Volume 268, number 1, 235–237 FEBS 08706 July 1990

Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes

Alexander L. Klibanov[1,2], Kazuo Maruyama[1], Vladimir P. Torchilin[2] and Leaf Huang[1]

[1] *Department of Biochemistry, University of Tennessee, 37996 Knoxville, USA* and [2] *Institute of Experimental Cardiology, 121552 Moscow, USSR*

Received 31 May 1990

Incorporation of dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl)phosphotidylethanolamine (PEG-PE) into large unilamellar liposomes composed of egg posphatidylcholine:cholesterol (1:1) does not significantly increase the content leakage when the liposomes are exposed to 90% human serum at 37°C, yet the liposomes show a significant increase in the blood circulation half-life ($t_{\frac{1}{2}} = 5$ h) as compared to those without PEG-PE($t_{\frac{1}{2}} < 30$ min). The PEG-PE's activity to prolong the circulation time of liposomes is greater than that of the ganglioside GM_1, a well-described glycolipid with this activity. Another amphipathic PEG derivative, PEG stearate, also prolongs the liposome circulation time, although its activity is less than that of GM_1. Amphipathic PEGs may be useful for the sustained release and the targeted drug delivery by liposomes.

Liposome; Polyethyleneglycol; Drug delivery system

1. INTRODUCTION

Liposomes have been used as a drug delivery system in the last two decades [1]. Most of the applications are based on the fact that liposomes are avidly taken up by the RES cells in liver and spleen [1,2]. However, liposomal drug delivery to cells or tissues other than the RES has been very difficult. Recently, we have shown in a model system that optimal target binding and retention of the immunoliposomes can only be achieved with liposomes which show a relatively low rate of RES uptake [3]. In these studies, ganglioside GM_1 was used as a molecule which endows the liposomes with a lower level of RES uptake and hence a prolonged circulation half-life [3]. Allen and coworkers were the first to show this unusual activity of GM_1 [4,5] and the name 'stealth liposomes' ('stealth liposome' is a registered trademark of Liposome Technology, Inc.) has been given to this new class of liposomes [6]. Gabizon and Papahadjopoulos have further examined a number of other natural and synthetic lipids for the 'stealth' activity [7]. Importantly, they have shown that liposomes with a prolonged circulation time accumulate efficiently in a solid tumor model system [7]. Since it has been shown that conjugation of PEG to proteins significantly prolongs the circulation half-life of the protein [8], we have investigated if the lipophilic PEG derivatives can significantly prolong the circulation half-life of liposomes.

Correspondence address: L. Huang, Dept. of Biochemistry, Univ. of Tennessee, Knoxville, TN 37996-0840, USA

Abbreviations: PEG, polyethyleneglycol; PEG-OSu, monomethoxy polyethyleneglycol succinimidyl succinate; PE, phosphatidylethanolamine; PEG-PE, dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl) PE; chol, cholesterol; PC, phosphatidylcholine; DTPA, diethylenetriaminepentaacetic acid; DTPA-SA, DTPA-stearylamide; RES, reticuloendothelial system; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Materials*

PEG-OSu (M_r = 5000), DTPA cyclic anhydride, cholesterol, and calcein were purchased from Sigma. Ganglioside GM_1 was purchased from Calbiochem. Triethylamine (>99% purity) was a product of Aldrich. Dioleoyl PE and egg yolk PC from Avanti Polar Lipids were used. Myrj 59 (polyethyleneglycol stearyl ester, M_r = 4500) was from Serva. ^{14}C-labeled dioleoyl PE was purchased from Amersham. $^{111}InCl_3$ (carrier-free) was from New England Nuclear. Synthesis of DTPA-SA is described elsewhere [9]. Pooled normal human serum was supplied by Scantibody, Inc.

3.2. *Synthesis of PEG-PE*

An aliquot of PEG-OSu in $CHCl_3$ was added to a solution of PE in $CHCl_3$, followed by addition of triethylamine (PEG-OSu/PE/triethylamine = 3:1:3.5, m/m). When necessary, radiolabeled [^{14}C]PE was added as a tracer. The reaction mixture was incubated overnight at room temperature and $CHCl_3$ was evaporated with a stream of nitrogen gas. Full conversion of the PE primary aminogroup was confirmed by the negative ninhydrin reactivity after the products were separated by thin layer chromatography. Phospholipid phosphorus assay [10] showed the appearance of a new phosphate-positive spot at a higher R_f value than PE. The reaction mixture was redissolved in 0.145 M NaCl. Unreacted PEG-OSu is rapidly hydrolyzed in the aqueous media. The resulting mixture in saline was applied to a Bio-Gel A1.5M column, preequilibrated with saline. Peak fractions containing PEG-PE micelles eluted in the void volume were pooled, dialyzed against water and lyophilized.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

3.3. *Liposome preparation*

Liposomes were prepared with PC and chol (1:1 molar ratio). As a tracer marker ^{111}In was used [11], which was bound to DTPA-SA (1 mol% incorporated into the lipid bilayer). In some cases, 7.4 mol% of ganglioside GM_1, Myrj 59 or PEG-PE was added to the lipid mixture. Each mixture of lipids in $CHCl_3$/EtOH was placed in a round-bottom glass tube and the organic solvent was evaporated with a stream of nitrogen gas. The lipid film was additionally vacuum desiccated for 2 h. Normal saline was added and the lipid was allowed to hydrate for 1 h. The lipid suspension was vortexed for 2 min, sonicated in a bath sonicator for 1 min and extruded 10 times through two stacked Nucleopore filters (0.4 and 0.2 μm). When necessary, a 62 mM solution of calcein in 10 mM sodium phosphate buffer, pH 8, was used instead of the normal saline as the liposome aqueous phase. Liposome size was measured in a Coulter N4SD submicron particle analyzer (Coulter, Hialeah, FL).

3.4. *Liposome stability*

Non-entrapped fluorescent dye was removed by gel filtration on a Sephadex G-75 column. Liposome stability was measured as the quenching of the calcein fluorescence after incubation in phosphate-buffered saline or in the presence of 90% human serum at 37°C. Samples from the incubation mixture were diluted with phosphate-buffered saline and the fluorescence was measured in the presence and absence of 0.1% Triton X-100, using a Perkin-Elmer LS-5 fluorescence spectrophotometer. The percent dye leakage was calculated as described earlier [12].

3.5. *Biodistribution studies*

Biodistribution studies were performed on male Balb/c mice as described previously [11]. Liposomes labeled with ^{111}In were injected in the tail vein (0.12–0.14 mg PC per mouse). Weight of mouse blood was assumed to be 7.3% of the body weight. Data were expressed as percent of the injected dose accumulated per organ. Radioactivity accumulated in lung, heart and kidneys did not exceed 3% of the injected dose.

3. RESULTS AND DISCUSSION

The conjugate of PEG with PE was purified by gel filtration on a Bio-Gel A1.5M column as micelles. Using ^{14}C-labeled PE with known specific radioactivity and the dry weight of the purified PEG-PE, the molar ratio of PEG to PE in the conjugate was estimated to be 0.97:1, assuming that the molecular weight of PEG-OSu was 5000 Da as indicated by the manufacturer. Stable liposomes can be prepared from egg PC and chol (1:1 molar ratio) incorporating up to 25 mol% PEG-PE and the fluorescent dye calcein can be entrapped in the liposomes.

The stability of PEG-PE containing liposomes in human serum is shown in Fig. 1. Liposomes composed of egg PC/chol (1:1, m/m) with or without 7.4 mol% PEG-PE were incubated at 37°C in phosphate-buffered saline or 90% normal human serum and the leakage of the entrapped calcein was measured over 12 h. It is clear from the data in Fig. 1 that PEG-PE did not increase the leakage rate of the liposomes incubated either in saline or in 90% serum. Only about 10% of the originally entrapped calcein had leaked in 12 h.

Large unilamellar liposomes ($d \sim 0.2$ μm) of four different compositions were prepared for in vivo studies: egg PC/chol (1:1, m/m), egg PC/chol/GM_1 (1:1:0.16, m/m), egg PC/chol/Myrj 59 (1:1:0.16, m/m) and egg PC/chol/PEG-PE (1:1:0.16, m/m). The liposomes were labeled with ^{111}In tracer bound to a lipophilic derivative of DTPA chelator which is incorporated in the liposome membrane. The biodistributions of the liposomes were studied in the mouse after i.v. injection (Fig. 2). Liposomes composed of egg PC and chol were very rapidly taken up by the RES (liver and spleen). By 5 h after injection, these liposomes had completely cleared from the blood and resided almost entirely in the liver and spleen. Liposomes of this size range and composition are readily taken up by the RES [2]. The activities of PEG-PE, Myrj 59 and GM_1 in

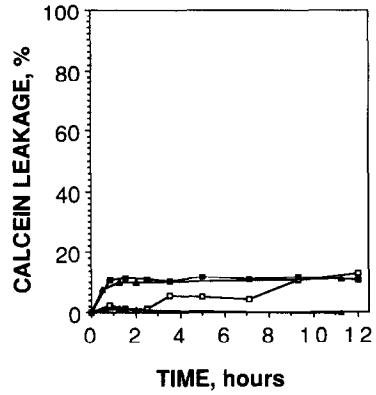

Fig. 1. Calcein leakage from liposomes in serum or PBS. (■) Liposomes composed of PC/chol/PEG-PE (1:1:0.16) in the presence of serum. (□) Liposomes composed of PC/chol/PEG-PE (1:1:0.16) in PBS. (▲) Liposomes composed of PC/chol (1:1) in the presence of serum. (△) Liposomes composed of PC/chol (1:1) in PBS.

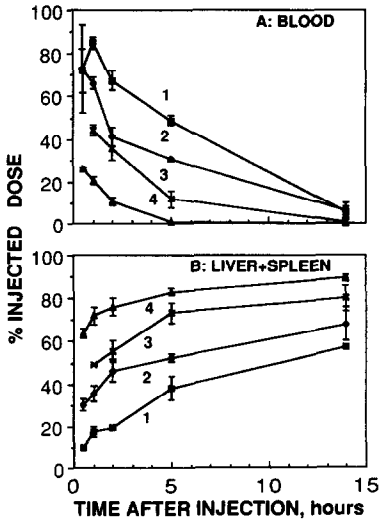

Fig. 2. Blood clearance of liposomes after intravenous injection. (A) Radioactivity of lipid in blood. (B) Radioactivity of lipid in liver and spleen (combined). Data are expressed as mean ± SE (n = 3). (■) Liposomes composed of PC/chol/PEG-PE (1:1:0.16). (♦) Liposomes composed of PC/chol/GM_1 (1:1:0.16). (×) Liposomes composed of PC/chol/Myrj 59 (1:1:0.16). (▲) Liposomes composed of PC/chol (1:1).

prolonging the circulation halflife of the liposomes can also be seen in Fig. 2. The activity of PEG-PE was striking. At 1 h after injection, 85% of the injected dose was found in the blood and only 18% in the liver and spleen. Even at 5 h, there were still more liposomes in the blood (49%) than in the RES (38%). Myrj 59 also showed some activity although it was not stronger than that of GM_1. The estimated $t_{1/2}$ for liposome blood clearance is <30 min, 0.5 h, 1.5 h and 5 h for PC/chol liposomes and liposomes additionally containing Myrj 59, GM_1 and PEG-PE, respectively. Thus, both amphipathic PEG significantly enhanced the blood residence time of the liposomes, although the activity of PEG-PE was superior to that of Myrj 59.

Conjugation of PEG to proteins prolongs the circulation time of the protein [8,13]. Adsorption of PEG-containing polymers to polystyrene microspheres results in reduced RES uptake [14,15]. There may be two mutually non-excluding factors which govern the affinity of liposomes to the RES: nonspecific hydrophobic interactions of liposomes with RES cells, and a specific opsonization reaction involving some blood component(s) [16–18]. Coating a liposome surface with PEG may increase the hydrophilicity of the liposome surface such that the nonspecific interaction of liposomes with RES cells is reduced. Additionally, PEG may sterically prevent the coating of opsonins to the liposome, resulting in a reduced specific interaction with the RES cells.

We have recently shown that the prolonged circulation time allows the immunoliposomes to bind with the target cells very effectively [3]. This is an important feature of using immunoliposomes as a target-specific drug delivery vehicle. Our present data indicate that amphipathic PEG can significantly enhance the liposome concentration in the circulation at a given time. Its use in the liposome-mediated drug targeting should be seriously evaluated.

Acknowledgements: This work is supported by NIH grants CA 24553 and AI 25834. We acknowledge that Judith Senior and M.C. Woodle have independently performed similar experiments and arrived at similar conclusions. We appreciate their sharing of the data before publication.

REFERENCES

[1] Liposomes as Drug Carriers: Recent Trends and Progress (1988) (Gregoriadis, G. ed.) John Wiley and Sons, Chichester.
[2] Hwang, K.J. (1987) in: Liposomes: From Biophysics to Therapeutics (Ostro, M.J. ed.) pp. 109–156, Marcel Dekker, New York.
[3] Maruyama, K., Kennel, S.J. and Huang, L. (1990) Proc. Natl. Acad. Sci. USA (in press).
[4] Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.
[5] Allen, T.M., Hansen, C. and Rutledge, J. (1989) Biochim. Biophys. Acta 981, 27–35.
[6] Allen, T.M. (1989) in: Liposomes in the Therapy of Infectious Diseases and Cancer (Lopez-Berenstein, G. and Fidler, I.J. eds) pp. 405–413, Alan R. Liss, New York.
[7] Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 18, 6949–6953.
[8] Beauchamp, C.O., Gonias, S.L., Menapace, D.P. and Pizzo, S.V. (1983) Anal. Biochem. 131, 25–33.
[9] Kabalka, G.W., Buonocore, E., Hubner, K., Moss, T., Norley, N. and Huang, L. (1987) Radiology 163, 255–258.
[10] Kates, M. (1972) Techniques of Lipidology, p. 421, Elsevier, New York.
[11] Holmberg, E., Maruyama, K., Litzinger, D., Wright, S., Davis, M., Kabalka, G.W., Kennel, S.J. and Huang, L. (1989) Biochem. Biophys. Res. Commun. 165, 1272–1278.
[12] Liu, D. and Huang, L. (1988) Biochemistry 28, 7700–7707.
[13] Knauf, M.J., Bell, D.P., Hirtzer, P., Luo, Z.-P., Young, J.D. and Katre, N.V. (1988) J. Biol. Chem. 263, 15064–15070.
[14] Illum, L. and Davis, S.S. (1984) FEBS Lett. 167, 79–82.
[15] Illum, L. and Davis, S.S. (1987) Life Sci. 40, 367–374.
[16] Moghimi, S.M. and Patel, H.M. (1988) FEBS Lett. 233, 143–147.
[17] Moghimi, S.M. and Patel, H.M. (1989) Biochim. Biophys. Acta 984, 384–387.
[18] Senior, J.H. (1987) CRC Crit. Rev. Ther. Drug Carrier Systems 3, 123–193.