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Elastase activated liposomal delivery to nucleated cells

Charles C. Pak, Ravi K. Erukulla, Patrick L. Ahl, Andrew S. Janoff, Paul Meers *

The Liposome Company, 1 Research Way, Princeton, NJ 08540, USA

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Abstract

The specific activation of liposomes for delivery has been explored by enzyme mediated cleavage of a peptide substrate covalently conjugated to a fusogenic lipid. We have previously shown an elastase sensitive peptide conjugated to 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) could be activated by enzymatic cleavage, triggering liposome-liposome lipid mixing and fusion with erythrocyte ghosts (Pak et al., Biochim. Biophys. Acta, 1372 (1998) 13–27). Further optimization of this system has been aimed at obtaining substrate cleavage at or below physiological elastase levels and to demonstrate triggered delivery to living cells. Therefore a new peptide-lipid, MeO-suc-AAPV-DOPE (*N*-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE), has been developed that exhibits greater sensitivity and selectivity for elastase cleavage and subsequent conversion to DOPE. This peptide-lipid was used with DODAP (dioleoyl dimethylammonium propane, a pH dependent cationic lipid) in a 1:1 mol ratio with the expectation that endocytosis would lead to a liposome with an overall positive charge if enzymatic cleavage had occurred. Elastase treated liposomes displayed pH dependent enhancement of binding, lipid mixing, and delivery of 10 000 MW dextrans, relative to untreated liposomes, when incubated with HL60 human leukemic cells. Heat denatured elastase did not activate DODAP/MeO-suc-AAPV-DOPE liposomes, indicating enzymatic activity of elastase is necessary. Liposomes bound to ECV304 endothelial cells at physiological pH could be activated by elastase to deliver an encapsulated fluorescent probe, calcein, into the cell cytoplasm. These results suggest enzyme substrate peptides linked to a fusogenic lipid may be used to elicit specific delivery from liposomes to cells.

Keywords: Liposome; Fusion; Elastase; Peptide; Lipid; Cleavage

1. Introduction

A paradox of liposomal delivery vehicles is the requirement for liposomal membranes that are both stable and yet capable of undergoing fusion or destabilization at the desired site, so as to elicit delivery of

Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; MeO-suc-AAPV-DOPE, *N*-methoxy-succinyl alanyl alanyl prolyl valyl 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; *N*-Ac-AA-DOPE, *N*-acetyl alanyl alanyl 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine; *N*-Rho-PE, *N*-lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; TMR-dextran, tetramethyl rhodamine dextran; DODAP, dioleoyl dimethylammonium propane; C12E8, octaethylene glycol monododecyl ether; HBSS, Hanks' balanced salt solution; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; RET, resonance energy transfer; RFU, relative fluorescence unit; TLC, thin layer chromatography; FDQ, fluorescence dequenching

* Corresponding author.

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the contents of the liposome into the cell. We have previously described the use of enzymatic cleavage to trigger liposomal fusion and contents delivery [1]. Although other triggers have been utilized to induce liposomal fusion or membrane destabilization [2–7], activation by enzyme cleavage has several advantages over these methods.

Elevated enzyme activity is associated with numerous pathological conditions. Metastatic cancer cells display enhanced extracellular activity of several degradative enzymes, such as matrix metalloproteinases and urokinase-type plasminogen activator (for review see [8]). Elevated enzymatic activity facilitates the extravasation of these cells from the circulation and increases their invasive potential. Inflammatory conditions such as cystic fibrosis [9–12], rheumatoid arthritis [13,14], and emphysema [15,16] are accompanied by an increase in extracellular elastase activity due to release of elastase from phagocytic cells. Elevated elastase activity appears to be due, in part, to an imbalance in the elastase/anti-protease ratio [9,17,18]. Elastase has also been associated with tumor progression and development [19–21]. The ubiquitous yet specific nature of disease-associated enzymatic activity, its localization near or on the membranes of cells involved in tissue remodeling [22,23] and its association with several pathologies provide numerous opportunities for triggering specific liposomal delivery to desired targets using the activity of such enzymes. The triggering event would be expected to convert the liposome from a relatively inert state to a fusogenic state and may even trigger specific binding depending on the design.

The selectivity of liposomal activation can be modulated by the choice of an enzyme substrate conjugated to a fusogenic lipid so that enzymatic cleavage releases or unmasks fusogenic lipids. Thus liposomes may be designed for a selected site of activation and hence liposomal delivery could be targeted. We have chosen to use elastase mediated triggering of liposomal fusion as a model for the general principle of enzymatically activated delivery via liposomes.

We have previously described the activation of liposomal lipid mixing and fusion by enzyme triggering [1]. A peptide-lipid consisting of *N*-Ac-Ala-Ala-, an elastase sensitive peptide sequence, was conjugated to the headgroup of DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine), a known fusogenic lipid. The fusogenic potential of this peptide-lipid, *N*-Ac-AA-DOPE, was limited until enzymatic cleavage of the peptide regenerated DOPE. Liposomes containing *N*-Ac-AA-DOPE could be triggered to lipid mix with target liposomes and fuse with RBC ghosts.

Although *N*-Ac-AA-DOPE was capable of demonstrating the utility of enzyme-triggered fusion, physiological relevance requires greater sensitivity to enzyme activation, demonstration of delivery to nucleated living cells and ultimately the ability to deliver in the physiological milieu which may include serum proteins. We chose to address the first two of these problems in this report.

First, it is necessary to demonstrate that the choice of an appropriate peptide can be used to optimize the cleavage and triggering for the desired target at physiological levels of the enzyme. Powers et al. [24] had shown that the peptide sequence MeO-suc-Ala-Ala-Pro-Val- was highly sensitive to elastase cleavage. Therefore we hypothesized that this sequence when conjugated to DOPE would create a triggerable peptide-lipid with greater sensitivity. In this study a new peptide-lipid, MeO-suc-AAPV-DOPE, was tested for elastase mediated DOPE generation. Included in this effort was the introduction of a lipid that would reduce the surface charge of the liposome compared to the previous version to enhance interaction of the positively charged enzyme, elastase, with the substrate. A second goal was to demonstrate delivery to nucleated cells, where at least some delivery may occur via an endosomal compartment. Liposomes were designed to take advantage of the low pH in that compartment in a manner that would also enhance elastase triggering. Here we report a liposomal system that could be shown for the first time, to be triggered by physiological levels of elastase to undergo lipid mixing with and aqueous contents delivery to nucleated cells.

2. Materials and methods

2.1. Reagents

Human leukocyte elastase was purchased from Calbiochem (San Diego, CA, USA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL,

USA) and were of 99% or greater purity. Calcein (>95% pure), tetramethyl rhodamine dextran (TMR-dextran), and streptavidin were from Molecular Probes (Eugene, OR, USA). Biotinylated-wheat germ agglutinin (WGA) was obtained from Pierce (Rockford, IL, USA). MeO-suc-Ala-Ala-Pro-Val-OH used for covalent linkage with DOPE was from Bachem Bioscience (King of Prussia, PA, USA). RPMI 1640, fetal bovine serum (FBS), and HBSS (Hanks' balanced salt solution) were purchased from Life Technologies (Gaithersburg, MD, USA). Medium 199 was purchased from BioWhittaker (Walkersville, MD, USA). 1,3-Dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), *p*-nitrophenol and triethylamine were obtained from Sigma (St. Louis, MO, USA). TMD-8 ion exchange resin was purchased from Aldrich (Milwaukee, WI, USA). HPLC-grade solvents, tetrahydrofuran (THF), chloroform and methanol were purchased from Baxter (McGaw Park, IL, USA). All chemicals and solvents were used without further purification.

2.2. Cells

HL60 human leukemia and ECV304 human endothelial cells were obtained from ATCC (Rockville, MD, USA). HL60 cells were passaged as suspension cultures in RPMI 1640 supplemented with 10% heat inactivated FBS. Adherent ECV304 cells were grown in medium 199 supplemented with 10% heat inactivated FBS. Greater than 98% viability was observed during routine tissue culture.

2.3. Synthesis and characterization of N-methoxy-succinyl alanine alanine proline valine 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (MeO-suc-AAPV-DOPE)

2.3.1. MeO-suc-AAPV-p-nitrophenyl ester

To a solution of MeO-suc-AAPV-H peptide (540 mg, 1.15 mmol) was added 142 mg (1.38 mmol) of *p*-nitrophenol, 175 mg (1.38 mmol) of DCC and a catalytic amount (a few crystals) of DMAP in 10 ml of dry chloroform. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. At this point TLC (thin layer chromatography) analysis showed that the reaction had gone to completion. The precipitate, dicyclohexylurea, from the reaction mixture was filtered using a G-2 funnel and the filtrate concentrated under reduced pressure. The residual material was used in next step without purification.

2.3.2. MeO-suc-AAPV-1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine

To a solution of *p*-nitrophenyl ester of MeO-suc-AAPV-OH (600 mg, 1.01 mmol) was added 604 mg (0.81 mmol) of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 82 mg (113 ml, 0.81 mmol) of triethylamine in 20 ml of chloroform:tetrahydrofuran (1:4 v/v). The reaction mixture was stirred under nitrogen atmosphere at room temperature overnight. TLC analysis showed that the reaction had gone to the completion. The reaction mixture was concentrated under reduced pressure and passed through activated TMD-8 ion exchange resin in THF:H_2O (9:1 v/v). The phosphorus positive fractions were pooled and concentrated to get a residual product. The residual material was purified with silica gel column chromatography (the column was washed with 5% methanol in chloroform, then eluted with chloroform:methanol:ammonium hydroxide 65:25:4 v/v/v), giving 915 mg (95% yield on the basis of DOPE), which on lyophilization gave a white solid. The lipopeptide molecule tested positive with a molybdenum reagent and negative with a ninhydrin reagent. By TLC the lipopeptide gave a single spot and >99% purity. The lipopeptide was also characterized by NMR and FAB mass spec analysis. Some characteristic ^{1}H-NMR signals (300 MHz, $CDCl_3$) are shown here: d 0.87 (t, 3H, $J=7.15$ Hz), 1.27 (40H), 1.56 (4H), 2.0 (8H), 2.23 (t, 4H, $J=7.15$ Hz), 5.17 (1H), 5.32 (4H, $J=3.12$ Hz). The ^{31}P-NMR spectrum (121.5 MHz, $CDCl_3$) gave a single signal. FAB (M^+) calculated for $C_{62}H_{109}N_5O_{15}P$ was 1195.55, and masses of 1196.8 (MH^+) and 1234.9 (MK^+) were observed.

2.4. Liposome preparation

Large vesicles were prepared by aliquoting desired amounts of lipid from chloroform stocks into 13×100 mm pyrex tubes and drying under a nitrogen stream. After exposure to high vacuum 4 h overnight the lipid film was hydrated in 10 mM

TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 154 mM NaCl, 0.1 mM EDTA (ethylenediamine tetraacetic acid), pH 7.4. Unless specified otherwise, this buffer was used for all experiments. After vortexing, samples were freeze-thawed 8× and extruded under pressure 10× (Lipex, Vancouver, BC, Canada) through 0.1 μm polycarbonate filters (Nucleopore, Pleasanton, CA, USA). Liposomes were stored at 4°C until used.

Fluorescent dextran containing liposomes were prepared by hydrating the DODAP/MeO-suc-AAPV-DOPE lipid film with a 50 mg/ml solution of 10 000 MW TMR-dextran in TES/NaCl/EDTA buffer. Liposomes were then vortexed, freeze-thawed, and extruded through 0.1 μm filters as described above. To remove unencapsulated dextran the liposome solution was extensively dialyzed with the TES/NaCl/EDTA buffer using a Biodialyser (Sialomed, Columbia, MD, USA) fitted with 50 nm pore size filters. Calcein loaded liposomes were prepared by hydrating the lipid film in the presence of buffer containing 50 mM calcein. The calcein solution was pH and osmolarity adjusted prior to preparation of liposomes. Calcein loaded liposomes contained 0.75 mol% *N*-Rho-PE to monitor liposome binding. After preparation of large vesicles as described above, calcein loaded liposomes were transferred to a 10 000 MWCO Slide-A-Lyzer (Pierce, Rockford, IL, USA) and extensively dialyzed with TES/NaCl/EDTA buffer. The encapsulated volume of these liposomes was 0.8 l/mol of lipid. Sonicated vesicles were prepared by drying lipid in the same manner as described above but preparations were vortexed then water bath sonicated for >10 min at room temperature. Lipid concentration was monitored by phosphate assay [25]. The size of liposomes was verified by quasi-elastic light scattering using a Nicomp submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA). Freeze-thaw-extrusion vesicles and sonicated vesicles were 70–80 nm and 35–45 nm in diameter, respectively, as determined by number weighted Gaussian analysis.

2.5. Detection of MeO-suc-AAPV-DOPE cleavage

2.5.1. TLC detection of MeO-suc-AAPV-DOPE cleavage

100 nmol of MeO-suc-AAPV-DOPE sonicated vesicles were incubated with 0, 5, or 10 μg elastase in 0.4 ml TES/NaCl/EDTA buffer, pH 7.4, overnight at 37°C. Lipid was extracted by organic phase separation [26], dried under N_2 stream, and exposed to vacuum for 4 h. Samples were resuspended in chloroform and spotted onto TLC plates. TLC was run using chloroform:methanol:ammonium hydroxide (65:25:5), air dried, sprayed with molybdenate blue, and charred on a hot plate.

2.5.2. ^{31}P-NMR analysis

DODAP/MeO-suc-AAPV-DOPE (1:1 mol ratio) freeze-thaw-extrusion vesicles were prepared and treated with or without elastase (0–50 μg protein/1000 nmol lipid/4 ml) for 2 h in TES/NaCl/EDTA buffer, pH 7.4, at 37°C. Liposomes were transferred to 13×64 mm polyallomer centrifuge tubes (Beckman, Palo Alto, CA, USA), and pelleted by ultracentrifugation at 149 000×*g* for 1 h at 4°C with a L5-50E ultracentrifuge (Beckman, Palo Alto, CA, USA). The liposome pellet (approximately 90% of the total) was resuspended in 100 μl TES/NaCl/EDTA buffer, to which 400 μl of 10% deoxycholate, 100 mM EDTA, 20 mM HEPES, pH 7.4, buffer and 200 μl of deuterium oxide (Cambridge Isotope Laboratories, Woburn, MA, USA) were added. After transfer to 5 mm NMR tubes samples were monitored at room temperature in a Bruker AC300 spectrometer operating at 121.5 MHz, with 110 μs 90° radio frequency pulse for proton decoupling and set to 2 s interpulse delay to avoid signal saturation. Sweep width was set at 50 kHz. 1 Hz line broadening was applied to all spectra. Peaks were identified by comparison with standards run under identical conditions.

2.6. Binding and lipid mixing of liposomes to HL60 cells

Lipid mixing was monitored by the *N*-NBD-PE/*N*-Rho-PE resonance energy transfer assay, as described [27]. Liposomes were prepared with 0.75 mol% *N*-NBD-PE and 0.75 mol% *N*-Rho-PE, which results in quenching of the *N*-NBD-PE fluorescence signal. Membrane fusion results in probe diffusion and relief from self-quenching, which is monitored as an increase in *N*-NBD-PE fluorescence. DODAP/MeO-suc-AAPV-DOPE (1:1 mol:mol) lipo-

somes were incubated in TES/NaCl/EDTA buffer ± elastase (5 μg/100 nmol lipid, 250 μM lipid concentration) for 2 h at 37°C, pH 7.4. HL60 cells were washed with TES/NaCl/EDTA buffer and incubated with liposomes (1×10^6 cells, 10 nmol liposome) in 200 μl TES/NaCl/EDTA buffer. Samples were either at pH 7.4 or adjusted to pH 5 by the addition of dilute HCl. All samples were shaken in an Eppendorf Thermomixer (Brinkmann Instruments, Inc., Westbury, NY, USA), 700 rpm, for 30 min at 37°C. There was no reduction in cell viability following this procedure, as detected by trypan blue exclusion (unpublished data). Cells were then washed with TES/NaCl/EDTA buffer, pH 7.4, and transferred to Falcon 24 well plates (Becton Dickinson, Lincoln Park, NJ, USA). Fluorescence was monitored in a Cytofluor II multiwell fluorescence plate reader (Perseptive Biosystems, Framingham, MA, USA) with a quartz halogen lamp using 450 nm excitation/530 nm emission or 560 nm excitation/620 nm emission wavelengths for *N*-NBD-PE or *N*-Rho-PE fluorescence, respectively. Liposome binding was determined as the amount of *N*-Rho-PE fluorescence associated with washed cells relative to total fluorescence of liposomes added. This percentage was converted to number of liposomes bound by multiplying by the number of liposomes added (assuming all liposomes were 100 nm in diameter and 10^5 lipid molecules/0.1 μm diameter liposome. Therefore 6.02×10^{10} liposomes of 0.1 μm diameter were added per sample). The % fluorescence dequenching (FDQ) was calculated by the following formula:

$$[(F_t/F_{\text{max cells}})-(F_{\text{o alone}}/F_{\text{max alone}})]/$$

$$[1-(F_{\text{o alone}}/F_{\text{max alone}})]\times100$$

where F_t = *N*-NBD-PE fluorescence of liposomes incubated with cells at a given time, $F_{\text{o alone}}$ = initial *N*-NBD-PE fluorescence of liposomes only, $F_{\text{max cells}}$ and $F_{\text{max alone}}$ = maximal *N*-NBD-PE fluorescence of liposomes incubated either with cells or alone, respectively, as determined by addition of 0.5% C12E8 detergent. FDQ was assumed to result from all-or-none lipid mixing of liposomes with cells. Therefore % FDQ could be converted to number of liposomes mixed by simple multiplication of the total. This was done to take into account both the enhancement of binding and the lipid mixing after elastase activation.

2.7. DODAP/MeO-suc-AAPV-DOPE liposome-ECV304 binding, lipid mixing, and calcein delivery

Liposomes were bound to adherent ECV304 cells via a biotin-streptavidin linkage. To this end DODAP/MeO-suc-AAPV-DOPE (1:1 mol:mol) liposomes were prepared with 0.3 mol% *N*-biotinyl caproylamine-PE as well as fluorescent lipid probes or with encapsulated calcein. ECV304 cells were washed with HBSS buffer and then incubated sequentially at room temperature with biotin-WGA (20 μg/ml) and streptavidin (40 μg/ml) prepared in HBSS, 30 min/treatment. Cells were washed after each treatment. Liposomes were treated with or without elastase as described above. Certain aliquots of pretreated DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) liposomes were freeze-thawed after dialysis and prior to the addition to cells to release the liposomal contents. Such freeze-thawed liposomes were exposed to a liquid nitrogen-37°C water bath for 5 cycles. The self-quenching of calcein was reduced by approximately 85% (maximal FDQ determined by detergent solubilization) after freeze-thawing, indicating the release of encapsulated calcein. In all cases, 50–100 nmol of liposomes were added to confluent ECV304 cell monolayers (1×10^5 cells/well of a 24 well plate) and incubated in HBSS for 30 min at room temperature to promote *N*-biotinyl cap-PE binding to streptavidin. Unbound liposomes were removed by repeated washes. After the final wash, fresh HBSS buffer was added to all wells and cells were incubated at 37°C for given times. Fluorescence was quantitated as described above.

2.8. Fluorescence microscopy of liposome-cell lipid mixing and aqueous contents delivery

DODAP/MeO-suc-AAPV-DOPE (1:1 mol/mol) liposomes were incubated for 2 h at 37°C without or with elastase (5 μg protein/100 nmol lipid). Liposomes containing the fluorescent lipid probes *N*-NBD-PE and *N*-Rho-PE were bound to HL60 in solution as described above. TMR-dextran loaded

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