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Murine plasma fibronectin depletion after intravenous injection of liposomes

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Summary

Liposomes (0.5 μm diameter), of a variety of lipid compositions, are capable of transiently decreasing murine plasma fibronectin levels when administered intravenously. Onset of fibronectin depletion was rapid; fibronectin levels returned to control levels within 8 h. There was no detectable dose–response effect to lipid doses in the range of 1.2–15.3 μmol/animal of plasma fibronectin. Small-diameter liposomes (0.05 μm) have no effect upon plasma fibronectin levels, which may be a factor contributing to their relatively slow removal from the circulation. The degree of plasma fibronectin depletion correlated well with concomitant accumulation of liposomes in the liver. The results may be explained by postulating that an association of fibronectin with the liposomal surface occurs (1 molecule of fibronectin per 70 molecules of lipid was detected on circulating liposomes). This surface-associated fibronectin may opsonize the liposomes for uptake by the reticuloendothelial system, or fibronectin depletion may be fortuitous resulting from liposome uptake by reticuloendothelial cells.

Introduction

Liposomes (lipid vesicles enclosing an aqueous space) are potential drug vehicles (Yatvin and Lelkes, 1982). Intravenous administration of liposomes ultimately results in the localization of the majority of the dose in the organs of the reticuloendothelial system (RES), especially liver and spleen (Abra and Hunt, 1981), a pattern of distribution similar to that obtained with a variety of colloids and emulsions (Saba, 1970).

In vitro a large number of plasma components have been demonstrated to bind to the liposomal surface, including: serum albumin, immunoglobulin, fibronectin and various apolipoproteins (Guo et al. 1980; Juliano and Lin, 1980; Rossi and Wallace, 1983). Enhanced receptor-mediated endocytosis of fibronectin-coated liposomes has been demonstrated in macrophage culture (Hsu and Juliano, 1982). Determination of the in vivo fate of liposomes by surface-bound plasma protein has been suggested (Scherphof et al., 1981); however, no direct evidence has been provided yet.

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It is therefore of interest to determine the effects of liposome doses on plasma components in vivo. We have concentrated on plasma fibronectin levels in view of their known function to opsonize blood-borne foreign particles or biological debris followed by removal of these particles by macrophages of the RES (Kaplan, 1980). Decreased plasma fibronectin levels have been found under experimental and pathological conditions (trauma, cancer, coagulation, hepatic failure) (Kaplan and Saba, 1978; Saba et al., 1980; Saba and Jaffe, 1980; Gonzales-Calvin et al., 1982).

To this end we have investigated the in vivo effect of liposome doses on mouse plasma fibronectin levels as a function of liposome size, composition, dose and time postinjection.

Materials and Methods

Materials

Egg yolk phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham, AL). Sodium dipalmitoyl phosphatidate, cholesterol (Ch), α-tocopherol (αT), stearylamine (SA), inulin (5000 Da) and aprotinin (protease inhibitor) were obtained from Sigma (St. Louis, MO). Inulin, carboxyl[^{14}C], spec. act. 19.6 mCi/g, was supplied by New England Nuclear (Boston, MA). Dipalmitoyl phosphatidic acid (DPPA) was prepared by chloroform extraction of the sodium phosphatides in 0.4 N HCl/20% methanol aqueous solution. Phosphate-buffered saline (PBS) contained: 92 mM sodium chloride, 43 mM anhydrous dibasic sodium phosphate, 11 mM monobasic sodium phosphate monohydrate and was routinely filtered through 0.22-μm pore size Millipore filters (Millipore Corp., Bedford, MA). PCS scintillation fluid (Amersham, Arlington Heights, IL) was used.

Preparation of liposomes

Multilamellar liposomes were prepared by the extrusion method as described previously (Abra and Hunt, 1981) and had a mean diameter of 0.5 μm. Small unilamellar liposomes were prepared by sonication of multilamellar liposomes in a bath-type sonicator (Laboratory Supplies, Hicksville, NY) for ca. 1 h until the dispersion appeared optically translucent. These liposomes had a mean diameter of 0.05 μm. Liposomes were labelled by encapsulating 2 μCi/dose [^{14}C]inulin as aqueous space marker (Abra and Hunt, 1981). The lipid mixtures employed are given in Table 1.

TABLE 1

Liposomal lipid compositions

Designation	Lipid composition	Molar ratio	Charge
A	PS/Ch	1:1	(−)
B	PC/Ch/αT	5:5:0.1	neutral
C	PC/DPPA/Ch/αT	4:1:5:0.1	(−)
D	PC/SA/Ch/αT	4:1:5:0.1	(+)
E	PC/DPPA/αT	8:2:0.1	(−)

Quantitative assay of plasma fibronectin

Groups of 3–5 mice (20–25 g) (ICR from Simonsen Labs., Gilroy, CA) received i.v. doses in the range of 1.2–15.3 μmol total lipid per animal in 0.2 ml PBS, controls received 0.2 ml PBS only. The kinetics of plasma fibronectin depletion were followed for 1 h, at 20 min intervals, and for one composition (C), additional measurements were made at 8 and 24 h. Blood samples (0.6 ml) were taken from the jugular vein whilst the animals were under ether anaesthesia and were added to solid citrate to give a final chelator concentration of 20 mM. Plasma was prepared by centrifuging these samples at 4° C for 10 min at 500 *g*. Plasma was diluted to 10% with Tris/tricine buffer, pH 8.6 (containing 0.08 M Tris-base, 0.024 M tricine, 0.02 M citrate and 40 kallicrein inhibitory units/ml, corresponding to 2.6 μg aprotinin/ml). Samples were stored on dry ice and plasma fibronectin assayed by electroimmunoassay ("rocket" electroimmunophoresis) as described in detail by Blumenstock et al. (1977).

In vivo disposition studies

Liposome doses used in the depletion studies were labelled with [^{14}C]inulin (except composition C), thus in vivo disposition of the encapsulated marker could be followed simultaneously. Blood samples (0.2 ml) were bleached overnight in scintillation vials by adding 0.4 ml 0.1 N KOH and

0.4 ml *t*-butyl hydroperoxide. Scintillation fluid was then added and samples counted on a Beckman LS 7800 scintillation counter. Liver samples (0.3–0.5 g) were assayed for total radioactivity by combustion as described previously (Abra and Hunt, 1981). Blood and organ levels were calculated as percent of encapsulated dose and expressed either per 2 ml blood (approximately the blood volume of a 23-g mouse) or per organ.

Recovery of liposome-bound plasma fibronectin

Two 20-min plasma samples from mice which had received a high liposome dose (9.1 μmol total lipid/animal; composition C) were pooled, and a plasma sample from control mice obtained. Liposomes were separated from plasma proteins by centrifugation of both samples on a Ficoll gradient (Fraley et al., 1980). Plasma fibronectin concentration and ^{14}C-radioactivity were assayed in experimental and control fractions and control values subtracted as background. The specific activity of administered liposomes was used to calculate the total lipid present in the sample. The following experimental data and literature values were used to calculate the number of fibronectin molecules associated per liposome: an average liposome radius of 0.23 μm (Abra and Hunt, 1981), 5.6×10^8 liposomes per 0.1 μmol total lipid (Abra and Hunt, 1981), 7.4×10^{-19} m^2 surface area per lipid head group (Huang and Mason, 1978) and a molecular weight of 450000 Da for plasma fibronectin (Saba and Jaffe, 1980).

Results

Cholesterol-containing liposomes (A–D) were all capable of depleting plasma fibronectin (Table 2). The largest depletion was caused by the electrostatically neutral compositions B, the smallest by the strongly negatively charged composition A. Composition C and D (negative and positive, respectively) depleted plasma fibronectin to intermediate levels. Composition E (a zero cholesterol version of C) had only a slight effect.

Maximum depletion was reached at the earliest time-point monitored in all cases (20 min) and approximately maintained over at least 1 h (Table 2). When 15.3 μmol total lipid (composition C) were administered to animals, plasma fibronectin values had returned to control levels (337 ± 29 μg/ml) after 8 h (301 ± 30 μg/ml) and remained constant for up to 24 h (326 ± 31 μg/ml).

TABLE 2

Plasma fibronectin depletion as a function of liposome composition

Values are plasma fibronectin levels, μg/ml plasma (mean ± S.D.), $n = 3$.

Lipid composition (dose; μmol total lipid/animal)	Time (min) [a]		
	20	40	60
Control	358 ± 27	n.d.	n.d.
A (1.6)	289 ± 54 *	286 ± 60 **	276 ± 40 **
B (2.2)	191 ± 29 ****	156 ± 38 ****	171 ± 15 ****
C (3.9)	n.d.	n.d.	215 ± 29 ****
D (1.7)	188 ± 34 ****	260 ± 57 ****	235 ± 42 ***
E (2.3)	306 ± 52 *	304 ± 28 *	301 ± 57 *

[a] Time post-injection of liposomes.

Student's *t*-test vs. control: * $P > 0.1$, ** $P = 0.1$–0.05, *** $P = 0.025$–0.01, **** $P < 0.01$. n.d. = not determined.

Plasma fibronectin values for control animals injected with 0.2 ml PBS did not vary significantly, and in fact were not significantly different from the plasma fibronectin values of untreated mice.

Recovery of total radioactivity from blood and liver 1 h postinjection is shown in Table 3. The highest liver accumulation was found for the neutral composition B, followed by the positively charged composition D and the negatively charged composition A (composition C was not investigated in this series). The lowest level of liver accumulation was found for the cholesterol-free composition E. Blood levels were generally low ranging from 1.5 to 8.5% of the injected dose.

Plasma fibronectin depletion, and blood and liver values for large (0.5 μm diameter) and small (0.05 μm) liposomes of composition A are shown in Table 4. Large liposomes lowered plasma fibronectin levels; however, no detectable depletion was seen when small liposomes of identical

TABLE 3

One hour blood levels and liver association of liposome compositions A, B, D, and E as assayed using the aqueous space marker ^{14}C-inulin

Values are means (S.D.) for 3 animals. One hour plasma fibronectin depletion values from Table 2 are added for comparison

Lipid composition (dose; μmol lipid/animal)	% Encapsulated dose		Plasma fibronectin depletion (μg/ml plasma)
	Blood (per 2 ml)	Liver (per organ)	
A (1.6)	1.5 (0.4)	51 (3)	276 (40)
B (2.2)	8.5 (0.6)	91 (8)	171 (11)
D (1.7)	4.6 (1.0)	76 (5)	235 (42)
E (2.3)	2.3 (0.4)	10 (1)	301 (57)

composition were administered. Blood levels of the small-size version were much higher than those for the large-size version. Liver accumulation was lower for small liposomes.

When increasing doses of 1.2–15.3 μmol lipid per animal (composition C) were injected, plasma fibronectin levels were significantly reduced to 235 ± 34 μg/ml, 215 ± 29 μg/ml, and 249 ± 35 μg/ml for doses of 1.2, 3.9 and 15.3 μmol lipid respectively (control value: 337 ± 29 μg/ml). However, there was no obvious correlation of increasing lipid dose and extent of depletion.

When liposomes were separated from plasma by Ficoll gradient centrifugation, 0.1 μmol total lipid and 5.6 μg plasma fibronectin were recovered in the liposome-containing band. Using the data and assumptions described under Materials and Methods, a rough calculation yielded ca. 13 000 molecules of plasma fibronectin associated with 900 000 molecules total lipid (number of lipid molecules per liposome surface), or 1 plasma fibronectin associated with 70 molecules of lipid.

TABLE 4

Plasma fibronectin depletion and ^{14}C blood and liver levels 1 h after administration of large and small liposomes of composition A

All values are means (S.D.) for 3–5 animals.

Liposome size (dose; μmol lipid/animal)	% Encapsulated dose		Plasma fibronectin depletion (μg/ml plasma)
	Blood (per 2 ml)	Liver (per organ)	
Large; 0.5 μm (1.6)	1.5 (0.4)	51 (3)	276 (40) **
Small; 0.05 μm (0.42)	29 (3)	39 (2)	384 (53) *
Control (no liposomes)	–	–	358 (57)

Student's *t*-test vs. control: * $P > 0.1$; ** $P = 0.1$–0.05

Discussion

Our results demonstrate that a variety of liposome compositions (negatively, positively charged and neutral) are capable of depleting plasma fibronectin levels in a reversible fashion when administered intravenously. The magnitude of plasma fibronectin depletion appears to be related to all 3 variables investigated in this study: the stability (presence or absence of cholesterol), charge density, and liposome size.

The fraction of cholesterol present in the liposomal membrane determines in vivo stability. The approximate stabilities of the liposomes used in this study are expected to be: composition A–D equally stable, and much more stable than composition E (Kirby et al., 1980; Hunt 1982). The smallest depletion was found for composition E, and this vesicle composition is known to disintegrate upon interaction with plasma (Juliano and Lin, 1980); presumably, the resultant vesicle fragments are no longer a suitable substrate for fibronectin interaction.

Since compositions A–D have approximately the same expected in vivo stabilities, a similar degree of depletion would be expected. However, the largest plasma fibronectin depletion is observed for composition B liposomes (neutral). An intermediate depletion is seen for composition C (negatively charged), and D (positively charged), followed by composition A (strongly negatively charged). The foregoing differences in observed fibronectin depletion may therefore be a result of differences in charge densities of the respective compositions.

Liver accumulation correlates well with the degree of depletion, i.e. composition B which caused

the strongest depletion was found sequestered by the liver to 91% after 1 h, whereas only 51% of composition A and 76% of composition D were associated with the liver at this time-point. The low liver association of encapsulated marker from composition E cannot be taken into account in this correlation since it is a function of disintegration and release of the marker into the bloodstream. (Liposomal lipid of composition E may be sequestered by the liver.)

Depletion is also dependent upon liposome size, large vesicles (composition A) giving rise to depletion whilst small vesicles of the same composition did not. Differences in lipid packing density arising from differences in vesicle radius of curvature may be affecting the ease of fibronectin–lipid interaction here.

Liposome blood concentrations cannot be correlated with plasma fibronectin depletion, since they are the net result of interaction of the dose with all organs in the body. However, blood concentrations of small liposomes are much higher compared to those of large liposomes of the same composition, and concurrently, no plasma fibronectin depletion was detectable for small liposomes.

Surprisingly, we were not able to detect any dose–response relationship between plasma fibronectin depletion and amount of liposomal lipid administered, although one might be expected from the literature (Saba, 1970). Two possible explanations are offered. Fibronectin depletion may occur rapidly in an initially dose-dependent manner, followed by a restoration of fibronectin levels to those seen by 20 min, as a result of mobilization of plasma fibronectin pools. Alternatively, plasma interaction with liposomes may occur via an intermediate blood component whose pools are rapidly exhausted at our lowest liposome dose. Whether either of these hypotheses is correct awaits further experimental investigation.

Despite the good correlation of plasma fibronectin depletion and liver accumulation of stable liposome compositions, it should be pointed out that other factors such as non-specific binding of liposomes to the vascular cell lining, binding of other plasma components (the overall binding 'pattern"), and, in the case of small vesicles, reduced mechanical filtration by the liver and a different flow rate may also contribute to the degree and site of liposome removal from the bloodstream.

The fact that fibronectin binding has been linked to the opsonization of biological and foreign particles for macrophage engulfment (Saba, 1970; Hsu and Juliano, 1982), together with our findings, lead us to postulate that plasma fibronectin may associate with the liposome surface and be removed from the circulation as the liposomes are sequestered by cells of the RES. Our results do not permit us to distinguish between non-specific fibronectin association with liposomes concomitant with liposome uptake by the reticuloendothelial system, and fibronectin binding leading to opsonization of the liposomes followed by uptake. The latter remains an intriguing possibility.

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