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Letter to the Editor

Milk fat globule epidermal growth factor 8 (MFG-E8) binds to oxidized phosphatidylserine: implications for macrophage clearance of apoptotic cells

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Dear Editor,

Milk fat globule epidermal growth factor 8 (MFG-E8) (also known as lactadherin) is a secreted glycoprotein, and consists of two EGF-like domains, a mucin-like domain, and tandem discoidin-like domains (C-domains); in addition, an RGD motif that binds to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins is present in the second EGF domain.[1] The C2 domain of MFG-E8 shares homology with the lipid-binding domains of blood coagulation factors VIII and V that have been shown to mediate Ca^{2+}-independent, high affinity ($K_d \sim 2$ nM) binding to phosphatidylserine (PS).[2] Similarly, MFG-E8 is known to bind to PS via its C2 domain.[3] Apoptotic cells express PS on the cell surface, and recent studies have suggested that MFG-E8 may act as a 'bridging' molecule that links apoptotic cells to phagocytes via its C1C2 domain and integrin-binding motif, respectively.[4] However, MFG-E8 binding to phospholipids was not examined in detail. We have previously documented the occurrence of cell surface externalization of oxidized PS (PS-OX) during apoptosis, and have provided evidence that both species of PS (oxidized and nonoxidized) function as recognition signals for macrophages.[5,6] We now show that MFG-E8 preferentially interacts with PS-OX and, to a lesser degree, with non-oxidized PS and PC (phosphatidylcholine).

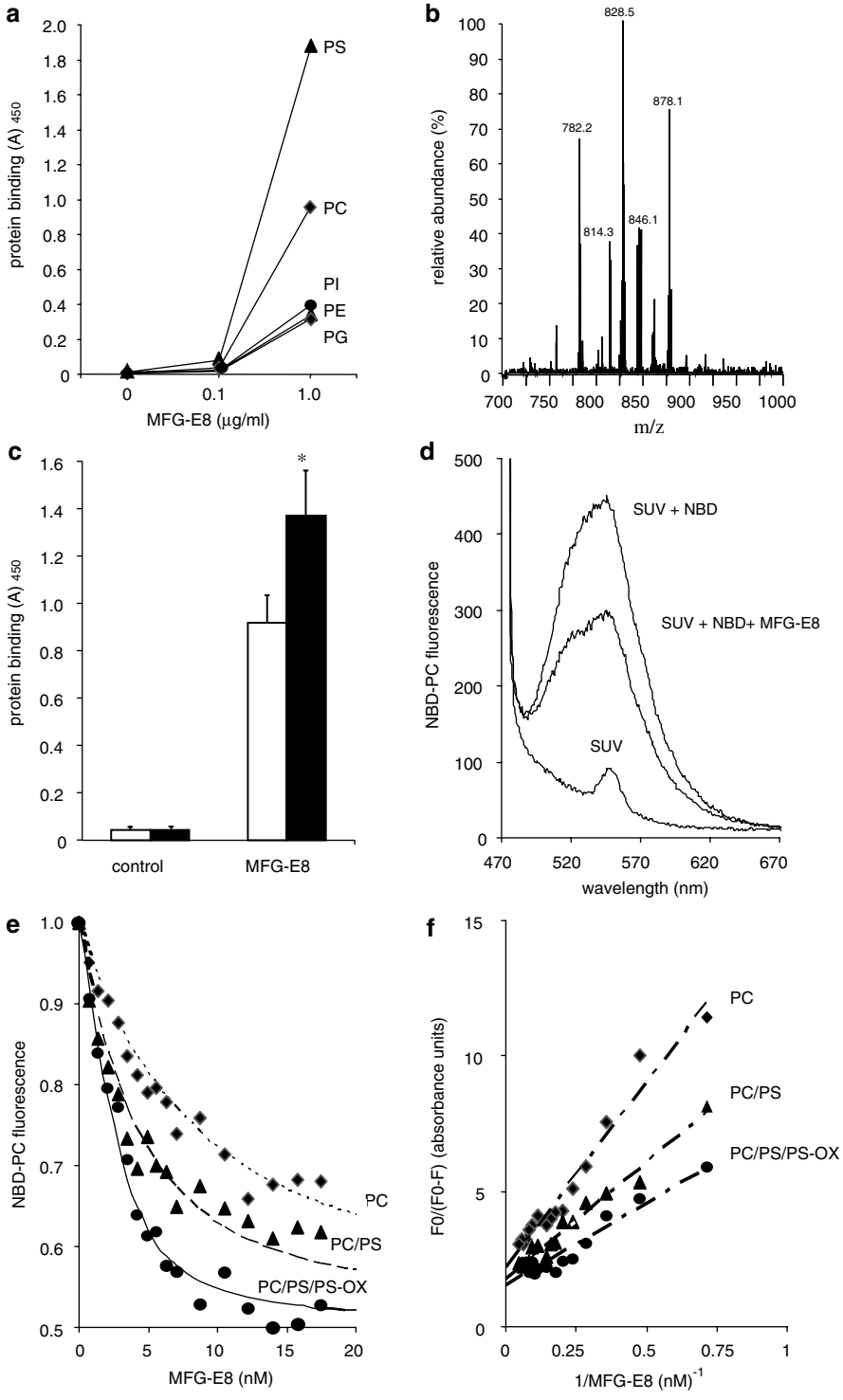
To determine the pattern of MFG-E8 binding to phospholipids, ELISA plates were coated with various phospholipids and incubated with recombinant, FLAG-tagged MFG-E8. As shown in Figure 1a, MFG-E8 bound preferentially to PS (i.e. two-fold more binding as compared with PC). Binding to other phospholipids (phosphatidylinositol, PI; phosphatidylethanolamine, PE; phosphatidylglycerol, PG) was negligible, even at higher concentrations of FLAG-tagged protein. We then asked whether MFG-E8 binds to immobilized PS-OX. To this end, PS was oxidized prior to addition to the microtitre plate using Fe^{2+}/*tert*-butylhydroperoxide (THP); approximately 60% of the PS molecules were determined to be oxidized by this method (Figure 1b). Quantification of the amount of MFG-E8 bound to the wells revealed a preferential binding of MFG-E8 to PS-OX as compared to nonoxidized PS (Figure 1c). Although the degree of binding of MFG-E8 to individual oxidation products of PS was not determined, our mass spectroscopic analyses revealed that PS hydroperoxides were dominating among the oxidation products (Figure 1b).

To validate our observations, binding of MFG-E8 to small unilamellar liposomes (SUVs) was determined by quenching of fluorescence of NBD-PC incorporated into the liposomes. NBD fluorescence is extremely sensitive to alterations in the local environment, and changes of NBD-PC fluorescence caused by lipid segregation or interactions with amino-acid groups can thus be detected upon protein attachment to the SUVs. As shown in Figure 1d, there was a considerable decay in NBD-PC fluorescence upon addition of MFG-E8 to PS-containing SUVs. Quenching was insensitive to the presence of Ca^{2+} (data not shown). Moreover, quenching of fluorescence was dependent on the phospholipid composition of the SUVs (PC > PC/PS > PC/PS-OX), as well as on the amount of MFG-E8 (Figure 1e). Importantly, the quenching curves were nonlinear and saturable, and therefore suggestive of binding curves. Taken together, these data show that MFG-E8 interaction with liposomes containing PS-OX is more pronounced than with the PS-expressing vehicles.

Quenching of NBD-PC fluorescence in SUVs could depend on dynamic (collisional) or static (complex formation) mechanisms. Based on the assumption of dynamic quenching with partially available fluorophore, our quenching data can be described by the Stern–Volmer law as $F_0/(F_0-F) = 1/f_a + 1/(f_a K[M_t])$ where K is a dynamic quenching constant and f_a is the fraction of total fluorophore accessible to the quencher (MFG-E8). As seen in Figure 1f, dependence of $F_0/(F_0-F)$ on $1/[M_t]$ (i.e. the inverse protein concentration) is essentially linear, thus making it possible to derive values for f_a and K. Our analyses reveal that the f_a values for the different liposomes were not significantly different and on an average were equal to 0.53. This indicates that the fraction of phospholipids located in the outer leaflet of the SUV and involved in interactions with MFG-E8 is slightly higher than the corresponding fraction in the inner leaflet. The quenching constants were calculated to be in the range of 3×10^9–$7 \times 10^9\,M^{-1}$. The lifetime (τ) of exited NBD-labeled phospholipids in membrane bilayers is approximately 4–6 ns[7] and the established relationship ($K = K_q \times \tau$) between K, τ and quenching rate (K_q) thus yields a K_q in the range of 10^{17}–$10^{18}\,M^{-1}\,s^{-1}$. These values greatly exceed the diffusion rates ($< 10^{10}\,M^{-1}\,s^{-1}$) for small molecules, suggesting a static nature of NBD-PC quenching;

in other words, our results are likely to reflect direct binding of MFG-E8 to PC, PS, and PS-OX, respectively.

We further analyzed our data based on the noncooperative model of protein binding to liposomes of different composition.[8] Several assumptions were made for these calculations: all phospholipids including the fluorescent probe are distributed equally between the inner and outer bilayer and are distributed homogenously within the same bilayer, and the number of binding sites on the protein (n) is independent of the type of phospholipid. In addition, we did not discriminate between the dissociation constant (K_d) of particular classes of phospholipids, but considered K_d values for different types of



liposomes (K_{dPC}, $K_{dPC/PS}$, and $K_{dPC/PS/PS\text{-}OX}$). The most satisfying fitting of quenching curves for all types of liposomes and all experimental data reported in Figure 1f was found at $n = 550$ and the following values for K_d: $K_{dPC} = 3.9\ \mu M$; $K_{dPC/PS} = 1.6\ \mu M$; $K_{dPC/PS/PS\text{-}OX} = 0.6\ \mu M$ (here, n is relatively large because it corresponds to the number of phospholipid molecules interacting with one protein, as opposed to the actual number of binding sites on the protein). Thus, MFG-E8 can be estimated to bind 8–20-fold stronger to PS-OX and 2.5–4-fold stronger to PS as compared with PC.

MFG-E8 was identified as one of the most abundant proteins of membranes of milk fat globules in human milk,[1] and is known to be expressed in the lactating mammary gland as well as in various other tissues.[2] Moreover, recent studies have shown that thioglycolate-elicited macrophages and phagocytic-competent immature dendritic cells express MFG-E8, and it was suggested that MFG-E8 serves as an opsonin for apoptotic cells by recognizing phospholipids such as PS.[4,9] The current experiments indicate that MFG-E8 binds to PC as well as to PS, albeit with higher affinity for PS. However, the specificity of this binding may not be sufficient to explain recognition of apoptotic cells by macrophages. PS-OX represents a significantly more favorable site for MFG-E8 interaction with lipid bilayers as compared to other phospholipids. Therefore, oxidatively modified phospholipids, such as PS-OX, may serve as preferential ligands for MFG-E8. Exposure of PS-OX during apoptosis could thus promote clearance of cell corpses at least in part through interaction with the 'bridging' molecule, MFG-E8. Moreover, since the overall binding affinity reported herein of MFG-E8 for PS is not as strong as for other PS-binding proteins (e.g. annexin V and factor VIII), it is conceivable that phospholipids are not the sole ligands for MFG-E8. Indeed, MFG-E8 could bind to another molecule(s) on the surface of apoptotic cells, thus increasing the stability of the protein–membrane interaction (for comparison, β_2-glycoprotein I, a phospholipid-binding protein, has been shown to interact with annexin II on endothelial cells[10]). One candidate for such a cofactor is annexin I, a cytosolic protein that is recruited to the cell surface during apoptosis, and colocalizes with PS in discrete membrane patches.[11] Further studies are required to determine the full spectrum of putative apoptosis-specific binding partners of MFG-E8.

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Figure 1 MFG-E8 interacts preferentially with oxidized phosphatidylserine (PS-OX). (**a**) 96-well plates coated with PS, PC, PI, PE or PG (3 μg/ml) were incubated with recombinant human MFG-E8-FLAG, and MFG-E8 bound to the wells was quantified by ELISA as previously described.[4] Data shown are the mean values of triplicate determinations. Similar results were obtained in 3–4 independent experiments. (**b**) Electrospray mass spectrometry of oxidized PS was performed in negative ion mode using a Micromass Quattro micro™ mass spectrometer.[12] Data were collected between 750–900 mass units with a sweep time of 2 s. Total ion current mass chromatograms depicting individual molecular species are shown. Mass-to-charge ratio (*m/z*) 782.2 corresponds to the parent compound (i.e. nonoxidized PS); *m/z* 814, *m/z* 846, and *m/z* 878 correspond to mono-, di- and trihydroperoxides, respectively. The peak at *m/z* 828 is also an oxidation product of PS, most likely resulting from redox cycling of the PS dihydroperoxide. (**c**) Quantification of MFG-E8 binding to PS (□) *versus* PS-OX (■). MFG-E8-FLAG was added at 2 μg/ml, and ELISA was performed as above. The phospholipid concentration was 10 μg/ml. Data shown are mean ± S.D. ($n = 3$). *$P < 0.05$ (PS *versus* PS-OX, by Student's *t*-test). (**d**) Fluorescence spectra of palmitoyl-2-[6-[(7-nitro-2,1,3-benzoadiazol-4-yl)amino]caproyl]-3-phosphatidylcholine (NBD-PC). SUVs were prepared from PC, PS and PS-OX (2 : 1 : 1) as previously described,[5] with or without the addition of NBD-PC (ratio 0.5 mol%). MFG-E8 (7 nM) was then added to NBD-PC-containing liposomes (*SUV* + *NBD* + *MFG-E8*) and quenching of NBD-PC fluorescence was determined using a Shimadzu RF-5301PC spectrofluorimeter (instrumental conditions: excitation wavelength 460 nm, excitation and emission slits 5 and 10 nm, respectively). (**e**) Dependence of NBD-PC fluorescence quenching on the concentration of MFG-E8. Liposome composition: *PC*–PC only; *PC/PS*–PC:PS = 1 : 1; *PC/PS/PS-OX*–PC : PS : PS-OX = 2 : 1 : 1. NBD-PC fluorescence was determined as described above. (**f**) Same data as in (**e**), but the extent of quenching is plotted *versus* the inverse protein concentration (consult text for details). Data depicted in (**d**), (**e**) and (**f**) are representative of five independent determinations. All phospholipids utilized herein were from Avanti Lipids (Alabaster, AL, USA). Recombinant MFG-E8-FLAG was produced and purified as described previously.[4] Anti-FLAG antibodies were obtained from SIGMA (St. Louis, MO, USA)