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Inhibitory effects of hybrid liposomes on the growth of synoviocyte causing rheumatoid arthritis

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ABSTRACT

Inhibitory effects of HL-*n* composed of 95 mol % L-α-dimyristoylphosphatidylcholin (DMPC) and 5 mol % polyoxyethylenedodecylether ($C_{12}(EO)_n$, n = 21, 23, or 25) on the growth of human rheumatoid arthritis (RA) fibroblast-like synoviocytes (HFLS-RA) in vitro were examined. Remarkably high inhibitory effects of HL-*n* on the growth of HFLS-RA cells were obtained. The induction of apoptosis by HL-*n* was revealed on the basis of TUNEL method. Furthermore, the therapeutic effects of HL-23 using mouse models of arthritis were investigated. Therapeutic effects without joint swelling were obtained in mouse models of RA treated with HL.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the overgrowth of synoviocytes. Synoviocytic overgrowth after the supersecretion of inflammatory cytokine such as TNF α and interleukin-1 forms pannus in a chronically inflamed microenvironment, which induces the destruction of articular cartilage and subchondral bone.[1,2] In the process of synovial pannus formation, cells of the synovial intima (macrophage-like and fibroblast-like synoviocytes (FLS)) which form the inner layer of the joint capsule wall, acquire transformed phenotype, undergoing rapid proliferation and invasion into the intracapsular fracture.[3,4]

Therapeutic effects of anti-rheumatic drugs (disease modifying anti-rheumatic drugs, DMARDS) which prevent articular destruction by inhibiting or halting the immune process in rheumatic pharmacotherapy have been reported.[2,5,6] However, there were patients who could not suppress the growth of synoviocyte with resistance to various DMARDS.[7,8] Furthermore, severe side-effects of DMARDS such as digestive organs dysfunction, liver dysfunction, kidney dysfunction, stomatitis, depilation, and myelosuppression have been reported.[2,9,10] 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.

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.[11,12] 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) Inhibitory effects of HL including antitumor drugs,[13] sugar surfactants[14] or polyunsaturated fatty acids[15] have been observed on the growth of tumor cells in vitro and in vivo. (b) High inhibitory effects of HL on the growth of tumor cells in vitro[16,17] and in vivo[18,19] along with the induction of apoptosis have been obtained without using any drugs. (c) The mechanistic details of apoptosis of tumor cells induced by HL[16] and the correlation between antitumor effects and membrane fluidity of HL have been clarified.[20] (d) After receiving the approval of the Bioethics Committee, successful clinical chemotherapy with drug-free HL to patients with lymphoma has been reported.[21] In this study, we examined the inhibitory effects of HL-*n* composed of 95 mol % L-α-dimyristoylphosphatidylcholin (DMPC) and 5 mol % polyoxyethylenedodecylether ($C_{12}(EO)_n$, n = 21, 23, or 25) on the growth of human RA fibroblast-like synoviocytes (HFLS-RA) in vitro. Furthermore, the therapeutic effects of HL in a mouse model with arthritis were examined.

HL were prepared by using sonication (VELVO VS-N300, 300 W) of a mixture containing 95 mol % L-α-dimyristoylphosphatidylcholine (DMPC, NOF Co. Ltd, purity >99%) and 5 mol % polyoxyethylenedodecyl ether ($C_{12}(EO)_{23}$, Sigma–Aldrich Japan, Tokyo, Japan; $C_{12}(EO)_{21}$ and $C_{12}(EO)_{25}$, Nikko chemicals Co. Ltd, Japan) in 5% glucose solution at 45 °C with 300 W, followed by filtration with a 0.20 μm filter. Physical properties of HL composed of 95 mol % DMPC, 5 mol % $C_{12}(EO)_n$ (n = 21, 23, or 25, HL-*n*) were examined (Scheme 1). Apparent mean hydrodynamic diameters (d_{hy}) of HL were measured using light scattering spectrometer (ELS-8000,

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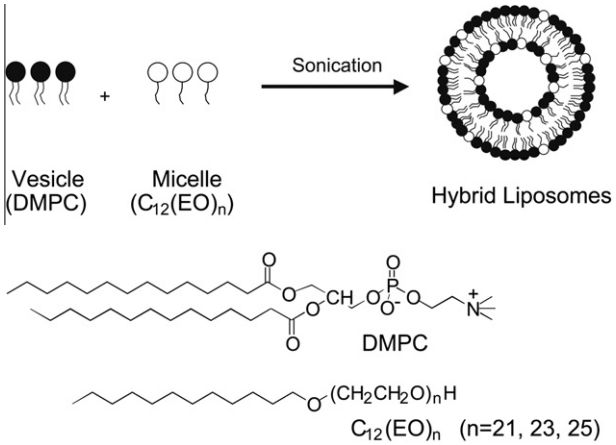

Scheme 1.

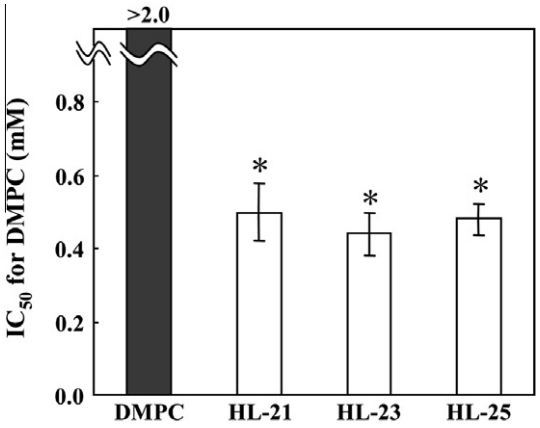

Figure 1. 50% inhibitory concentration (IC_{50}) for HL-*n* on the growth of HFLS-RA. * Significant difference (p <0.01) compared with DMPC liposomes (Student's t-test). Data represent the mean ± SD.

Otsuka Electronics Co. Ltd, Japan) with He–Ne laser light source (633 nm). The diameter was calculated by Stokes–Einstein equation ($d_{hy} = (\kappa T)/(3\pi\eta D)$), where κ is Boltzmann constant, T is absolute temperature, η is viscosity and D is diffusion coefficient. Hydrodynamic diameter (d_{hy}) of HL-*n* (n = 21, 23, or 25) was about 50 nm, which were preserved for a period remaining stable for more than one month. On the other hand, DMPC liposomes were unstable and precipitated after 14 days.

We examined the 50% inhibitory concentration of HL-*n* (n = 21, 23, or 25) on the growth of human synoviocytes from rheumatoid arthritis (HFLS-RA) in vitro. HFLS-RA, derived from RA patients after synovectomy, were purchased from Cell Applications (San Diego, USA).[3] HFLS-RA cells were routinely cultured in synoviocyte growth medium (Cell Applications). Fifty-percent inhibitory concentration (IC_{50}) on the growth of tumor cells was determined on the basis of WST-1 [2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] assay (Cell Counting Kit-1, Dojindo Laboratories, Kumamoto, Japan). Cells (5.0×10^4 cells/ml) were seeded in 96-well plates and cultured in a 5% CO_2 humidified incubator at 37 °C for 24 h. Cells were cultured for 48 h after adding DMPC and HL-*n* (n = 21, 23, or 25). WST-1 solution was added and incubated for 3 h. Absorbance at wavelength of 450 nm was measured by spectrophotometer (E_{max}, Molecular Devices Co., USA). The inhibitory effects of HL-*n* (n = 21, 23, or 25) on the growth of HFLS-RA cells were evaluated by $A_{mean}/A_{control}$, where A_{mean} and $A_{control}$ denote the absorbance of water-soluble formazan, in the presence and absence of HL, respectively. The results are shown in Figure 1. The IC_{50} values of HL-*n* (n = 21, 23, or 25) on the growth of HFLS-RA cells were remarkably smaller than those of the DMPC liposomes.

We examined the induction of apoptosis by HL-*n* (n = 21, 23, or 25) for HFLS-RA cells using the TUNEL method and flow cytometry. Apoptotic DNA rates in HFLS-RA cells were measured on the basis

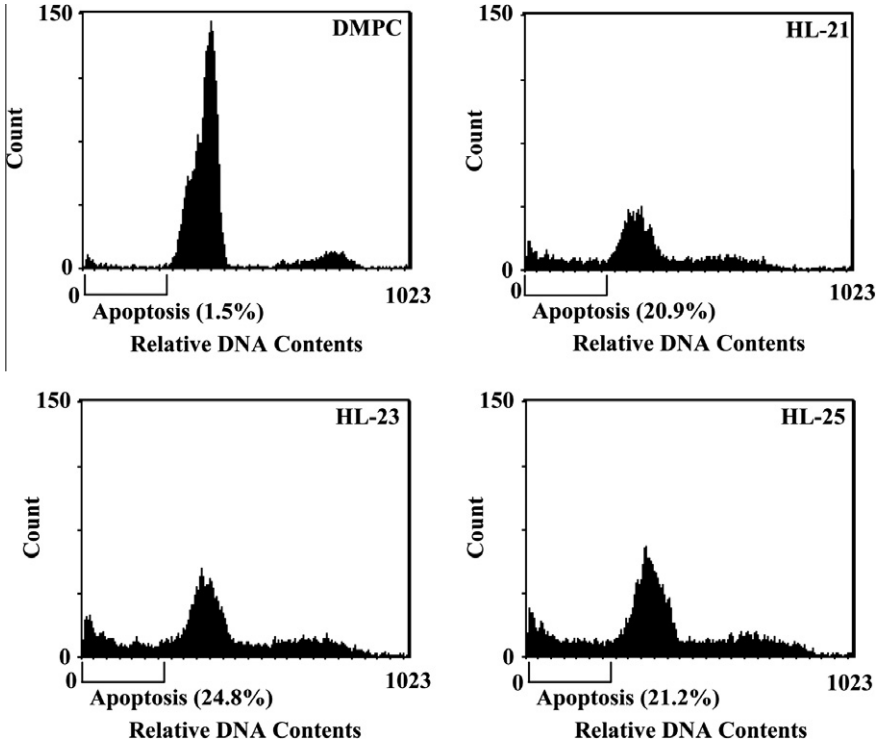

Figure 2. Apoptotic DNA rate of HFLS-RA cells treated with DMPC or HL-*n*.

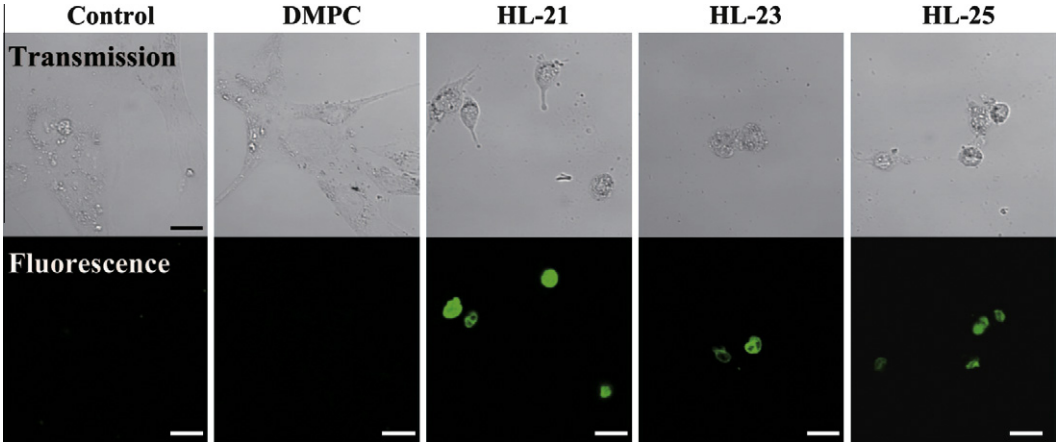

Figure 3. Fluorescence micrographs of HFLS-RA cells treated with HL-*n* using TUNEL method. Scale bar: 10 μm. Magnification: ×40.

of flow cytometry. Apoptotic DNA rates in HFLS-RA cells treated with HL-*n* (*n* = 21, 23, or 25) were measured using flow cytometry.[22] The results are shown in Figure 2. A high apoptotic DNA rate was obtained after the treatment with HL-*n* (*n* = 21, 23, or 25), although fairly low apoptotic DNA rates were obtained in the case of DMPC liposomes.

Detection of apoptotic cells was performed by TUNEL method using an in situ cell death detection kit (Roche Diagnostics K.K., Switzerland). HL-*n* (*n* = 21, 23, or 25) were added to the cell suspension (5.0×10^4 cells/ml) and cultured for 24 h. The medium including the dead cells was centrifuged and the cells were fixed with 4% paraformaldehyde solution, and then processed for TUNEL according to the manufacturer's instructions. The stained cells were observed using confocal laser microscope (TCS-SP, Leica Microsystem, Germany) with a 488 nm Ar laser line (detection, 515–565 nm). Fluorescence micrographs of HFLS-RA cells after the treatment with HL-*n* (*n* = 21, 23, or 25) on the basis of TUNEL method are shown in Figure 3. Interestingly, HFLS-RA cells were dyed in green after adding HL-*n* (*n* = 21, 23, or 25), indicating that HL-*n* (*n* = 21, 23, or 25) induced apoptosis for HFLS-RA cells, although the cells were not dyed when using the DMPC liposomes.

These results indicate that the HL-*n* (*n* = 21, 23, or 25) could induce apoptosis for HFLS-RA cells.

We examined the therapeutic effects of HL-23 using SKG mice as models of RA in vivo.[23] Male SKG mice (SKG/Jcl; 5 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan).[24] SKG mice express the human RA-like such as invasion of inflammatory cells and synovial cell proliferation with pannus formation and neovascularization. Purified beta-glucans such as curdlan and laminarin can trigger severe chronic arthritis in SKG mice.[25] The results are shown in Figure 4. No joint swelling was obtained in the mice treated with HL-23, although joint swelling and deformity of fingers in all feet were observed in the control group. It is noteworthy that remarkable therapeutic effects were obtained in the mouse models of RA intravenously treated with HL-23 without drugs.

In conclusion, we clearly demonstrated that HL-*n* showed inhibitory effects on the growth of HFLS-RA cells in vitro along with apoptosis and therapeutic effects for mouse models of RA in vivo. The noteworthy aspects are as follows. (a) The IC_{50} values of HL-*n* (*n* = 21, 23, or 25) on the growth of HFLS-RA cells were remarkably smaller than those of the DMPC liposomes. (b) The induction of apoptosis by HL-*n* (*n* = 21, 23, or 25) was verified for HFLS-RA cells on the basis of flow cytometry, and TUNEL method. (c) Therapeutic effects of HL-23 were obtained in mouse models of RA. It is noteworthy that remarkable inhibitory effects of drug-free HL-*n* (*n* = 21, 23, or 25) leading to apoptosis on the growth of synoviocyte causing rheumatoid arthritis were obtained for the first time.

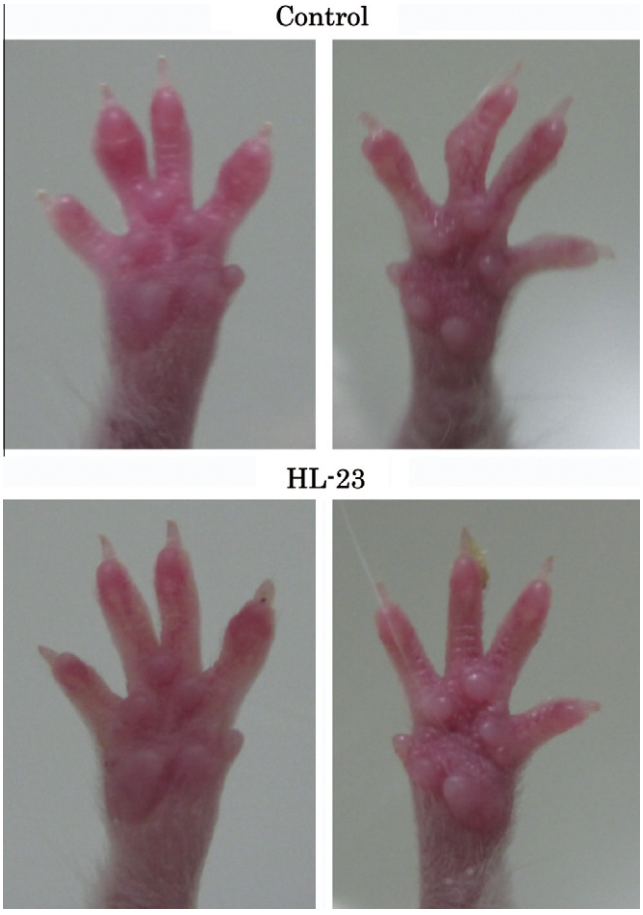

Figure 4. Therapeutic effects of HL-23 for SKG mice of human RA model. HL-23 (dose for DMPC, 136 mg/kg) were intravenously administered in mice for 36 weeks.

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22. The HFLS-RA cells treated with HL for 24 h were centrifuged at 3000 rpm for 5 min and washed with PBS(−), then fixed in chilled ethanol. The cells were washed again, treated with RNase (0.25 mg/ml) and then stained with propidium iodide (PI, 0.5 mg/ml) that has 493 nm excitation and 635 nm emission wavelength. The samples were analyzed using flow cytometer (Epics XL system, Beckman Coulter, California, USA) with a single excitation 488 nm of 15 mW argon laser. The PI signals were detected by FL3 sensor in 605–635 nm. Apoptotic DNA rates were calculated by apoptotic DNA rates = (apoptotic DNA content/DNA content) × 100.
23. After a quarantine and acclimatization period, the mice were randomly grouped on the basis of body weight using the stratified randomization method. The number of mice was three in each group. Laminarin (Sigma–Aldrich Japan, Tokyo, Japan) was dissolved in sterile saline at 100 mg/ml. Mice were intraperitoneally injected with 0.3 ml (30 mg) laminarin solution. HL-23 (dose for DMPC, 136 mg/kg) in 5% glucose solution or 5% glucose solution alone (control) were intravenously administered in mice once each day for 14 days after the injection of Laminarin, and then HL in 5% glucose solution or 5% glucose solution alone (control) was administered once every two days for 36 weeks. Joint swelling was monitored by macroscopic inspection.
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