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Evidence for the presence of functional lipid rafts in immune cells of ectothermic organisms

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ABSTRACT

The role of lipid rafts in non-mammalian leukocytes has been scarcely investigated. We performed biochemical and functional analysis of lipid rafts in fish leukocytes. Fish Flotillin-1 and a fish GM1-like molecule (fGM1-L) were found in low density detergent-resistant membranes (LD-DRM) in goldfish macrophages and catfish B lymphocytes, similarly to mammals. The presence of flotillin-1 and fGM1-L in LD-DRM was sensitive to increased detergent concentrations, and cholesterol extraction. Confocal microscopy analysis of flotillin-1 and fGM1-L in fish leukocytes showed a distinctive punctuated staining pattern, suggestive of pre-existing rafts. Confocal microscopy analysis of macrophages showed that the membrane of phagosomes containing serum-opsonized zymosan was enriched in fGM1-L, and zymosan phagocytosis was reduced after cholesterol extraction. The presence of flotillin-1 and fGM1-L in LD-DRM, the microscopic evidence of flotillin-1 and fGM1-L on fish macrophages and B-cells, and the sensitivity of phagocytosis to cholesterol extraction, indicate that lipid rafts are biochemically and functionally similar in leukocytes from fish and mammals.

1. Introduction

Over the last decade lipid rafts have been shown to have several functions, including lipid and protein transport (Ait Slimane and Hoekstra, 2002), signal transduction (Simons and Toomre, 2000), and pathogen invasion (Hartlova et al., 2010). Lipid rafts have a prominent role in the mammalian immune system, acting as signaling platforms that efficiently orchestrate cells functions initiated by different types of immune receptors (Holowka and Baird, 2001; Langlet et al., 2000; Triantafilou and Triantafilou, 2010). Importantly, lipid rafts are involved in the activation of T- (Kabouridis and Jury, 2008) and B-lymphocytes (Gupta and DeFranco, 2007), as well as in facilitating the activation of cells involved in innate immunity (Yoshizaki et al., 2008). The functions attributed to lipid rafts, within the immune system, are derived from studies performed solely on mammalian leukocytes, and to date there is no evidence of functional lipid rafts in immune cells of ectothermic organisms.

Recently, the integration of various findings yielded a consensus definition of lipid rafts (Pike, 2006). The lipid rafts were defined as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions" (Pike, 2006). Although the existence of lipid rafts in biological membranes it is now generally accepted (Brown, 2006; Brown and London, 1998; Lingwood et al., 2009; Simons and Toomre, 2000), a lively debate about their existence and function was primarily fueled by the differences in the experimental techniques used to characterize them, and the apparent lipid rafts' heterogeneity.

Initial evidence supporting the existence of membrane microdomains (later to be known as lipid rafts) in biological membranes came from the observation that cell membranes are not fully solubilized by non-ionic detergents (e.g. Triton X-100) at low temperatures, and that this detergent-insoluble membrane fraction could be isolated from low density fractions of sucrose gradients (Brown, 2006). This early operative definition of lipid rafts was subject of much debate (Munro, 2003). It is now widely accepted that these low density detergent-resistant membranes (LD-DRM) are not equivalent to lipid rafts, but are actually derived from them (Brown, 2006; Lingwood et al., 2009; Lingwood and Simons, 2007). More importantly, detergent resistance is firmly recognized as a valuable tool for the study of lipid rafts function (Brown, 2006; Lingwood and Simons, 2007). The analysis of the partitioning of molecules to LD-DRM has proved very useful for the evaluation of many lipid raft related phenomena, and also for the identification of rafts-targeting signals, required for proper protein function (Brown, 2006; Lingwood and Simons, 2007).

Abundant evidence supports the notion that cholesterol is critical for the maintenance of lipid raft structure and function

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(Barenholz, 2002). Therefore the ability of cholesterol modulators to affect cellular functions, thought to be raft-dependent (e.g. due to the association of the involved molecules with LD-DRM), has become another valuable tool for lipid rafts research (Zidovetzki and Levitan, 2007).

In addition to the biochemical manipulations, microscopic analysis of biological membranes has yielded important information about the structure of lipid rafts. Using relatively simple microscopic techniques, the formation of micro-domains can be readily visualized in model membranes (Dietrich et al., 2001), due to phase separation of different lipids. The visualization of membrane micro-domains (i.e. lipid rafts) in living cells has proven more difficult, and has required the development of more sophisticated microscopic techniques (Chen et al., 2004; de Almeida et al., 2009; Duggan et al., 2008; Owen et al., 2009; Rao and Mayor, 2005). Confocal microscopy analysis of well characterized lipid rafts markers, the ganglioside GM1 (Janes et al., 1999), and the protein flotillin-1 (Rajendran et al., 2003), shows a distinctive punctuated pattern on the plane of the membrane; which is suggestive of stable large (over 100 nm) membrane micro-domains in living cells (Rajendran et al., 2003).

The most persuasive evidence for the existence and function of lipid rafts comes from studies that combined LD-DRM analysis, microscopy, cholesterol extraction, and loss of function experiments after cholesterol extraction (Brown, 2006; Lingwood and Simons, 2010). In this work, we used a combination of experimental approaches to obtain evidence for the presence of functional lipid rafts in immune cells of fish. Our results indicate that lipid rafts are biochemically and functionally similar in leukocytes from fish and mammals.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus* L.) from Mt. Parnell Fisheries Inc. (Mercersburg, PA) were kept at the University of Alberta, Biological Sciences Aquatic Facility; with a simulated 14 h light/10 h dark photoperiod. Fish were acclimated for at least 3 weeks at 17 °C prior to use for the generation of macrophage cultures. Animals were cared for according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada). All procedures performed on fish followed the guidelines dictated by the University of Alberta Animal Care and Use Committees, who approved this study with the protocol number 149/08/12.

2.2. Cells

Goldfish macrophages were generated in vitro as described (Neumann et al., 1998). All experiments were performed using day-7 primary kidney macrophage cultures. The murine macrophage cell lines P388D1 and J774A.1 were obtained from the ATCC, and maintained in DMEM (Invitrogen Life Technologies) containing 10% fetal calf serum as described (Guy and Belosevic, 1993; Haddad and Belosevic, 2009). The channel catfish (*Ictalurus punctatus*) B lymphocyte cell line 3B11 was a kind gift from Dr. Melanie Wilson, University of Mississippi Medical Centre and has been extensively characterized (Miller et al., 1994; van Ginkel et al., 1994). 3B11 cells were maintained in AL-3 medium consisting of equal parts AIM-V and L-15 (Invitrogen Life Technologies) adjusted to catfish tonicity with 10% (v/v) deionized water, supplemented with 1 μg/mL NaHCO3, 50 μM β-mercaptoethanol, and 3% heat inactivated, pooled, normal catfish serum as described (Miller et al., 1994). The human B lymphocyte cell line 721.221 was a kind gift from Dr. Deborah N. Burshtyn (Medical Microbiology and Immunology Department, University of Alberta). Cells were maintained in Iscove's medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum and 2 mM L-glutamine as described (Kirwan and Burshtyn, 2005).

2.3. Western blot

Caveolin-1 and flotillin-1 were detected in cell lysates prepared in RIPA lysis buffer as described (Garcia-Garcia et al., 2001). For SDS–PAGE 50 μg (B lymphocytes) or 30 μg (macrophages) of protein were loaded per lane. For Western blot analysis, the following antibodies were used, rabbit anti-Flotillin-1 (1:500; Santa Cruz Biotechnology, catalog sc-25506), rabbit anti-caveolin-1 (1:1000; Cell Signaling Technology, catalog 3238S), rabbit anti-caveolin-1 (1:200; Santa Cruz Biotechnology, catalog sc-7875). After electro-transference, PVDF membranes were incubated for 3 h in blocking buffer (PBS containing 5% milk, 1% BSA and 0.1% Tween-20) and the membranes were incubated overnight with different antibodies. Goat anti-rabbit IgG (Bio-Rad, catalog 172-1019) was used for the detection of the primary antibodies at a 1:3000 dilution in blocking buffer. Membranes were developed by enhanced chemiluminescence.

2.4. GM1 detection by flow cytometry

Mouse or goldfish macrophages, and human or catfish B lymphocytes (1×10^6 cells) were fixed for 30 min with 1% paraformaldehyde in PBS, washed, and then incubated for 30 min on ice with 20 μg/mL FITC-cholera toxin B subunit (Sigma–Aldrich, catalog C1655-250UG) in 100 μL PBS. Surface staining of GM1 was detected in the FL-1 channel, using a FACScalibur flow cytometer (Becton Dickinson).

2.5. Isolation of low density detergent-resistant membranes (LD-DRM)

Isolation of LD-DRM by Triton X-100 extraction, and ultracentrifugation on discontinuous sucrose density gradients, was performed essentially as described (Garcia-Garcia et al., 2007). Cell lysates were prepared from 3 to 4×10^7 P388D1 macrophages or 721.221 human B lymphocytes, or 6 to 8×10^7 goldfish macrophages or 3B11 catfish B lymphocytes, using the indicated Triton X-100 concentrations. Macrophages from day-7 primary kidney cultures from 3 to 4 fish were pooled for the preparation of each gradient. After 16 h centrifugation at 170,000×*g*, seven 400 μL fractions were collected (top to bottom), and pooled together: low density (5% sucrose) + low-to-medium density interphase (2 fractions), medium density (35% sucrose, 2 fractions), medium-to-high density interphase (1 fraction), and high density (40% sucrose, 2 fractions). In select experiments, macrophages were treated with the indicated concentration of methyl-β-cyclodextrin for 45 min in PBS, before detergent extraction.

2.6. Protein and GM1 detection in LD-DRM

Proteins in the pooled sucrose gradient fractions were detected by Western blot as described above. Sixty microliters of each pooled fraction were loaded per lane. For the detection of GM1 in the pooled gradient fractions, SDS–PAGE and electro-transfer were performed as for protein detection, but the SDS–PAGE run was stopped when the 10 kDa marker was half-way through the gel. After electro-transference, the section of the PVDF membrane close to the 10 kDa marker was cut, incubated overnight in blocking buffer, and then blotted for three hours with a 1:10,000 (P388D1 macrophages) or 1:500 (lymphocytes and goldfish macrophages) dilution of HRP-cholera toxin B subunit (Sigma–Aldrich, catalog C3741) in blocking buffer containing 0.3% Tween-20.