Evaluation of Phosphatidylserine-Specific Peptide-Conjugated Liposomes Using a Model System of Malaria-Infected Erythrocytes

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Malaria is one of the most prevalent parasitic diseases and is most widespread in tropical regions. The malarial parasite grows and reproduces in erythrocytes during its life cycle, resulting in programmed erythrocyte death, termed eryptosis. Lipid scrambling, which occurs following the exposure of anionic lipids such as phosphatidylserine (PS) on the outer surface of erythrocytes, is a characteristic physical change that occurs early during eryptosis. Here, we prepared “PS specific peptide (PSP)”-conjugated liposomes (PSP-liposomes) and investigated whether PSP-liposomes hold promise as a novel strategy for actively targeting eryptosis. Eryptosis was induced by exposing red blood cells (RBCs) to ionomycin, a known calcium ionophore. When PSP liposomes were mixed with either RBCs or RBCs undergoing eryptosis (E-RBCs), the amount of PSP-liposome bound to E-RBCs was much higher than the amount bound to RBCs. However, the amount of PSP-liposome bound to E-RBCs was significantly inhibited by the presence of annexin V protein, which binds specifically to PS. These results suggest that PSP-liposomes could be an effective drug nanocarrier for treating E-RBCs and malaria-infected erythrocytes.

Key words  liposome; malaria; eryptosis; phosphatidylserine; targeting

MATERIALS AND METHODS

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| **Materials** | 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipid (Avanti Polar Lipids, AL, U.S.A.). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG<sub>2000</sub>-DSPE) and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[maleimide-conjugated (Mal-PEG<sub>2000</sub>-DSPE) were generously donated by NOF Corporation (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The lipophilic fluorescent dye, DiI, was purchased from Invitrogen (CA, U.S.A.). The sequence of PSP was LSYYPSYC as previously described with minor modifications was synthesized by Biologica Co. (Nagoya, Japan). All other reagents were of analytical grade. |
| **Preparation of PSP-Liposomes** | The liposomes were prepared by the thin film hydration method, followed by membrane extrusion to produce liposomes of uniform size, as described previously. In order to prepare fluorescently-labeled liposomes, 1 mol% of DiI was added. The mean diameters of liposomes were determined by dynamic light scattering with a particle sizer (ZetaSizer Nano-ZS, Malvern Instrument Ltd., Malvern, U.K.). |
| **PSP-PEG-DSPE** | was inserted into liposomes by post-insertion methods, as described previously. The lipid ratio was DSPC/CHOL/PSP-PEG-DSPE=2/1/0.1 (molar ratio). A fluorescence dye (FAM)-conjugated PSP (synthesized by Biologica Co.) was used for the measurement of conjugation efficiency. The FAM-PSP-liposomal fraction and free FAM-PSP fraction was separated by gel filtration and the fluorescence intensity in each fraction was measured to calculate the conjugation efficiency of PSP into liposome. |
| **Animals** | Male Std-ddY mice (aged 5–6 weeks, approximately 20g) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were approved by the Animal |

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**Preparation of Eryptosis-Induced Red Blood Cells (E-RBCs)** Eryptosis was induced as described previously with some modification. Briefly, 100 µL of RBC pellet prepared was gently treated with 900 µL of Ringer’s solution in the presence of ionomycin solution (Sigma-Aldrich, MO, U.S.A.) or treated with high osmotic Ringer’s solution, then the mixture was incubated for 3h at 37°C. The surface-exposure of PS was evaluated using an Annexin V-FLUOR staining kit (Roche Applied Science, IN, U.S.A.) with flow cytometry (FACScan; BD Biosciences, CA, U.S.A.).

**Determination of Fluorescently-Labeled Liposomes Bound to Erythrocytes** Dil-labeled liposome suspension (10 µL) was gently mixed with 965 µL of Ringer’s solution and 25 µL of RBCs or E-RBC suspension, then the mixtures were incubated for 0.5–3h at 37°C. The erythrocyte samples with bound Dil-labeled liposomes were analyzed by flow cytometry (BD Biosciences). The fluorescence intensity was normalized by the mean fluorescence intensity of E-RBCs with bound PSP-liposomes.

**Statistical Analysis** All data are mean values±standard deviation (S.D.). A two way ANOVA with Bonferroni post-test was used to assess statistical significance by using GraphPad Prism (GraphPad Software Inc., CA, U.S.A.).

**RESULTS AND DISCUSSION**

The PSP-liposomes was characterized in Table 1. The induction of eryptosis was evaluated by detecting exposed PS on the surface of the erythrocytes. Approximately 50% of the erythrocytes were PS-positive in the presence of ionomycin (at least 1 µM), a calcium ionophore which could induce eryptosis by the influx of calcium ion into erythrocytes (Supplementary Fig. 1). Brand et al. reported that an influx of calcium ion was induced by the growth of the parasite, followed by PS exposure. These results are in agreement with our observations. Although our experimental model does not completely reproduce the infection of erythrocytes by the malarial parasite, they do mimic PS exposure caused by eryptosis. Similar eryptosis was observed by high osmotic pressure (900 mOsm).

The amount of liposome bound to RBCs or E-RBCs was evaluated using fluorescence dye (DiI)-labeled liposomes. PSP-liposomes bound significantly to E-RBCs (Fig. 1) compared with RBCs. In contrast, PEGLyated liposomes did not bind to RBCs or E-RBCs. Unexpectedly, maleimide-group-conjugated PEG liposomes, in which the maleimide group was used to conjugate PEG and PSP, non-specifically bound with E-RBCs. Various proteins containing a thiol group can bind to RBCs or E-RBCs. Unexpectedly, maleimide-group-conjugated PEG liposomes could interact with them. We previously have demonstrated the experiment by using cationic liposomes in our earlier trials. The liposomal composition containing cationic lipid and PEG-lipid could affect the binding amount of E-RBC. By testing various incubation times after mixing PSP-liposomes and erythrocytes (Fig. 2), we demonstrated that specific binding of PSP-liposomes with E-RBCs occurred within 3h. We speculate that PEGLyated liposome could bind with neither E-RBC nor RBC, in contrast, Mal-PEG-liposome might bind E-RBC at some extent.

To confirm the specific binding of PSP, PSP-liposomes were incubated with E-RBCs and RBCs in the presence of annexin V. As shown in Fig. 3, the fluorescence of bound PSP-liposomes to E-RBCs was significantly inhibited by annexin V, whereas the fluorescence of bound PSP-liposomes to RBCs

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Table 1. Formulation Parameters of PSP-Liposomes

| Parameter                | Value       |
|--------------------------|-------------|
| Diameter (nm)            | 132.6±7.7   |
| Zeta potential (mV)      | −33.9±0.3   |
| Conjugation efficiency (%)| 62.0±7.1   |

Data represent mean±S.D. (n=3).
was unchanged. In the point of incomplete inhibition of the binding of PSP-liposome in the presence of excess amount of annexin V, we do not have clear reasons. PSP-liposome has possibility to bind with other components which are induced by eryptosis. We speculate that non-fluorescence-labeled PSP-liposome also can inhibit the binding of DiI-labeled PSP-liposome.

CONCLUSION

We have demonstrated a new strategy for delivering drugs to eryptosis-induced erythrocyte which is a model of malaria parasite-infected erythrocytes. PS is likely a universal target for erythrocytes undergoing eryptosis. Although further improvements are necessary for the development of efficient and specific nanocarriers, the present study provides insights into the design of an effective and practical drug delivery system for malaria-infected erythrocytes.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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![Fig. 3. Competitive Inhibitory Effect of Annexin V against PSP-Liposomes](image-url)