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CYTOPLASMIC DELIVERY MEDIATED BY LIPOSOMES MODIFIED WITH A FUSOGENIC POLYMER

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Intracellular delivery of calcein mediated by egg yolk phosphatidylcholine (EYPC) liposomes modified with succinylated poly(glycidol), which is a poly(ethylene glycol) derivative having carboxyl groups, was examined. When CV-1 cells were incubated with the bare EYPC liposomes containing calcein at 37 °C, only weak and vesicular fluorescence of calcein was observed by using a fluorescence microscope. In contrast, the cells treated with the polymer-modified liposomes containing calcein displayed more intensive and diffuse fluorescence, indicating that calcein was transfered into the cytoplasm. Moreover, fusion assay by the resonance energy transfer method suggested occurrence of fusion between the polymer-modified liposomes and the endosome and/or lysosome. Results obtained in this study implies that after internalization through an endocytic pathway, the polymer-modified liposomes transfer the content into the cytoplasm by fusing with the endosomal and/or lysosomal membranes.

1. Introduction

Because polar molecules, ions, and macromolecules hardly permeate cellular membranes, systems which promote transfer of these molecules into cells are needed for cytoplasmic delivery of these molecules. As such systems, fusogenic liposomes have been developed. These liposomes fuse with cellular membranes and transfer their contents into the cytoplasm.

While several fusogenic liposomes have been reported, among them pH-sensitive liposomes are important for site-specific delivery of drugs. pH-Sensitive liposomes are stable under a neutral condition, but become fusogenic under an acidic condition. Therefore, after uptake by the target cells through an endocytic pathway, the liposomes destabilize and/or fuse with the endosomal membrane because inside of endosome is an acidic environment and then, release their contents into the cytoplasm.[1)]

So far, several pH-sensitive liposomes have been prepared by using phosphatidylethanolamine (PE) with unsaturated acyl chains[1)]. Unsaturated PE has strong tendency to form nonbilayer structure and hence, liposomes consisting of unsaturated PE alone can not be obtained under the physiological condition. However, addition of titratable amphiphiles, such as oleic acid, as another component gives PE liposomes with pH-sensitivity. Stability of these liposomes is, however, relatively poor.

Another approach for preparing pH-sensitive liposomes is modification of liposomes with polymers that generate fusogenic activity depending on pH. In a previous study[2)], we synthesized succinylated poly(glycidol) (SucPG) (Figure 1) as a fusogenic polymer with pH-sensitivity. This polymer has a main chain structure similar to that of poly(ethylene glycol) which is a well-known fusogenic polymer. Also this polymer has carboxyl groups on the side chains. It was shown that fusion ability of egg yolk phosphatidylcholine (EYPC) liposomes modified with SucPG increases with decreasing pH because carboxyl groups on the polymer chain become protonated and then the polymer chain interacts with the liposomal membrane strongly possibly due to hydrophobic interaction and hydrogen bonding between carboxyl group of the polymer and phosphodiester group of the

lipid[2].

Since fusion ability of SucPG-modified EYPC liposomes increases under an acidic condition, it is expected that after uptake by cells through an endocytic pathway, the liposomes transfer their content into the cytoplasm by fusing with the endosomal membrane. Thus, in this study, cytoplasmic delivery of a water-soluble molecule, calcein, mediated by EYPC liposomes modified with SucPG was investigated. Mechanism of the liposome-mediated delivery was also discussed.

2. Experimental

SucPG was synthesized by the reaction of poly(glycidol) (peak molecular weight: 4600) with succinic anhydride in N,N-dimethylformamide. Unit mol percent of succinylated residues in the resultant polymer was determined to be 94 by ^{1}H-NMR. Decylamine was attached to the polymer as anchor to the liposomal membrane (6 unit mol%) using a condensing agent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

EYPC liposomes modified with SucPG were prepared by sonication of mixture of the lipid and the decylamine-attached SucPG in aqueous solutions.

CV-1, an established line of African green monkey kidney cells, was incubated with the SucPG-modified liposomes in phosphate-buffered saline (PBS) containing 0.36 mM calcium and 0.42 mM magnesium. After incubation, the cells were washed three times with PBS containing calcium and magnesium and then viewed using a fluorescence microscope.

Fusion between the liposomes and the cells was detected by measuring resonance energy transfer between N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B-sulfonylphosphatidylethanolamine (Rh-PE).[3] After incubation with the liposomes labeled with NBD-PE (5 mol %) and Rh-PE (1 mol %), the cells were washed three times with PBS containing calcium and magnesium and removed from culture dishes by exposure to PBS containing EDTA. Fluorescence spectra associated with the cell suspension irradiated at excitation wavelength of 450 nm were measured. Percent fusion of liposomes with the cells was determined from the fluorescence spectra of the liposome-treated cells.[3]

Uptake of the liposomes by the cells was measured by using the liposomes labeled with NBD-PE (5 mol %). The cells treated with the labeled liposomes were suspended in PBS by the above procedure and fluorescence intensity associated with the cell suspension irradiated at excitation wavelength of 450 nm was measured.

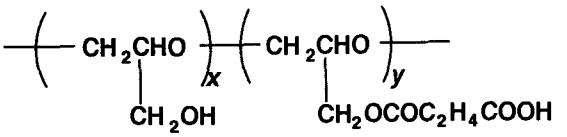

Figure 1. Structure of succinylated poly(glycidol).

3. Results and Discussion

When the cells were incubated with the bare EYPC liposomes containing calcein at 37 °C, weak and vesicular fluorescence was observed by using a fluorescence microscope. Similar vesicular fluorescence was also seen in the cells after incubation with an aqueous calcein solution at the same temperature. In contrast, the cells treated with the SucPG-modified liposomes containing calcein displayed diffuse fluorescence of calcein. These results suggest that calcein exists in endosome and/or lysosome of the cells treated with the bare EYPC liposomes or with calcein solution, whereas calcein is transferred into the cytoplasm of the cells treated with the SucPG-modified liposomes.

Effect of amount of SucPG fixed on EYPC liposomes on the liposome-mediated delivery of calcein was investigated. Four kinds of calcein-loaded liposomes consisting of EYPC and SucPG in the ratio of 10/0, 9/1, 8/2, and 7/3 (w/w) were prepared. Diameters of these liposomes were estimated to be 40.1, 40.3, 44.9, and 47.9 nm, respectively, by dynamic light scattering. The diameter increases

slightly with increasing amount of the polymer fixed. Since we used poly(glycidol) with peak molecular weight of 4600, molecular weight of SucPG was calculated to be ca. 10000. It is likely that fixation of the polymer onto the liposome increases apparent size of the liposome.

While the cells treated with any of the liposomes containing SucPG displayed diffuse fluorescence, the fluorescence increased as the cells were treated with the liposomes bearing a higher amount of the polymer.

Figure 2 shows the uptake kinetics for the SucPG-modified liposomes by the cells. Amount of the liposome associated with the cells is constant and independent of time when the incubation is performed at 0 °C. Since endocytosis is inhibited at the temperature, the liposomes are likely to bind onto the cell membranes under the condition. In contrast, at 37 °C amount of the liposome associated with the cells increases with time, indicating uptake by the cells through an endocytic pathway at 37 °C.

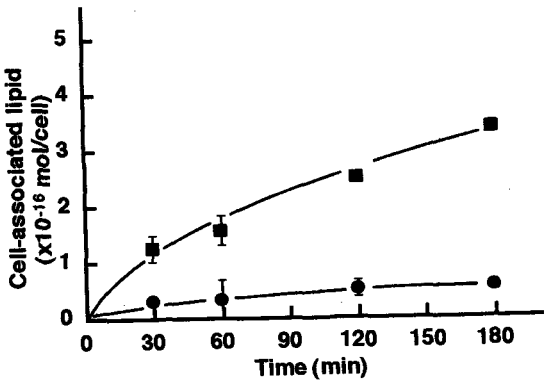

Figure 2. Uptake of SucPG-modified EYPC liposomes by CV-1 cells in phosphate-buffered saline at 37 °C (■) and 0 °C (●) as a function of time.

Figure 3 represents influence of SucPG content of the liposomes on uptake by the cells. Amount of the liposome taken up by the cells tends to decrease slightly as the polymer content in the liposomal membrane increases. Since surface of the liposomes bearing a higher amount of the polymer should be covered more effectively by the highly hydrophilic polymer chains, the polymer chains attached on the liposomes might reduce interaction between the liposomes and the cells and suppress uptake by the cells. Also difference in diameter of the liposome may affect uptake by the cells, although the difference is not significant.

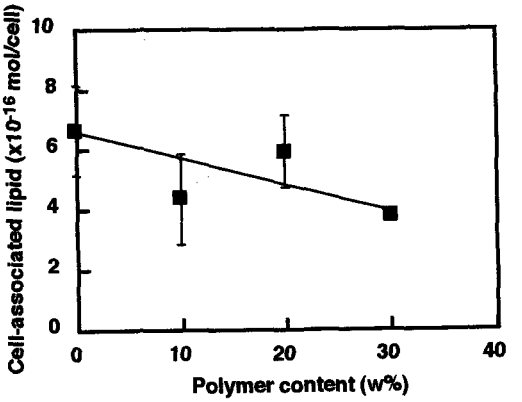

Figure 3. Effect of SucPG content of the liposome on uptake by CV-1 cells. After 3 h incubation in phosphate-buffered saline at 37 °C.

As is seen in Figure 3, the bare liposome-treated cells contain higher amount of the liposome than the cells treated with the polymer-modified liposomes do but the former cells displayed much weaker fluorescence than the latter cells, as previously mentioned. This result suggests that most calcein molecules are still trapped and quenched in the bare liposomes after internalization by the cells. In contrast, the cells treated with the liposomes consisting of EYPC and SucPG (7/3) contain the least amount of the liposomes but displayed the most intensive fluorescence, indicating that after internalization into the cells the liposomes transfer their content into the cytoplasm most efficiently.

If the SucPG-modified liposomes transfer their

content into cytoplasm by fusing with endosomal and/or lysosomal membranes, transfer of calcein should be interfered in the presence of chloroquine because it effectively elevates pH of endosome and lysosome and hence, inhibits the liposomes from becoming fusogenic. In fact, the diffuse fluorescence was weakened significantly in the cells treated with the SucPG-modified liposomes containing calcein in the presence of chloroquine.

In order to confirm the occurrence of fusion between the SucPG-modified liposomes and endosomal and/or lysosomal membranes, fusion assay by the resonance energy transfer method was performed. The SucPG-modified EYPC liposomes labeled with NBD-PE and Rh-PE were prepared. When these lipids exist in the liposomal membrane, resonance energy transfer between these fluorescent lipids occur. However, when the liposomes fuse with the cellular membranes, the fluorescent lipids should intermix with lipids present in the cellular membranes, resulting in decrease of energy transfer efficiency.[3)]

CV-1 cells were incubated with the liposomes for 3 h and percent of liposomes fused in the cells was evaluated by assuming that there are two distinct fraction of liposomes, namely those fused and those not fused, in the liposome-treated cell. The result is listed in Table 1. Clearly, the liposome with a higher content of the polymer reveals a higher value. While the values presented in this table may contain fraction of liposomes not fused but degraded. However, degradation of lipid of these liposomes might occur equally in the cell. Thus, this result suggests that the liposomes with a higher amount of the polymer fuse more intensively with cellular membranes.

On the other hand, the cells treated with the SucPG-modified liposomes labeled with the fluorescent lipids displayed vesicular fluorescence, suggesting that the fluorescent lipids exist mainly in endosome and/or lysosome.

Interaction of SucPG with endosomal and/or lysosomal membranes may induce destabilization of these membranes and enhance permeation of calcein through the membranes. However, large values of percent fusion presented in Table 1 implies that the polymer-modified liposomes transfer the content by fusing with endosomal and/or lysosomal membranes.

Table 1

Percent of liposome fused after uptake by CV-1 cells

Liposome	Percent
EYPC	30.9±0.6
EYPC/SucPG (9/1,w/w)	67.5±0.6
EYPC/SucPG (8/2,w/w)	73.9±0.9
EYPC/SucPG (7/3,w/w)	77.7±2.0

In conclusion, it was found that the SucPG-modified EYPC liposomes can transfer their content into cytoplasm possibly by fusing with endosomal and/or lysosomal membranes. In this polymer-liposome system, various kinds of lipid can be used as membrane components. If unsaturated PE is added as an additional membrane component, fusion ability of the liposomes is expected to increase. Also, conjugation of an antibody or saccharides to the polymer-modified liposomes is possible by using carboxyl groups on the polymer. Such conjugation will give the liposomes with target specificity. Therefore, this polymer-liposome system might have potential usefulness as a cytoplasmic delivery system with high stability and high efficiency.

References

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