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Liposome transduction into cells enhanced by haptotactic peptides (Haptides) homologous to fibrinogen C-termini

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Abstract

Haptides are 19–21mer cell-binding peptides equivalent to sequences on the C-termini of fibrinogen β chain (Cβ), γ chain (preCγ) and the extended αE chain of fibrinogen (CαE). In solution, Haptides accumulated in cells by non-saturable kinetics [Exp. Cell Res. 287 (2003) 116]. This study describes Haptide interactions with liposomes and Haptide-mediated liposome uptake by cells. Haptides became incorporated into negatively charged liposomes, changing their zeta potential. Atomic force microscopy and particle sizing by light scattering showed that the liposomes dissolved Haptide nanoparticles and absorbed them from solution. Pre-mixing fluorescent rhodamine-containing liposomes or "stealth" doxorubicin (DOX)-containing liposomes (Doxil) with Cβ, preCγ or to a lesser degree CαE, significantly enhanced their uptake by fibroblasts and endothelial cells. Confocal microscopy showed Haptide-induced liposome uptake saturated above ~ 40 μM Haptide. Cytotoxicity tests with lower concentrations of Doxil liposomes indicated that premixing with ~ 40 μM Cβ or preCγ increased their toxicity by one order of magnitude. It was evident that the liposomes complexed with an amphiphilic Haptide are transduced through cell membranes, probably by a non-receptor-mediated process. These results suggest that Cβ or pre-Cγ could be employed to augment the cellular uptake of drugs in liposomal formulations.

Keywords: Haptides; Fibrinopeptides; Liposomes; Cell-membrane; Transduction; Doxil; Doxorubicin; Rhodamine

1. Background

Fibrinogen exhibits substantial hydrophobic character, as demonstrated by its ability to bind to lipids and fatty acids. It has been proposed that the affinity of fibrinogen for hydrophobic lipid surfaces, particularly those rich in cholesteryl-esters, may predispose them to thrombosis [1–5]. Also, the ability of fibrin to interact with and entrap liposomes led to the evalua-

Abbreviations: Fibrin(ogen), fibrin and/or fibrinogen; FITC, fluorescein isothiocyanate; DOX, doxorubicin; Doxil, doxorubicin-loaded stealth (PEG-coated) liposomes; Haptides, 19–21mer synthetic peptides homologous to the carboxy-terminal sequence of the β chain (Cβ), including the C-termini of the γ chain (preCγ) and the αE chain (CαE), which elicit haptotactic responses from different cell types mostly of mesenchymal origin; HF, human fibroblasts; BAEC, bovine aortic endothelial cells.

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Table 1
Synthesized peptides and their homology to Cβ

Peptide	Sequence	Cβ Homology	Mole equiv.
Cβ*	KGSWYSMRKMSMKIRPFFPQQ	21/21	21/21
PreCγ*	KTRYYSMKKTTMKIIPFNRL	11/20	14/20
CαE*	RGADYSLRAVRMKIRPLVTQ	10/20	12/20
Cγ (contr)	GEGQQHHLGGAKQAGDV	0/17	5/17
Cα (contr)	SEADHEGTHSTKRGHAKSRP	0/20	7/20

*Defined as Haptide.
The homology to Cβ is represented by shadowed background.

tion of fibrin glue with entrapped liposomes as a topical, slow drug delivery system [6,7].

Normal fibrinogen (Fib 340) is a complex hexamer composed of two sets of three non-identical chains (α, β and γ) linked by multiple disulfide bonds. Both the structure of fibrinogen and the mechanism of its coagulation following activation by thrombin have been extensively studied [8]. A larger variant fibrinogen (Fib 420) with an extended α chain has also been described [9]. In search of epitopes that might mediate fibrinogen's ability to attract and attach cells [10], the conserved sequences at the C-termini of the fibrinogen β chain (Cβ) and their analogues on both the γ and the extended αE chain (CαE) were studied. The 19–21mer homologous peptides equivalent to the C-termini of the β chain (Cβ), the CαE and a sequence near the C-terminus of the γ chain (preCγ) were synthesized [11] (Table 1). When these peptides were bound to cell-inert matrices, such as sepharose beads (SB), they elicited cell attachment (haptotactic) responses from different cell types, including normal fibroblasts, endothelial and smooth muscle cells [11]. These haptotactic peptides were named "Haptides". None of the Haptides were toxic or altered the rate of proliferation of different cell types at a wide range of concentrations, and did not block antibodies directed against cell integrins β, αv, αvβ1 and αvβ3. Free Haptides were rapidly internalized into the cells by non-saturable kinetics and became distributed within the cell cytoplasm.

At low concentrations (<20 μM), the Haptides accelerated fibrinogen polymerization, as indicated by shorter thrombin-induced clotting time and more turbid fibrin clots. At higher concentrations (>120 μM), the Haptides induced spontaneous fibrinogen precipitation, even without the presence of thrombin. By contrast, up to 100 μM, Haptide did not bind to platelets and had no significant effect on platelet aggregation induced by ATP, epinephrine or collagen [12].

This study focused on the interactions of Haptides with liposomes. The core aqueous compartment of liposomes can contain a variety of materials or drugs [13–15]. Most attempts to use liposomes as intravenous drug delivery vehicles envisioned them as capable of circulating in blood, eventually to be taken up by certain tissues and slowly release their drug-containing cores. For systemic drug formulations, liposomes have been rendered less likely to be taken up by the liver or circulating leucocytes by formulating them with a polyethylene glycol (PEG) coating ("stealth liposomes") [14,16,17]. A number of drugs formulated with liposomes have been described [15–19]. The current study demonstrates that Haptides could significantly augment the uptake of liposomes by different types of cultured cells.

2. Materials and methods

2.1. Chemicals and reagents

Clinical grade human fibrinogen and thrombin were prepared from blood plasma by the New York Blood Center and Vitex (New York, NY). Tissue culture media, serum, bovine serum albumin (BSA) and other reagents were purchased from standard commercial sources for laboratory supply, mainly from Biological Industries (Beit-HaEmek, Israel), Sigma (Israel and St. Louis, MO) and GIBCO (Grand Island, New York, NY); other reagents were from Sigma.

2.2. Liposomes

Liposomes loaded with fluorescent rhodamine were provided by A. Gabizon (Hadassah University Hospital, Dept. of Oncology). The liposomes were prepared as previously described [17] with DPPE-rhodamine (Avanti Polar Lipids, Birmingham, AL) and contained hydrogenated soybean phosphatidylcholine/cholesterol/DSPEPEG/Folate/DPPE-rhodamine (molar ratio, 99.4:70:0.5:0.1).

Doxorubicin-loaded stealth (PEG-coated) liposomes (Doxil; Ortho Biotech, Raritan, NJ) is a generic name for a clinical grade "stealth PEGylated" lipo-

somal doxorubicin (DOX) supplied as a drug with 2 mg DOX/ml of liposomal suspension. As DOX is fluorescent, the resultant liposomes were capable of being monitored by UV fluorescence techniques.

The ratio between liposomal drug and Haptides was calculated based on the net weight of the drug (within the aqueous compartment of the liposomes) to the weight amount of added Haptide.

2.3. Synthesis of C-terminal peptides

The 19–21mer peptides sequences presented in Table 1 were synthesized at a few different facilities, such as by SynPep (Dublin, CA), the Inter-departmental Services of the Medical School at the Hebrew University (Jerusalem) or by Alpha Diagnostics International (San Antonio, TX). Most experiments employed peptides that were >90% pure as determined by HPLC/mass-spectrometry. Fluorescein isothiocyanate (FITC)-derivatized peptides were prepared with a single FITC-tag covalently bound on the amino terminal. No detectable release of free FITC from the intact peptides could be recorded.

2.4. Monitoring Haptide aggregates by particle counter

Previous experiments with gel filtration chromatography in phosphate buffer indicated that Haptides exist mainly as soluble multimers up to $n=20$. But about 10% of the Haptides were organized as bigger nanoparticles that could not pass through a 0.22-μm filter. The average size of Haptide nanoparticles was measured at 25 °C by dynamic light scattering with the 'Zetasizer-3000'. Malvern Instruments, UK (10 mW He–Ne laser, wavelength 633 nm, detector angle 90°, dispersant viscosity 0.89 cP, dispersant refractive index 1.33, sample refractive index 1.50). The samples were passed through a 0.45-μm filter prior to their measurements. The average particle size (by volume distribution) was taken as a mean value of three measurements.

2.5. Zeta potential measurements of charged Doxil liposomes

The binding of Haptides to the lipid bilayer surface of the liposomes was evaluated by measuring the change in the zeta potential of the liposomes. The Zeta potential is the electric potential at the shear plane of a particle. The Zeta potential of particles is calculated from their measured electrophoresis mobility by using the Henry equation [20]:

$$U_E = \frac{2z\varepsilon}{3\eta} * f(K_a) \quad (1)$$

where U_E is the electrophoretic mobility, z the zeta potential, ε is the dielectric constant, η is the viscosity and $f(K_a)$ is the ratio of radius of curvature to double-layer thickness. In the case of aqueous solution (high dielectric constant) and moderate ionic strength, the Smoluchowski approximation is applied where $f(K_a)=1.5$.

The stability of hydrophobic colloids depends on the zeta potential as follows: If the particles have a large negative or positive zeta potential, they will repel each other and the dispersion will most likely be stable. In particles with low zeta potential values, there is only little repulsion force and the particles will eventually aggregate, resulting in dispersion instability. The zeta-potential measurements were performed with a ZetaSizer 3000 HSA (Malvern Instruments, UK). The liposomes and peptide samples were diluted in 0.1 M PBS buffer at pH 7.0 followed by vigorous stirring. The zeta potential was determined at 25 °C, at least three times for each sample.

2.6. Atomic force microscopy

Tapping Mode imaging was carried out using a Solver P47 (NT-MDT, Moscow, Russia) scanning probe microscope. A 110-μm long 'Ultrasharp' silicon tip was used, with a radius of curvature of less than 35 nm (SC-12 series, NT-MDT). This tip has a typical resonance frequency of 155 kHz, and typical spring constant of 1.75 N/m. Height mode images were collected along with either an amplitude or phase image. A scan rate of 2–2.5 Hz was typically sufficient to maintain good signal-to-noise ratio. Multiple scans were imaged in each case, with a variety of areas examined for consistent sample morphology. Only features that were reproducible are reported. The samples were prepared by spin coating of the tested solution with particles on a mica surface freshly

cleaved prior to spin coating (Muscovite mica, Pelco International). A custom made spinner was used for all spin coating experiments, which were carried out at room temperature. Samples were filtered through 450-nm filter and dispensed onto the central portion of the spinning mica film, which was accelerated to the process speed of 3100 rpm. In each case, the total spin time was kept fixed (60 s). Upon cessation of spinning, coated substrates were dried horizontally at room temperature for 15 min before carrying out the AFM imaging.

2.7. Extracting free DOX from Doxil liposomes suspension

To remove free DOX that may have leaked from Doxil liposomal suspensions, Doxil was passed through a Chelex resin column (BioRad). The eluate contained only liposomal Doxil (~ 80 nm diameter) with no free DOX. The adsorbed DOX could be released from the column by a solution of 90% butanol + 10% HCl (0.75 N) and the OD_{492} used to calculate its concentration.

2.8. Cell cultures

The cell types used in the present study were obtained and cultured as previously described [10,11]. Briefly, normal human skin fibroblasts (HF) were isolated from skin biopsies of young normal volunteers and cultured for no more than 14 passages. Normal bovine aortic endothelial cells (BAEC) were isolated from fresh thoracic aortas collected at slaughterhouse from sacrificed young animals and kept in culture for up to 12–15 passages. The cell cultures were maintained at 37 °C in a water-jacketed CO_2 incubator, and were harvested by trypsin/versen solution with 1–2 passages/week in a split ratio of 1:10 for rapidly proliferating, transformed cells and 1:4 for normal cell types.

2.9. Monitoring Haptides and liposomes uptake by HF or BAEC

Cells were seeded and grown in a CO_2 incubator for 24 h in a four-cell chamber cover-system slide for cell culture (Nalge Nunk, Naperville, IL). In initial experiments, the cells were exposed to a dose of trace ^{FITC}peptide. In liposomes uptake experiments, cells were exposed to a pre-mixed combination of fluorescent liposomes and non-labelled Haptides. Typically, 20 μl of fluorescent liposomes and peptide were mixed with 180 μl PBS and added to the cell chamber. Incubation was continued at different time points, up to 1 h, at 37 °C. Uptake was stopped by washing the cells twice with PBS and fixing with 500 μl of 4% formaldehyde. Coverslips with the fixed cells were covered with a mounting solution of PBS-glycerol 80% and subjected to fluorescence and confocal fluorescence microscopic examination.

3. Results

3.1. Haptide uptake by cells

When cells were exposed to ^{FITC}Cβ and ^{FITC}preCγ, the Haptides accumulated in the cells. Fig. 1 shows the Haptide distribution, as monitored by computerized confocal microscope through the mid-section of the cells. The fluorescent signals revealed that, within 1 h of incubation, one could observe significant accumulation of either Haptide throughout the cell cytoplasm. It was previously demonstrated that addition of other non-active FITC labeled peptide resulted in no significant accumulation of fluorescence within the cells, relative to background [11].

3.2. Haptides aggregation and interaction with liposomes

When Haptides were dissolved in PBS about 10% of them appeared as nano-aggregates, as indicated by OD_{280} readings before and after removing the particles by either filtration (0.22 μ filter) or high-speed centrifugation (data not shown). When these Haptide solutions were examined by atomic force microscopy, the presence of nanoparticles, 80–300 nm in diameter (Fig. 2A) could be monitored. This was confirmed by size measurement based on dynamic light scattering, which showed an average hydrodynamic diameter of 145 nm (Fig. 2B, right-most column). The addition of liposomes to the Haptide nanoparticles dispersion with a Doxil/Haptide weight ratio of more than 10^{-3} disaggregated the Haptide nanoparticles, and possibly solubilized and adsorbed them onto the lip-

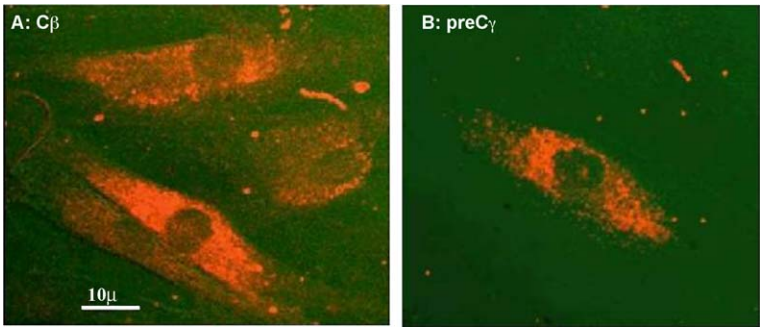

Fig. 1. Confocal fluorescence microscopy of ^{FITC}Cβ and preCγ (~ 40 μM) uptake by HF following 1-h incubation at 37 °C. The images show the distribution of ^{FITC}Haptide within the cytoplasm with very limited penetration into the nucleus.

osomes that are about 80 nm in diameter (see Doxil alone, Fig. 2B, left-most column) leaving no larger nanoparticles of aggregated Haptides.

In order to confirm the nature of the interactions between the liposomes and Haptide, the zeta potential of liposome-Haptide mixture solutions was measured. The zeta potential of 0.1 mg/ml Doxil liposome was −12.4 mV and nanoparticles from pure 1 mg/ml Haptide (preCγ) had the value of +2.2 mV (Fig. 3). When the liposomes were mixed with 0.1 mg/ml of preCγ in a liposome/Haptide weight ratio to 1:1, the zeta potential of the liposomes was shifted to a value of −7.9 mV. This zeta potential could be further shifted up to a value of −4.6 mV by adding preCγ to reach a liposome/Haptide weight ratio of 1:10. In both mixtures, there was no indication for the existence of more than one charged particle population. These results indicate that the Haptides aggregates were dissolved by the liposomes and merged into them to form Haptide–liposome complexes.

3.3. Haptide-mediated liposome uptake by cells

The uptake of rhodamine-liposomes into the cytoplasm of HF and BAEC was enhanced by pre-mixing the liposomes with Haptides. When ~ 40 μM of either Cβ or preCγ was mixed with the fluorescent

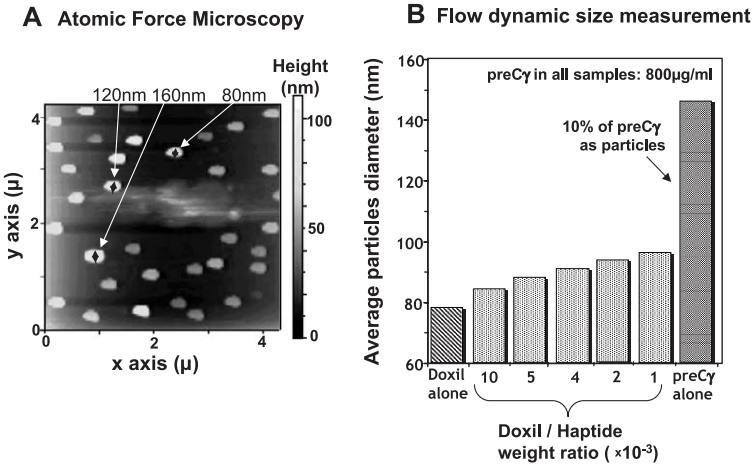

Fig. 2. (A) Atomic force microscopy of Haptide nanoparticles formed in PBS. (B) Hydrodynamic diameter measurements of Haptide nanoparticles and liposomes at different Doxil/Haptide weight *ratios*. At higher liposomal concentrations, the liposomes *disaggregated* the larger Haptides nanoparticles, leaving only the smaller ~ 80 liposomal particles (leftmost column).

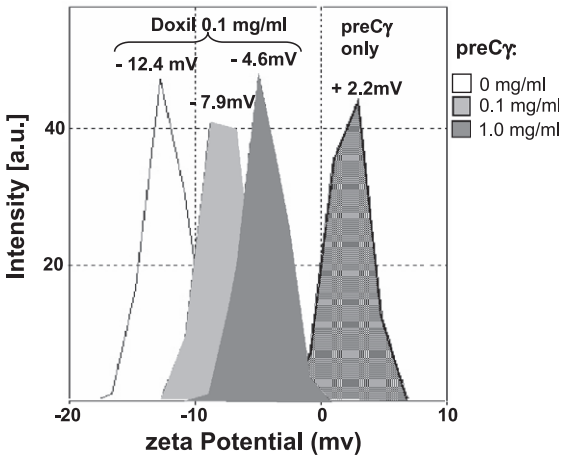

Fig. 3. Zeta-potential of the surface of Doxil liposomes without and with Haptide preCγ: The Doxil liposomes are highly negatively charged, while the Haptide is positively charged. At an increasing ratio of Haptide to Doxil, the charge of the liposomes became progressively more positive, indicating the integration of the Haptides into the external liposomal surface.

rhodamine-liposomes (final concentration ~ 5 μg/ml), enhanced uptake of fluorescent liposomes into HF was seen by confocal microscopy (Fig. 4). At these concentrations, the CαE exerted a lower effect and a control inactive peptide Cα exerted no effect on the liposome uptake by cells.

A dose response of Cβ-induced enhancement of rhodamine liposome uptake into HF is shown in Fig. 5. One hour after exposure of the cells to liposomes only (final concentration ~ 5 μg/ml), very little liposomal adsorption was observed in a section through the cells (by confocal microscopy). However, when the liposomes were pre-mixed with Haptides, high liposome uptake by the cells was observed, reaching a plateau at Cβ concentration above 33 μM. It was apparent that after 1 h, the liposomes became distributed mostly within the cell cytoplasm and the nuclei were less stained by the fluorescence dye. Due to the cytotoxicity of the liposomal formulations at this concentration, the measurement of the fluorescence was not extended for longer time intervals.

Doxil uptake into cells was also significantly enhanced by Haptides, as clearly shown by confocal microscopy. When these liposomes with a net concentration of 5 μg/ml rhodamine were pre-mixed with ~ 40 μM Haptides, the uptake of Doxil by the cells was significantly augmented. Both Cβ and preCγ, and to a lesser degree CαE, increased the entry of Doxil into the cytoplasm of BAEC. By contrast, inactive peptides exerted only a marginal baseline liposome uptake (Fig. 6). Haptide-augmented uptake of liposomes by cells could also be observed, though at a slower rate, at 4 °C (not shown).

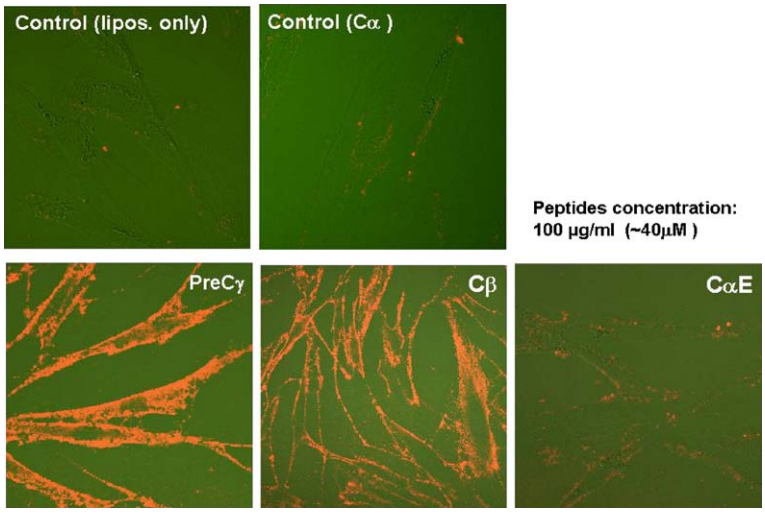

Fig. 4. Confocal fluorescence microscopy of Haptide-induced augmentation of the uptake of rhodamine liposomes by HF. Only a small fraction of the untreated fluorescent liposomes or those mixed with the control peptide Cα penetrated within 1 h into the cells (only the rhodamine was fluorescent). By contrast, Cβ and preCγ significantly augmented the uptake of rhodamine liposomes but CαE exerted lower effect.

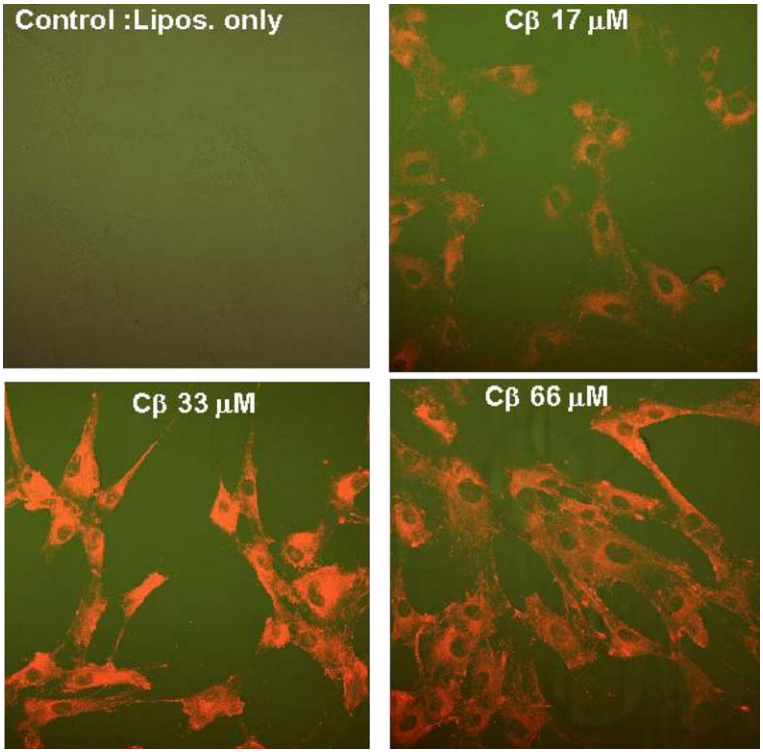

Fig. 5. Uptake of fluorescent rhodamine liposome by HF at different Haptide doses. When the liposomes (5 μg/ml) were premixed with Haptide at concentrations above 33 μM, a maximal liposomal uptake was observed (by confocal microscopy).

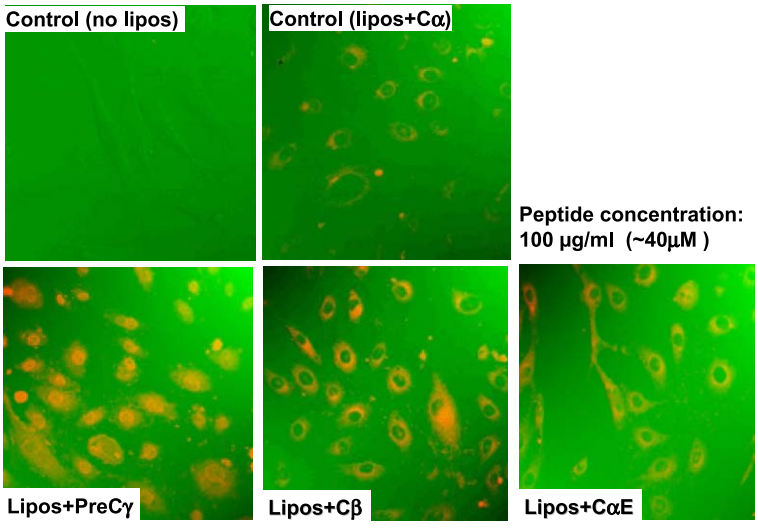

Fig. 6. Haptide induced augmentation of the uptake of fluorescent Doxil liposomes by BAEC (by confocal microscopy). Only the DOX in the Doxil was fluorescent. Premixing the liposomes (final concentration ~ 5 μg/ml) with Cβ or preCγ (~ 40 μM) resulted in higher liposome uptake whereas CαE had lesser effect and the control peptide Cα, exerted negligible effect. The transduced liposomes appeared to be distributed within the cell cytoplasm.

To confirm the Haptide-enhanced transduction of liposomes into cells, the cytotoxicity of Doxil on cells was monitored. HF and BAEC were incubated with Doxil at various concentrations and their survival was monitored by a colorimetric MTS assay. In a control experiment (Fig. 7A), when HF were exposed to free DOX, a sigmoidal dose response was recorded with high sensitivity to the drug, as measured in the median response, between maximum to minimal survival (LD_{50} of about 2×10^{-7} M). The addition of either 40 μM Cβ or preCγ did not change the profile of the free DOX toxicity (Fig. 7A). Doxil at equivalent drug concentrations was much less toxic ($LD_{50} \sim 2 \times 10^{-5}$ M). However, pre-mixing ~ 40 μM (final concentration) Cβ or preCγ with Doxil increased the drug toxicity by about one order of magnitude ($LD_{50} \sim 10^{-6}$ M), to achieve a toxic effect recorded for the free drug (Fig. 7B).

In order to rule out the possibility that the Haptides interaction with liposomes released free DOX from the liposomes and so enhanced the drug toxicity, the leakage of DOX from Doxil liposomes was tested with and without pre-mixing with Haptides. In either case, no significant drug leakage in control and in Haptide-exposed Doxil was recorded ($0.07 \pm 0.05\%$ and $0.12 \pm 0.08\%$, respectively). The results indicated that Haptides did not affect liposomal stability and did not cause any significant drug leakage from the liposomes. Therefore, Haptide-enhanced cytotoxicity could only occur via the enhanced uptake of the Doxil–Haptide complex.

4. Discussion

The Haptides, which consist of 19–21-amino acid homologues of the C-termini of the Cβ, the preCγ and the CαE, have been described as being haptotactic to different cell types [11]. In free form, Haptides could be rapidly internalized through cell membranes and become distributed within the cytoplasm. Haptides were also shown to be non-toxic to different normal cell types tested [11]. In addition, it was demonstrated that solubilized Haptides could accelerate fibrin polymerization and modulate fibrin turbidity [12].

Soluble Haptides tended to form multimers in solution, as originally determined by gel filtration [11]. About 10% of the Haptides appeared to aggregate as larger nanoparticles. Atomic force microscopy and dynamic light scattering particle measurements indicated that the Haptide nanoparticles exhibited an average diameter of ~ 145 nm (Fig. 2). The Haptides became adsorbed by liposomes. Particle size measurements confirmed that the liposomes could even disaggregate the larger Haptide nanoparticles, leaving only the liposomal-Haptide complexes (Fig. 2B). The interaction of the positively charged Haptides with liposomes reduced their net negative surface charge, indicating

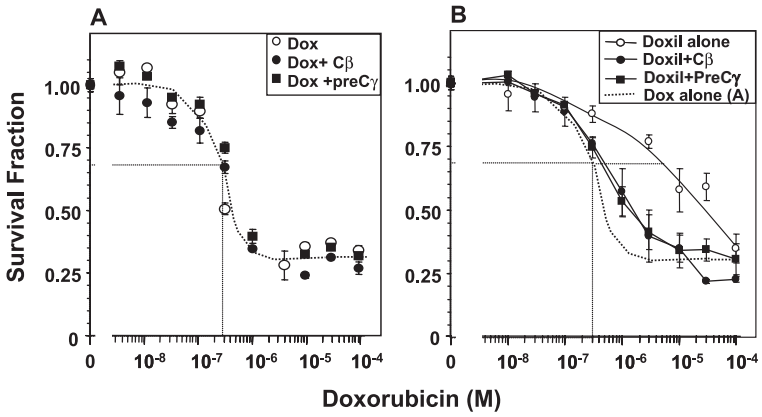

Fig. 7. Effect of Haptides on DOX and Doxil toxicity in HF. The LD_{50} was evaluated as the dose needed to reduce survival by half (range of survival in the sigmoid curve was calculated between 100% and the value at the lower plateau). (A) The toxicity of DOX was not affected by premixing the drug with ~ 40 μM Haptides. (B) By contrast, with pre-mixing the liposomal Doxil with the same concentration of either Cβ or preCγ significantly reduced the LD_{50} by more than one order of magnitude.

that the Haptides became distributed on the external membrane of the liposomal lipid bilayer (Fig. 3).

Fluorescent microscopy revealed that soluble FITC-Haptides tended to become rapidly internalized within the cell cytoplasm (Fig. 1). Moreover, the uptake of Doxil, as well as rhodamine-containing liposomes, by either HF or BAEC (Figs. 4–6 was much enhanced by premixing the fluorescent liposomes with Cβ or preCγ. CαE, which has a lower degree of homology with Cβ and which was previously shown to elicit a lower haptotactic response from different cell types [11], also was observed to be less potent in augmenting liposome uptake (Fig. 4).

Exposure of cells to free DOX is expected to result in accumulated of the drug in the nucleus. The higher fluorescent signal within the cytoplasm soon after exposure to Doxil + Haptide with much lower staining of the nucleus (Fig. 6) suggests that the drug was initially preserved mostly in a liposomal form. The eventual penetration of DOX from the Haptide-aided Doxil internalization into the nucleus was made evident along a few days follow-up by the elevated cytotoxicity following the exposure to this mixture (Fig. 7).

The amino acid sequences of Haptides (Table 1) are comprised of both hydrophobic and cationic residues (i.e. net 4–5 positively charged amino acids per 19–21mer). The liposomes tested here were composed of negatively charged hydrophobic residues, whereas the cells had polarized membranes negatively charged inward. Thus, the Haptides could be attracted to the liposomes (Fig. 2B), as well as to the cell membranes (Fig. 1) by a combination of hydrophobic and ionic interactions. The current study suggests that the Haptides augmented liposome insertion through the cell membrane into the cell cytoplasm (Figs. 4–6). As previously shown, Haptide uptake could also occur at 4 °C [11]. Thus, it does not appear that the liposome transduction was a metabolically driven process. Rather, the data presented suggest that by virtue of their attachment and their penetration through cell membranes, as well as their incorporation into the external liposomal lipid bilayer, Haptides Cβ and pre-Cγ augmented direct liposome insertion through the cell membrane into the cell cytoplasm without recourse to any specific membrane receptors. Interestingly, mixing Haptides with free DOX alone did not augment its toxicity, whereas Haptides did augment the uptake and toxicity of the liposomal formulation Doxil. A model for the liposomes transduction, based on these observations, is given in Fig. 8.

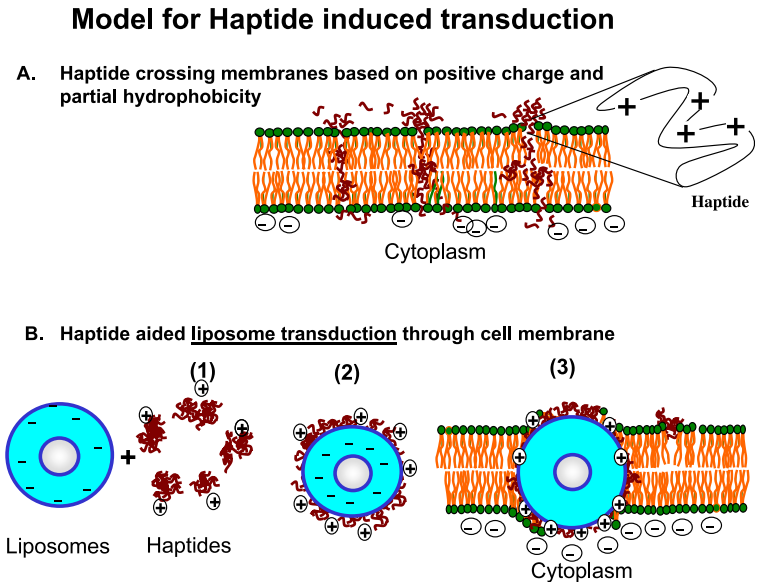

Fig. 8. A model for Haptide-mediated enhancement of liposomes uptake into cells. (A) Haptides become incorporated into the cell membrane and tranverse into the cells. (B) The incorporation of the Haptides into the liposomal lipid bilayer and the cathionic properties of the Haptides coated liposomes permit the entire complex to become transduced directly through the cell membrane.

Other peptides that augment drug penetration through cellular membranes have been recently described. Thus, an 18mer homologue of a viral gp41 and 16–20 mer peptides from antennapedia have been reported to translocate through cells [21–29]. The antineoplastic DOX coupled covalently to small peptide vectors L-SynB1 (18 mer), increased the amount of DOX transported into brain parenchyma 20-fold. Intravenous administration of such vectorized DOX into mice led to a significant increase in brain DOX concentrations for the first 30 min of post-administration, compared to the injection of free DOX [30,31]. These results suggested that the transport of vectorized DOX occurred via an adsorptive-mediated endocytosis mechanism [31].

Results obtained with cell penetration peptides (PPC) were recently reviewed [32]. For example, HIV-derived TAT peptide significantly enhanced intracellular liposome delivery into cells in a manner not affected by temperature or metabolic inhibitors [33,34]. However, the application of TAT peptides to improve tumor control in an animal model seemed to fail [35]. A mechanism was proposed for non-receptor and non-energy dependent trans-membrane transduction of molecules as well as particles with the aid of TAT peptides [35,36]. It was suggested that this type of transport might derive from the high arginine content in those peptides [36,37]. In the case of Haptides, their composition is more varied and their activity cannot be ascribed to high arginine content. Notwithstanding, it is proposed that their transduction into cells also occurs via a non-receptor mediated mechanism, as presented in Fig. 8.

Another advantage of the Haptides over TAT peptides is that they are homologous to the normal peptidic sequences of human fibrinogen which is most abundant in the circulation and does not normally induce any immunological response. By contrast, the family of cell penetrating molecules that include TAT are foreign antigens that can evoke an immunological response on the one hand and induce cell transformation on the other [38]. The TAT peptides were also shown to have some adverse effect with their interaction with growth factors, causing inhibition of angiogenesis and induction of apoptosis [39]. Therefore, the Haptides may be viewed as safer transduction agents for the use for in-vivo applications. Nevertheless, the use of these finding for clinical applications is still not straightforward. A possible major disadvantage of the use of Haptides for targeted delivery of drugs in-vivo is the apparent lack of evidence of cell specificity. This was demonstrated by seemingly equal enhanced transduction into HF and BAEC. Future combination of Haptides treated liposomes and targeting mechanisms, as demonstrated for the use of stealth liposomes [17], may be needed.

To conclude, this study showed that the new cell transduction Haptides (Cβ and pre-Cγ, and in a lesser degree CαE), were capable of binding to liposomes and were particularly potent in terms of augmenting the delivery of rhodamine or Doxil liposomes into normal cells in culture. Based on the data presented here, it is suggested that the Haptides Cβ or pre-Cγ could be employed to augment the cell uptake of drugs that are formulated in the form of liposomes or nanoparticles.

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