Membrane microdomains and their role in human disease: a novel strategy for characterization disease specific alteration

Doctoral Thesis

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Introduction

Monocytes and monocyte-derived macrophages are critically involved in innate immunity response during systemic inflammation, in the initiation and progression of atherosclerotic lesions and disorders of intracellular cholesterol trafficking. Recently, membrane lipid microdomains have been implicated in various monocyte functions, including scavenging and recycling of atherogenic lipoproteins, immune response, and cellular cholesterol influx/efflux and trafficking. The glycosylphosphatidylinositol (GPI)-anchored CD14 is a constituent of innate pattern recognition complex and seems to mediate various ligand induced cellular events through lipid microdomains.

Membrane lipid microdomains, also called lipid rafts, are liquid-ordered, laterally segregated dynamic assemblies of the plasma membrane, enriched in cholesterol, sphingolipids and other order-preferring membrane lipids. Lipid microdomains also present a unique protein composition. Proteins with high raft-affinity are anchored with GPI to the outer plasma membrane leaflet or to cholesterol itself, while proteins linked to the inner leaflet are mainly with double-acylated chains, such as the Src family tyrosine kinases (e.g. Lck, Fyn and Lyn), caveolins and heterotrimeric G proteins. These microdomains float freely within the liquid disordered bilayer of cellular membranes, but they can also coalesce to form larger, ordered clustering platforms.

These specialized membrane microdomains are thought to be involved in several cellular functions, including membrane sorting and trafficking, compartmentalization of receptors, cell signalling and serve as an entry site for various pathogens or toxins. They have been shown to mediate internalization of bacteria, viruses and parasites into the host cell, to initiate apoptosis of the host cell upon infection, and to regulate the release of cytokines from infected mammalian cells. Regarding the major role of lipid rafts in distinct cellular functions, they have a prominent impact on the pathogenesis of several human diseases, such as neurological diseases (Alzheimer´s, Parkinson´s and prion disease), infectious and inflammatory diseases (systemic inflammatory response syndrome (SIRS)/sepsis), cardiovascular and metabolic disease, autoimmunity (systemic lupus erythematosus and rheumatoid arthritis), lipid storage diseases (Niemann-Pick type C and Gaucher disease), angiogenesis and development of cancer.

Historically, lipid microdomains have been characterized by their insolubility to cold, nonionic detergents (Lubrol WX, Nonidet-P40, Brij-98), classically to Triton X-100 (TX-100). This property is resulted from the tightly packed acyl chains in lipid rafts, since the

lipid-lipid interactions are more stable than lipid-detergent interactions. Thus, a classical method to study lipid rafts is the isolation of detergent resistant fractions of membranes (DRMs) by gradient-ultracentrifugation, followed by immunoblotting. Despite controversial discussions regarding the existence of lipid microdomains or DRMs *in vivo* and TX-100-induced artificial changes in biological membranes, methods based on TX-100 insolubility are still widely used and accepted to investigate the composition of membrane lipid microdomains. To define membrane microdomains, DRMs are undoubtedly one of the most useful starting points.

Aim of the thesis

The main object of the thesis was to characterize peripheral blood monocytic membrane lipid microdomains based on their detergent resistance. Our goal was to optimize a newly developed flow cytometric assay for rapid analysis of constitutive and induced DRM association of plasma membrane proteins from freshly obtained whole blood samples. Further, we tested this analytical tool to screen disease specific alterations of membrane lipid microdomains in human peripheral blood monocytes. The detergent insolubility of cell surface antigens, previously demonstrated to play a crucial role in inflammatory response and lipid homeostasis, such as GPI-anchored proteins (CD14, CD55), Fcγ-receptors (CD16, CD32, CD64), scavenger receptors (CD36, CD91, CD163), constituents of the integrin/complement receptor 3 complex (CD11a, CD11b, CD18) and the pentaspanin/tetraspanin family (CD47, CD81), were analysed in detail.

The proposed project addresses the following issues:

I. To establish a flow cytometric detergent solubilisation assay for whole blood monocytes
II. To investigate the effect of *in vitro* stimulation with lipopolysaccharide (LPS) on CD14-dependent receptor clustering in human monocytes
III. To characterize the CD14-dependent receptor clustering *ex vivo* on peripheral blood monocytes in inflammatory diseases (SIRS/sepsis and CAD/myocardial infarction)
IV. To investigate the effect of disturbed cellular membrane trafficking on DRM partition of monocyte antigens
V. To investigate the effect of *in vitro* ezetimib treatment, a drug influencing cellular lipid homeostasis, on DRM association of monocyte proteins.

Methods

1. Blood samples

Peripheral EDTA-blood samples were obtained from healthy blood donors and from patients with SIRS/sepsis, CAD/myocardial infarction and Niemann-Pick type C disease.

2. Immunostaining

For flow cytometry analysis 100 μL aliquots of whole blood samples were incubated for 15 minutes on ice with saturating concentrations of fluorochrome-conjugated monoclonal antibodies (mAbs). Sample preparation and analysis were always carried out within 6 hours of vein puncture.

3. *In vitro* modifications of the plasma membrane microdomains

Methylated β-cyclodextrin (MBCD) and complexed cholesterol with MBCD were used for modifications of the plasma membrane cholesterol content. Cells were incubated with 10 mM MBCD to deplete membrane cholesterol. Reconstitution of cholesterol after depletion was performed with complexes of MBCD (4,6mg/mL) and cholesterol (100 μg/mL) at 37°C for 30 minutes. After incubation with MBCD or complexed cholesterol the cells were washed with phosphate-buffered salines (PBS) and labelled as described above. Control samples were incubated with PBS.

4. *In vitro* stimulation of cells with LPS

Monocytes were incubated with 100 ng/mL LPS at 37 °C for 15 minutes, then washed with ice-cold PBS in order to stop further activation, and finally labelled with mAbs. Control samples were incubated with PBS instead of LPS.

5. Flow cytometry of detergent resistance and data analysis

The measurements were carried out on a FACSCanto flow cytometer (BD Biosciences). Mean fluorescence intensity (MFI), forward scatter (FSC) and side scatter (SSC) signals of unstained and labelled peripheral blood cells were recorded before and 5 minutes after TX-100 treatment. Data were analyzed by FACSDiVa and CellquestPro softwares (BD Biosciences).

The TX-100 solubility of antigens was evaluated using the **f**low **c**ytometric **d**etergent **r**esistance **(FCDR)** index:

$$FCDR = (MFI_{Det} - MFI_{AfDet}) / (MFI_{Max} - MFI_{Af}),$$

MFI_{Det} indicates the fluorescence of labeled TX-100 treated cells, MFI_{AfDet} is the autofluorescence of TX-100 treated cells, MFI_{Max} is the fluorescence of stained untreated cells and MFI_{Af} is the autofluorescence of untreated cells.

6. Visualization of raft microdomains with confocal microscopy

For microscopy whole blood samples were treated with 0.05% cold TX-100 (15 min) at 4°C, and then fixed with 4% formaldehyde (15 min), followed by staining with FITC-conjugated mAb against GPI-anchored protein CD55. For visualization of lipid microdomains, monocytes were labelled with 50 μg/mL raft-specific lipid probe 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-tetramethylrhodamine (DMPE-TMR). Confocal images were acquired with an inverted Leica TCS-SP5 confocal microscope (Leica Lasertechnik) equipped with a ArKr 75 mV mixed-ion laser. Acquisition and analysis were performed with the Metamorph software package (Analysis Universal Imaging).

7. Western blot analysis

Cells were harvested and washed with PBS, frozen (-20°C). Cell debris was separated by centrifugation at 10.000 x g for 10min at 4°C. Proteins were separated by SDS-PAGE and transferred to PVDF (Pall, Dreieich) membranes. Detection of the proteins was carried out with the ECL Western blot detection system (Amersham/Pharmacia) following by quantification with the Lumi-Imager (Roche Diagnostics).

8. Fluorescence Resonance Energy Transfer (FRET) by flow cytometry

FACSCanto flow cytometer and FACSDiVa (BD Biosciences) software were used for the measurements. Measurements were performed without compensation. 30,000 leukocytes were acquired.

The principle of FRET first described by Förster in the late 1940s allows the measurement of distances between surface molecules. The energy transfer parameter (ET_p), which is proportional to FRET efficiency (ET), was calculated according to **(1)** where A is acceptor, D is donor, FL2 is mean fluorescence in channel 2 (488 →530 nm) (donor, R-PE), FL3 is mean fluorescence in channel 3 (488 →585 nm), FL4 is mean fluorescence in channel 4 (633 → 670 nm, acceptor, Cy5) (each value obtained after autofluorescence subtraction):

(1) $ET_p = FL3(D,A) - FL2(D,A)/a - FL4(DA)/b\ FL3(D,A)$

$a = FL2(A)/FL3(A)$

b = FL4(D)/FL3(D)

FRET efficiency (ET) was calculated from quenching according to **(2)**:

(2) ET = FL2(D) - FL2(D,A) FL2(D)

According to Szöllösi et al. an ET = 5% was defined as the threshold level for significant transfer efficiency in our experiments.

9. Statistical analysis

Data are given as mean ± SD. For statistical comparison Mann-Whitney-U or Wilcoxon signed-ranks test was used. Values for $p < 0.05$ were considered as statistically significant (*).

Results

I. Establishment and evaluation of a flow cytometric method to assess detergent resistance of plasma membrane proteins in monocytes

The purpose of our initial studies was the determination of the optimal detergent concentration (0.01%, 0.05% and 0.1% TX-100) for human peripheral blood monocytes. Our results showed that very low concentration of the detergent (0.01% TX-100) is sufficient for extracting non-raft proteins from peripheral monocytes. We used the GPI-anchored LPS (CD14)-receptor to identify the monocyte population during detergent treatment. Solubilisation with TX-100 slightly changes the light scatter characteristics of the cells, indicating cell shrinkage upon detergent extraction. Visualisation of detergent treated monocytes by confocal microscopy confirmed that monocytes remain morphologically intact cells after mild TX-100 treatment.

Cholesterol is essential for microdomain formation, though, comparison of detergent solubility of control and cholesterol-depleted membranes can differentiate, whether a given membrane protein is associated to or excluded from lipid rafts/microdomains. Removal of cholesterol with MBCD treatment resulted in increased TX-100 solubility of usually DRM associated proteins, such as GPI-anchored protein CD14 and CD55. These changes were reversed by reconstitution of plasma membrane cholesterol of MBCD-depleted cells by providing exogenous complexed cholesterol. Cholesterol depletion and loading did not affect detergent solubility of the transferrin receptor CD71, a well-known non-raft protein. Our results presented that flow cytometry is capable of detecting constitutive DRM association. Hence, we determined the DRM association of several surface proteins of human peripheral blood monocytes, implicated in cellular signalling, inflammatory reactions and/or lipid metabolism. All FCDR data were in accordance with data obtained with other methods, such as classic immunobiochemical detection, confocal microscopy or FRET analysis.

II. Investigation of the effect of *in vitro* stimulation with LPS from different bacterial species on CD14-dependent receptor clustering in human monocytes

One of the most important functions of membrane microdomains/rafts is recruiting and concentrating of receptors upon extracellular stimulation and enhancing the efficacy of signalling events. Lipid rafts are intimately involved in endotoxin-mediated activation of human monocytes/macrophages. We investigated the effect of LPS stimulation isolated from different bacteria, such as *Salmonella minnesota, Escherichia coli* and *Francisella tularensis,*

and compared with each other. Constitutive (CD14, CD32, CD55), or activation-induced (CD81 and TLR-4) DRM association were determined. All three LPS species induced the translocation of CD81 or TLR-4 into lipid microdomains. FRET analysis could also confirm the LPS-induced clustering of CD14, TLR-4 and CD81 in human monocytes within lipid rafts, irrespectively of LPS species.

Our results demonstrated that all of the examined LPS species induce approximately equally the clustering of the given pattern recognition receptors within lipid microdomains.

III. Characterization of CD14-dependent receptor clustering on peripheral blood monocytes in inflammatory diseases (SIRS/sepsis and CAD/myocardial infarction)

Although LPS and related molecules serve as a major ligands for CD14, the activation of CD14-dependent receptor complex by atherogenic lipoproteins (e.g. E-LDL, Ox-LDL etc.) are also involved in certain signalling events in monocyte/macrophages. *In vivo* LPS activation in SIRS/sepsis patients resulted in an increased detergent insolubility of CD14 and CD55, CD64, CD36, CD91, CD163, CD11a, CD11b/CD18, and CD81 suggesting their stronger association to DRMs due to clustering, while the increased detergent solubility of CD47 indicates its disassembly from lipid microdomains. A similar phenomenon was observed for *in vivo* atherogenic lipoprotein activation in patients with CAD/myocardial infarction. Almost all of the investigated receptors presented an elevated detergent resistance (e.g. CD14, CD55, CD16, CD32, CD64, CD36, CD91, CD163, CD11a, CD11b, CD18, and CD47), with the exception of the tetraspanin CD81, which translocates to DRMs only upon LPS stimulation.

IV. Investigation of the influence of disturbed cellular membrane trafficking on DRM partition of monocyte antigen

DRM association of given antigens was monitored *ex vivo* on whole blood monocytes from human mutant cells with altered cellular cholesterol processing, such as NPC disease (altered late endosomal cholesterol influx) and Tangier-disease (impaired cholesterol efflux).

Each investigated surface antigen showed highly elevated partititon to DRMs in NPC monocytes. These data suggest that the accumulation of cholesterol and sphingolipids, due to the altered endocytic trafficking results in the enrichment of microdomains in the plasma membrane, as well. This phenomenon may have functional significance in the pathogenesis of NPC disease.

In contrast to the NPC-deficient cells, ABCA1-deficient monocytes, which accumulate cholesterol in the trans-Golgi network-compartment, fail to release cholesterol to the plasma membrane upon ApoAI/HDL3-induced cholesterol efflux. ABCA1-deficient monocytes did not show significant changes in DRM associations compared to controls.

V. Investigation the effect of *in vitro* administration of ezetimib, a drug influencing cellular lipid homeostasis

Structural and functional integrity of membrane microdomains/ lipid rafts strictly depends on their lipid content. Lessons from the application of MBCD showed that modification of raft cholesterol content is useful for investigating raft-associated function. Ezetimib was initially described as a selective cholesterol absorption inhibitor, blocking the absorption of dietary and biliary cholesterol at the level of enterocytic brush-border in the small intestine. The binding site for ezetimib is CD13, which is a constitutively expressed, raft-associated antigen, and also presented on the surface of monocytes/macrophages.

We found, that ezetimib rapidly disrupts lipid microdomain assembly within short time (15 minutes). Highly detergent-insoluble proteins, such as CD32, CD64, CD14, and the ezetimib target CD13 exhibited significantly decreased DRM association upon ezetimib treatment by FCDR assay, irrespectively of staining priority. Our findings obtained by FCDR assay were confirmed with other methods, including flow cytometric FRET, confocal microscopy and immunoblot studies. These data indicate that ezetimib likely modifies lipid microdomain/raft structure on peripheral blood monocytes, like other cholesterol lowering agents.

Conclusion

This work established and applied a novel flow cytometric assay for rapid analysis of surface antigens association to membrane microdomains/ lipid rafts on circulating monocytes. The method is based on the property that membrane microdomains exhibit insolubility to cold nonionic detergent, such as TX-100.

Constitutive and activation-induced DRM-association of certain antigen was demonstrated upon *in vitro* LPS stimulation. We showed a specific clustering of tetraspanin CD81 and TLR-4 receptors into lipid microdomains in response to LPS activation. Furthermore, a strengthened DRM association of surface antigens upon CD14-dependent receptor clustering were observed, as well as an inverse DRM partition of penta-/tetraspanin family member CD47 and CD81 in SIRS/sepsis and CAD/myocardial infarction patients. Moreover, Fcγ receptor CD16, which is primarily involved in phagocytosis and atherogenic lipoprotein clearance, showed enhanced DRM association only in CAD/myocardial infarction patients, but not in SIRS/sepsis patients. Our observation regarding ligand-induced receptor clustering also supports the concept of lipid raft heterogeneity, namely rafts exist in small distinct microdomains with a diverse receptor composition and upon activation they selectively fuse to form large signalling platforms.

Our data demonstrate that defective NPC1 bearing monocytes reveal a highly elevated DRM association of all investigated cell surface antigens, suggesting the enrichment of lipid microdomains in the plasma membranes. Interestingly, ABCA1 deficient monocytes do not show a significant difference in the detergent resistance of surface antigens.

Finally, we presented that ezetimib, a lipid lowering drug, rapidly disrupts lipid microdomain assembly on peripheral blood monocytes, likely through disturbing the association of CD13 with its co-receptors CD36 and CD64.

In conclusion, our data indicate that rapid flow cytometric assay provides a simply and powerful analytical tool to screen disease specific alterations of membrane microdomains from freshly obtained blood samples. Hence, this feasible tool could play a role in identifying disease marker(s) in disorders with supposed involvement of membrane lipid microdomains or may contribute to monitor effects of therapeutic agents on monocyte membrane constituents.

Summary

Lipid rafts resemble cholesterol- and glycosphingolipid-enriched, liquid-ordered plasma membrane microdomains, allowing specific interactions that modulate signal transduction, membrane trafficking and pathogen entry. Recently, lipid rafts have been also implicated in a range of monocyte/macrophage functions, including endotoxin-mediated activation, scavenging and recycling of atherogenic lipoproteins, cellular cholesterol influx/efflux and trafficking. Due to their tightly ordered lipid phase, membrane microdomains show a relative resistance to nonionic detergents (classical to Triton X-100) providing an easy analytical tool to study them.

The aim of the thesis was to analyze specific antigens of lipid microdomains in monocytes that establish the raft-associated, ligand induce CD14-dependent specific activation of these cells. The studies established and applied a rapid flow cytometric detergent resistance-based (FCDR) assay to investigate microdomain association of proteins on circulating monocytes from whole blood samples. By using FCDR assay, constitutive and activation-induced detergent resistant membrane (DRM) associations of certain antigen were demonstrated upon *in vitro* LPS stimulation. Characteristic alterations and different patterns of CD14-dependent receptor co-assembly within microdomains could be detected *ex vivo* in different disease states, such as inflammatory response (SIRS/sepsis and CAD/myocardial infarction) or disorder affecting cellular cholesterol trafficking (Niemann-Pick type C disease). Moreover, we provided evidence that this assay is capable to detect effects of therapeutic agents affecting membrane microdomains structure and/or function, such as ezetimib a lipid lowering drug.

Our results demonstrate that flow cytometric analysis of short time in situ detergent extraction provides a powerful tool for rapid examination of blood monocyte DRMs. This may contribute to screen patients with potential microdomain abnormalities and/or monitor effects of therapeutic agents on membrane constituents. In addition, multi-color analysis allows the possibility to explore parallel different surface antigens on different cell populations without the need to purify them by physical separation.

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