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Liposomes for Solubilization and Delivery of Curcumin into Leukemia Cells

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ABSTRACT – Curcumin is a phytochemical compound with anticancer activity. Although curcumin has substantial pharmacological effect against various cancers, the low solubility of curcumin has hindered its development. For an organic solvent-free injectable formulation, we encapsulated curcumin in various liposomes. Due to its lipophilic property, curcumin was placed in the membrane region of liposomes. Curcumin was stably encapsulated in all formulations tested in this study. The cellular uptake of curcumin delivered in liposomal formulations or free form was measured in K562 human leukemia cell lines using a flow cytometry and MTT viability assay, respectively. Although all the liposomes could solubilize curcumin, the cellular levels and the anticancer effects of liposomal curcumin varied with the composition of liposomes. Moreover, liposomal curcumin down-regulated the expression of Notch-1, the molecule involved in the carcinogenesis, to the similar extent to free curcumin dissolved in dimethyl sulfoxide. These results warrant the development of liposomal curcumin as an injectable formulation for leukemia treatment.

Key words – curcumin, liposomes, leukemia, solubilization

Curcumin (diferuloylmethane), a natural phenolic compound isolated from the plant *Curcuma longa* Linn., is one of major active components of the food flavor. Recently, curcumin has been reported to be pharmacologically safe and capable of preventing early stages of cancer in clinical trials.[1] Curcumin is known to induce apoptosis in various cancer cell lines including human hepatoma and leukemia cells,[2] exert anti-inflammatory effects by inhibiting cyclooxygenase 2 and 5-lipoxygenase[3] and inhibit the activation of transcription factors such as nuclear factor kappa B (NF-kB) involved in regulation of cytokine synthesis or activating protein 1 (AP-1).[4-6] Curcumin promotes oxidation-mediated apoptosis as an antioxidant such as alpha-tocopherol, superoxide dismutase and catalase in leukemia cell lines.[7]

Despite its potential as an anticancer drug, curcumin has suffered from its limited solubility. Currently, curcumin is dissolved in dimethyl sulfoxide for *in vitro* experiments. The use of dimethyl sulfoxide is, however, not acceptable in clinical studies, requiring the advent of new technologies for solubilizing curcumin in the absence of organic solvents.

In this paper, we aimed to solubilize the insoluble anticancer drug, curcumin, using liposome-based delivery system. Liposomes are relatively easy to prepare, biodegradable, and capable of encapsulating versatile molecules of therapeutic entity as compared to other delivery vehicles. Since liposomes have hydrophilic as well as lipophilic features, they can envelop hydrophilic substances in their inner compartment and insert lipophilic substances in their membrane. Here, we report the cellular uptake and anticancer activity of liposomal curcumin in K562 human leukemia cell line.

Materials and Methods

Chemicals

Phospholipids were purchased from Avanti Polar Lipid (Birmingham, AL). Curcumin and cholesterol (Chol) were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals used in this study were of reagent grade.

Cell culture

K562 cell lines, derived from human chronic myelogenous leukemia, were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. As antibiotics for the culture medium, penicillin (100 units/mL) and streptomycin (100 μg/mL) were used. The cells were cultured at 37°C under 5% CO_2.

Preparation of liposomes

L-α-Phosphatidylcholine (PC), L-α-Phosphatidyl-DL-glyc-

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erol (PG), and Chol were dissolved in chloroform. The molar ratio of total lipids and curcumin was 10:1. Lipid films were prepared by removing chloroform under vacuum using a rotary evaporator at room temperature. Liposomes, composed of PC/curcumin (molar ratio, 10:1), PC/Chol/curcumin (molar ratio, 10:4:1), PC/PG/curcumin (molar ratio, 7:3:1) or PC/PG/Chol/curcumin (molar ratio, 7:3:4:1) were prepared by rehydrating the lipid films with 1 mL of phosphate-buffered solution (PBS) and vortexing the resulting suspension for a few minutes. The sizes of liposomes were controlled by extruding the liposomes three times through 0.2 μm polycarbonate membranes under nitrogen gas.

FACS analysis

Cells were seeded in 6-well plates (3×10^5 cells/well), treated with 50 μM of free and liposomal curcumin, and incubated for 24 hr. The cells were then collected and washed twice with PBS using centrifugation. The cells loaded with curcumin were analyzed by using FACSCalibur flow cytometer equipped with CELLQUEST PRO software (Becton Dickinson, NJ). Two independent experiments were performed.

Cell viability assay

Proliferation and survival of cells after free or liposomal curcumin treatment were assessed by the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Cells were plated in 24-well (2×10^4 cells/well) in 500 μL of medium, and incubated at 37°C for 1 day before treatment. Next day, free curcumin dissolved in dimethyl sulfoxide or liposomal curcumin was added to the culture medium (500 μL) at 50 μM of curcumin concentrations and incubated in a humidified tissue-culture chamber (37°C, 5% CO_2) for 24 hr. 50 mM stock solution of curcumin in DMSO was prepared. Final DMSO concentration was 0.1% and this concentration had no effect on cell viability. As control, untreated cells were used. The cell survival in each well was compared with that of control. The mean value and standard error for each treatment were determined, and converted to the percent relative to control. Eight independent experiments were performed.

Total RNA extraction and RT-PCR

K562 cells were seeded in a 6-well plate, incubated for 1 day, and followed by the treatment of 50 μM of free or liposomal curcumin for 24 hr. Then, the cells were harvested, centrifuged and washed with PBS two times. The total RNA from each sample was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The amount of the total RNA was determined by spectrophotometer at a wavelength of 260 nm. One microgram of total RNA from each sample was subjected to first-strand cDNA synthesis by using the *AccuPower* RT PreMix from Bioneer (Daejeon, Korea). Reverse transcription reaction was performed at 42°C for 1 hr and 95°C for 5 min. The primers used in the PCR analysis were as follows: for Notch-1, the forward primer was 5'-CAA CAT CCA GGA CAA CAT GG-3' and the reverse primer was 5'-GGA CTT GCC CAG GTC ATC TA-3'. For human GAPDH, the forward primer was 5'-AGC CAA AAG GGT CAT CAT CTC T-3' and the reverse primer was 5'-AGG GGC CAT CCA CAG TCT T-3'. PCR amplification was performed for 32 cycles of denaturation (94°C, 40 sec), annealing (55°C, 40 sec), and extension (72°C, 1 min), which yielded a PCR product of 229 bp size.

Results and Discussion

Liposomal formulation of curcumin

Curcumin was encapsulated in liposomes of various compositions. Since curcumin is a hydrophobic compound showing high solubility in chloroform, we dissolved both curcumin and phospholipids in chloroform, and encapsulated curcumin in the lipid membrane part of liposomes. When it comes to the charges of liposomes, neutral compositions (PC, or PC/Chol) and anionic (PC/PG, or PC/PG/Chol) liposomes have been used in this study. These compositions were chosen because they were known to be less toxic as compared to cationic formulations. The liposomal and free curcumin affected the morphology of K562, human leukemia cell line. The cells were observed by phase-contrast microscopy 24 hr after treatment with free or liposomal curcumin (Figure 1). As compared to untreated cells (Figure 1A), the cell treated with curcumin showed the increased population of irregularly shaped cells. Elongated, swollen, or shrunken cells were observed after curcumin treatment, indicating the damaged cells. Moreover, the change of cell morphology after treatment with liposomal curcumin provided indirect evidence that the cellular level of curcumin might be high enough to affect the viability of the cells.

Cellular delivery of curcumin by FACS analysis

To test the cellular delivery of curcumin, FACS analysis was used. Thanks to the high fluorescence of curcumin at the excitation wavelength near 550 nm,[8] cellular level of curcumin was detected at 484 nm wavelength using FACS analyzer. Flow cytometry analysis evidenced the uptake of curcumin into the leukemia cells (Figure 2). PC/PG liposomal formu-

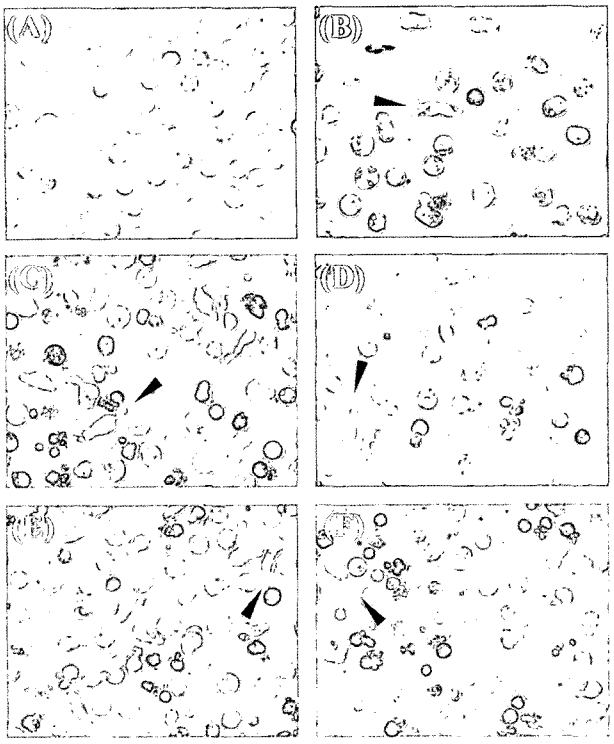

Figure 1–The phase-contrast microscope pictures of cells following treatment with curcumin in free form or various liposome formulations. Damaged cells were indicated by arrows.
(A) untreated control, (B) free curcumin dissolved in dimethyl sulfoxide, (C) PC/curcumin (10:1), (D) PC/Chol/curcumin (10:4:1), (E) PC/PG/curcumin (7:3:1), (F) PC/PG/Chol/curcumin (7:3:4:1).

lation exhibited the highest cellular level of curcumin in K562 cells. The percentage of the cells positive with fluorescence of curcumin was 82% for free curcumin (Figure 2B), 85% for PC/curcumin (Figure 2C), 79% for PC/Chol/curcumin (Figure 2D), 87% for PC/PG/curcumin (Figure 2E), and 77% for PC/PG/Chol/curcumin (Figure 2F). Although the cellular curcumin level did not significantly differ between neutral (PC/curcumin, PC/Chol/curcumin) and anionic (PC/PG/curcumin, PC/PG/Chol/curcumin) liposomes, the liposome formulations lacking Chol showed higher cellular level of curcumin than did those with Chol. Currently, we can not explain the mechanism by which the presence of Chol reduced the cellular delivery of liposomal curcumin. However, the possibility exists that curcumin may be fitted to the spaces between phospholipids, providing the liposome with the balanced rigidity and fluidity. The enhanced rigidity of Chol-containing liposome might affect the extent of liposomal endocytosis by cancer cell lines.

Anticancer activity of liposomal curcumin to leukemia cells

The anticancer activity of liposomal curcumin was affected

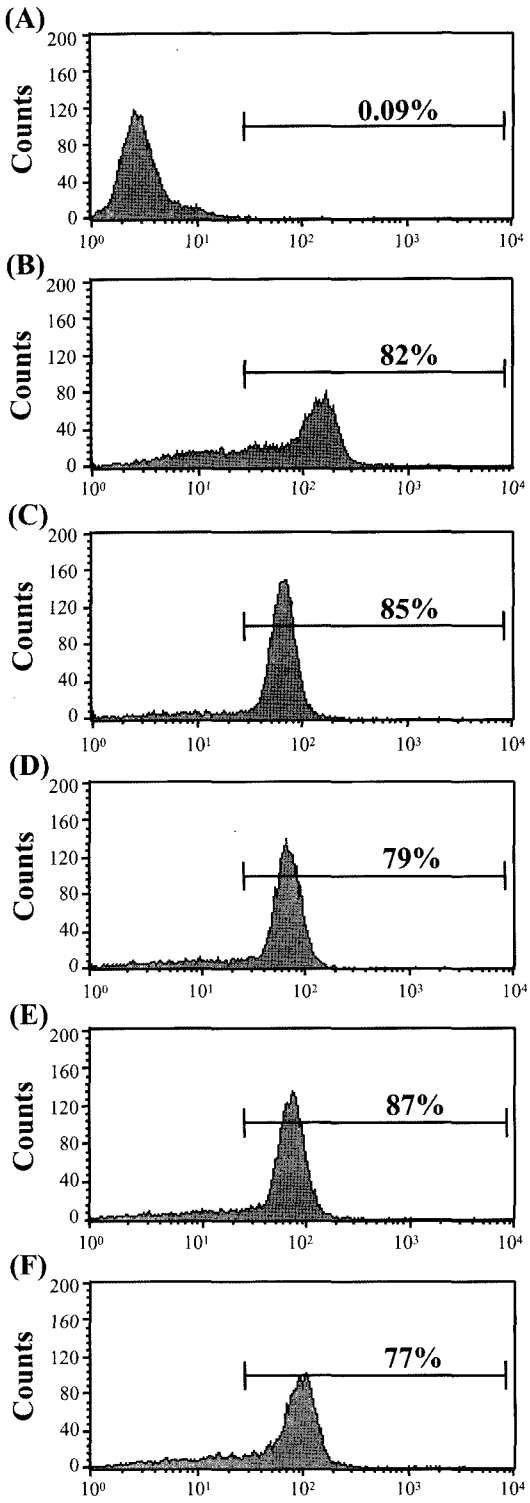

Figure 2–FACS analysis of K562 cells following treatment with curcumin in free form or various liposome formulations.
(A) untreated control, (B) free curcumin dissolved in dimethyl sulfoxide, (C) PC/curcumin (10:1), (D) PC/Chol/curcumin (10:4:1), (E) PC/PG/curcumin (7:3:1), (F) PC/PG/Chol/curcumin (7:3:4:1). For each group, representative data have been displayed. Similar results were observed in repeated experiments. The percentage in the histograms is presented as the mean (n=2).

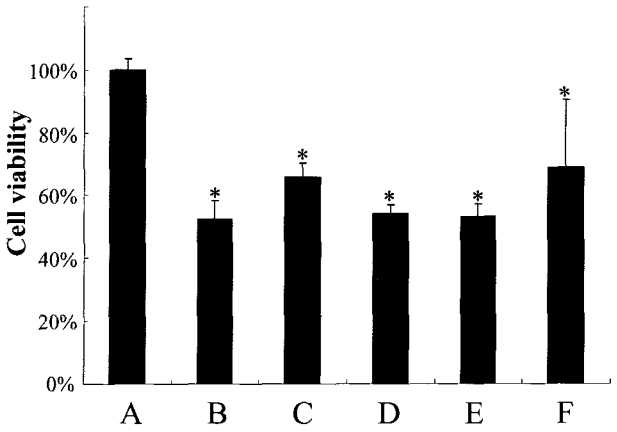

Figure 3–*In vitro* toxicity of free or liposomal curcumin in K562 leukemia cells.
The cells were incubated for 24 hr with curcumin in free or various liposomal formulations. Cell viability was assessed by the MTT assay. As control, untreated cells were used. *: Significantly different from other groups (ANOVA & Student Newman Keuls test, $P<0.05$). The results are expressed as the mean±S.D. ($n=8$). (A) untreated control, (B) free curcumin dissolved in dimethyl sulfoxide, (C) PC/curcumin (10:1), (D) PC/Chol/curcumin (10:4:1), (E) PC/PG/curcumin (7:3:1), (F) PC/PG/Chol/curcumin (7:3:4:1).

by the compositions of liposomes. Exposure to free or liposomal curcumin (50 μM, for 24 hr) inhibited the growth of K562 cells (Figure 3). Although the growth inhibitory activity of liposomal curcumin varied depending on the formulations, liposomal curcumin consistently reduced the growth of leukemia cells. These results agree with the FACS data providing the high population of curcumin-loaded cells after liposomal curcumin treatment. Nevertheless, in neutral liposomal compositions, PC/Chol/curcumin composition showed more toxicity than PC/curcumin composition. Given the higher rigidity of Chol-containing liposomes, it could be speculated that Chol in neutral liposomes might have extended the retention of curcumin in the cells. In this regard, we assumed that variable MTT results in neutral liposome compositions might contribute to FACS analysis.

Notch-1 mRNA downregulation after treatment of free or liposomal curcumin

In addition to the growth inhibitory functions of curcumin, we tested the effect of free or liposomal curcumin on the expression of carcinogenesis-related genes. Among various carcinogenesis-related markers, Notch-1 has been known to play important roles in the carcinogenesis of human cancers including K562 cells.[9] Notch-1 and NF-kB pathways have been reported as key modulators in cellular events related with apoptosis. It has been shown that there is a close interaction between Notch-1 and the NF-kB pathway. Given the cellular

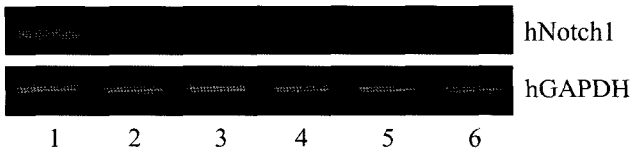

Figure 4–The mRNA expression of Notch-1 in K562 leukemia cells after treatment of free and liposomal curcumin.
Lane 1, untreated control; lane 2, free curcumin dissolved in dimethyl sulfoxide; lane 3, PC/curcumin (10:1); lane 4, PC/Chol/curcumin (10:4:1); lane 5, PC/PG/curcumin (7:3:1); lane 6 PC/PG/Chol/curcumin (7:3:4:1).

interaction, consistently elevated expression levels of Notch-1 might be required to maintain NF-kB activity in cancer cell lines.

Figure 4 shows the down regulation of Notch-1 mRNA expression following the treatment of curcumin in K562 cell. Untreated cells showed the expression of Notch-1. In contrast, the cells treated with curcumin in free or liposomal forms showed similar reduction in the expression of Notch-1. The inactivation of Notch-1-mediated cell growth inhibition observed in this study may be related in part with the inactivation of NF-kB activity. Moreover the inactivation of Notch-1 may contribute to the apoptosis of leukemia cells. Our observation on the down regulation of Notch-1 by curcumin agrees with the previous study which showed the reduced expression of Notch-1 in pancreatic cancer cells *in vitro*.[10]

Our results suggest that liposome-based delivery systems would be further used for injectable formulations of insoluble anticancer drugs like curcumin in the future. Since liposomal curcumin presented anticancer effects similar to free curcumin dissolved in dimethyl sulfoxide, the liposomes could be used for organic solvent- or detergent-free curcumin formulations. Moreover, the liposomes may serve as a base for targeted delivery of macromolecules into cancer cells by modifying the surface with specific markers. To date, liposomes coupled with antibodies, folate or transferrin have been extensively studied for site-specific delivery.[11] Hyaluronic acid, a linear, negatively charged polysaccharide, can be also used as a targeting moiety of liposomal leukemia therapy because hyaluronic acid receptor are known to be overexpressed in various cancer cell lines including leukemia cells.

Conclusion

Our results suggest that curcumin can be solubilized via liposomal formulation, and that the optimized liposomal formulation has potential as injectable formulation of curcumin for leukemia treatment in the future.

Acknowledgements

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