molecular Devices, CA, USA). The IC_{50} values of HL were determined from the concentration-dependency of HL on the cell viability. The results are shown in Figure 1. The IC_{50} values of HL were a third of that of DMPC liposomes on the overgrowth of SW982 cells. These results indicate that HL should be effective for inhibiting the growth of synovial sarcoma cells.

Next, we examined the induction of apoptosis by HL in SW982 cells using a confocal laser scanning microscope on the basis of TUNEL method. The results are shown in Fig.2. The nuclei of all SW982 cells were stained by TOPRO-3 were shown by red fluorescence. Green or yellow (overlay) fluorescence in cells treated with HL using TUNEL method were observed, indicating the presence of fragmented-DNA by induction of apoptosis. In contrast, apoptotic cells in SW982 cells treated with DMPC liposomes were not observed. These results suggest that HL should induce apoptosis for synovial sarcoma cells.

Furthermore, we examined pathway of induction of apoptosis by HL for SW982 cells. Activation of caspases is an indispensable process in the execution phase of apoptosis. To investigate the apoptotic pathways of SW982 cells induced by HL, the activation of caspase-3, -8 and -9 were measured as the protease activity of caspase using the cell-permeable substrate of PhiPhiLux G1D2 (caspase-3), CaspaLux 8-L1D2 (caspase-8) and CaspaLux 9-M₁D₂ (caspase-9) (OncoImmunin, Inc., Gaithersburg, MD, USA) using a confocal laser microscope according to the manufacturer's instructions. SW982 cells (1.0×10^5 cells) were treated with HL ([DMPC]=10 mM, [C₁₂(EO)₂₃]=0.53 mM) for 3 h. The cells were centrifuged at 3000 rpm for 5 min, and resuspended in 50 μ l of chilled cell lysis buffer. The cell lysates were incubated with reaction buffer (50 μ l) and respective caspase substrate (75 μ l) at 37 °C for 1 h. After washing twice with 1 ml of ice-cold PBS (-), the cells were resuspended in 1 ml PBS(-). Results are shown in Figure 3. Fluorescence micrographs of SW982 cells stained with the cell-permeable fluorescence caspase-3 (Figure 3A), caspase-8 (Figure 3B) and caspase-9 (Figure 3C) substrate after the treatment with HL. The caspase-3, -8, and -9 activity in SW982 cells were observed after the treatment with HL for 2 h. These results suggest that the apoptosis induced by HL for SW982 cells were attained through the activation of caspase-3, -8, and -9.

We examined fusion and accumulation of HL (HL/ICG) including a fluorescence probe (indocyanine green (ICG); Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) into the membrane of SW982 cells, was performed using fluorescence cell imaging system (EVOS; Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells (5.0×10^5 cells/ml) were cultured in a 5% CO₂ humidified incubator at 37°C for 24 h. The cells were treated with HL ([DMPC]=10 mM, [C₁₂(EO)₂₃]=0.53 mM), [ICG]=0.1 mM, including fluorescence probe for 0.5h, and then stained at Hechst333442 for 0.5h. Stained cells were observed using fluorescence cell imaging system (710/40 nm Excitation; 775/46 nm Emission). The results are shown in **Figure 4**. The nuclei of all SW982 cells were stained by Hoechst 33342 was shown by blue fluorescence. A significant accumulation of HL/ICG (yellow color) into SW982 cells was observed. In contrast, no accumulation of DMPC/ICG was observed in WiDr cells. These results suggest that HL could selectively fuse and accumulate into SW982 cells.

In conclusion, we found for the first time that HL inhibited the overgrowth of synovial sarcoma (SW982) cells, which are model cell of RA by the induction of apoptosis in vitro. This study suggests that HL could be a promising novel nanomedicine for the treatment on overgrowth of synovial cells in RA.

Figure 1: Inhibitory effects of HL on the overgrowth of SW982 cells. Showed anti-proliferation activity in prostate cancer cells. HL inhibited the growth of SW982 cells for 48 h. Data represent the mean (n=3) $\pm$ S.E. * p<0.05 (vs. DMPC)

Figure 2: Induction of apoptosis in SW982 cells by HL. Fluorescence micrographs of SW982 cells treated with HL for 48h using TUNEL method. [DMPC] = 300 μ M. Scale bar: 20 μ m.

Figure 3: Activation of caspases in SW982 cells treated with HL. (A) Fluorescence micrographs of SW982 cells treated with HL for 3h using the cell-permeable substrate of PhiPhiLux G1D2 (caspase-3). [DMPC] = 10 mM. Scale bar: 20 μ m. (B) Fluorescence micrographs of SW982 cells treated with HL for 3h using the cell-permeable substrate of CaspaLux 8-L1D2 (caspase-8). [DMPC] = 10 mM. Scale bar: 20 μ m. (C) Fluorescence micrographs of SW982 cells treated with HL for 3h using the cell-permeable substrate of CaspaLux 9-M₁D₂ (caspase-9). [DMPC] = 10 mM. Scale bar: 20 μ m.

Figure 4: Fusion and accumulation of HL (HL/ICG) including a fluorescence probe into the membrane of SW982 cells. HL fused and accumulated into SW982 cells. [DMPC] = 10 mM. Scale bar: 200 μ m.

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