Towards understanding the role of membrane cholesterol and sialic acid in the function of immune cells: studies with monoclonal antibodies

Ph.D. thesis

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Budapest, Hungary 2010.
INTRODUCTION

For their normal functions, cells require membrane integrity. Beside proteins, lipids and carbohydrates are also important cell membrane components that separate cells from the surrounding area. At the same time, they also connect cells with the extracellular environment by receiving signals from the external milieu. Cholesterol is an important structural lipid of lipid rafts (microdomains) in the mammalian plasma membrane. These membrane microdomains seem to play essential role in organizing cell surface molecules, in regulation of transmembrane signal transduction processes or controlling internalization/endocytosis of the bound molecules or pathogenic organisms (e.g. influenza virus, HIV-1, several bacteria and also parazites). Oligosaccharide chains can be attached to raft-associated as well as 'raftophobic' membrane proteins or lipids. Sialic acid is typically found on the exposed termini of this glycoconjugates and serves as ligand for Sia-binding receptors. These receptors are expressed by different immunocytes and control migration, adhesion and signal transduction. Thus, cholesterol and sialic acid are both important, non-protein membrane components that can significantly influence function of immune cells.

OBJECTIVES

Cholesterol is a key, stabilizing molecule of membrane microdomains. Therefore, studying its role in lipid raft-mediated immune processes may help better understanding how lipid rafts function. Furthermore, based on these properties, membrane cholesterol and other raft components became potential new targets for immunotherapies. In spite of the considerable knowledge on lipid rafts, there are still only a few reliable and properly selective markers for these microdomains. No in situ modulators of rafts are available so far either, which could affect biological role of rafts in immune processes without depleting/depriving cholesterol from the membrane. Having in hand two novel IgG type anti-cholesterol monoclonal antibodies (ACHAs), developed in our laboratory, we set the following goals and questions:

1. Characterization of the novel cholesterol-specific monoclonal antibodies (ACHAs) in terms of their specificity, affinity and plasma membrane or intracellular binding sites.

Monoclonal IgG3-type cholesterol-specific antibodies (AC1, AC8) have recently been developed in our laboratory by immunizing mice with cholesterol-rich liposomes. Our aim was to characterize in details their binding to membrane cholesterol in liposomes and various immune cells. We investigated the potential of monoclonal IgG ACHAs as selective markers of lipid rafts/caveolae, as well.

2. What is the molecular background behind the capacity of monoclonal ACHAs to inhibit in vitro HIV infection/production?

Our collaboration partners (Z. Beck and co-workers: University of Debrecen, Hungary/Walter Reed Army Institute of Research, Rockville, MD, USA) found that treatment of HIV-1 target cells, but
not of the virus, with AC1 or AC8 antibodies prior to *in vitro* infection significantly diminished the virus production. Our goal was to study the background mechanism of this inhibitory potential of ACHAs, with special attention to their effects on distribution/interaction pattern, accessibility, mobility or raft association of the primary (CD4) and coreceptors (CXCR4) of HIV-1.

Another important membrane component is sialic acid, a carbohydrate, attached to glycoconjugates (glycoproteins and glycolipids), serving as ligands to Sia-binding proteins that are known to regulate cell signalling and cell-cell interactions. GL7 antibody has been shown to bind to the surface of mouse lymphocytes and human B cell lines. It has been also revealed that the antibody’s epitope have a Sia moiety. The cell specific expression and membrane localization of the GL7 epitope on immune cells as well as the functional role of the molecule(s) bearing the epitope still remained unresolved. Therefore, here we set the following goal:

**3. Characterization of the GL7 epitope: its expression and localization in human immunocytes.**

The expression of GL7 epitope by human immune cells has not yet been sufficiently characterized. Therefore, we propose to characterize the expression of the epitope by various human lymphoid and myeloid cells and the localization of the epitope-bearing cells in lymphoid organs. Furthermore, we analysed the membrane localization and the nature of the GL7 epitope. These investigations may contribute to identify GL7 epitope-bearing molecule(s) and, in this way, assign a function to the epitope.

**METHODS APPLIED**

- flow cytometry technique to detect expression, immune phenotype, antibody binding
- standard and special confocal microscopic imaging
- isolation of detergent resistant membrane fractions by gradient ultracentrifugation and asaying by immuno-dot blot
- fluorescence resonance energy transfer (FRET) measurements by confocal microscopy
- fluorescence correlation spectroscopy imaging (FCS microscopy; diffusion/mobility assay)
- surface plasmon resonance (SPR) technique for characterization of binding properties of antibodies
- flow cytometric detergent resistance (FCDR) method
RESULTS

1. Characterization of the novel cholesterol-specific monoclonal antibodies (ACHAs) in terms of their specificity, affinity and plasma membrane or intracellular binding sites.

- Binding properties of the generated monoclonal ACHAs to cells were determined by flow cytometry and confocal microscopy. AC1 and AC8 antibodies bound to different murine and human intact lymphocyte and monocyte-macrophage cell lines, which was enhanced substantially by a limited papain digestion of the cell surface that removes most of the largely protruding extracellular protein domains.
- The AC8 antibody strongly colocalized with markers of caveolin$^+$ and caveolin cholesterol-rich lipid rafts at the cell surface and intracellularly with markers of ER and Golgi apparatus, suggesting that monoclonal ACHAs recognize clustered cholesterol in live cells.
- Specificity was further confirmed by cholesterol modulating agents, cholesterol oxidase or filipin III antibiotics, by which treatments the binding of our antibodies could be decreased either to cells or to isolated DRMs.
- Using surface plasmon resonance (SPR) technique, a medium affinity ($K_d = 7.8 \times 10^{-10} \text{ M}$) binding of AC8 monoclonal ACHA to cholesterol-rich liposomes was found.

2. What is the molecular background behind the capacity of monoclonal ACHAs to inhibit in vitro HIV infection/production?

- Upon binding to human T cells or monocytes, AC8 induced lateral lipid raft clustering, assessed by confocal microscopy. This clustering was characterized with an increased average raft-size (from 200-300 to 500-1000 nm) and elevated number (by five-fold) of highly ‘patchy’ cells.
- AC8 monoclonal ACHA promoted the association of CXCR4 with both CD4 and lipid rafts, as shown by the increased (by approximately two-fold) colocalization or FRET efficiency between CXCR4 and CD4 or CTX, calculated from confocal images. Consistent with this, the lateral mobility of CXCR4, measured by FCS microscopy, also significantly decreased. Fab fragments of ACHAs and antibodies against non-raft membrane proteins did not show these effects.


- Our results clearly demonstrate that the sialic acid-containing GL7 epitope is exclusively expressed by lymphocytes among human blood cells, similarly to mouse and rat. However, an important difference is that while in rodents the GL7 epitope appears only after 48h activation of T and B cells, in human the epitope is constitutively expressed.
- Fluorescence immunohistochemical staining of human tonsil showed that GL7 epitope is maximally highly expressed in the B cell follicles.
by CD19^+ IgD^+ IgM^<low> naïve B lymphocytes. Other B cell subpopulations and T cells have lower expression level of the epitope.

- The GL7 epitope strongly colocalized with the lipid raft marker G_{M1} ganglioside in human primary lymphocytes and B or T cell lines, whereas the non-raft protein CD71 showed only a moderate colocalization with GL7. Flow cytometric detergent resistance measurements (FCDR) further confirmed this raft-association.

CONCLUSIONS

Our monoclonal cholesterol-specific IgG antibodies upon binding to intact immunocytes are capable of marking lipid rafts and modulating cholesterol/raft-dependent functions of immune cells. Based on our results, the novel ACHAs, recognizing ‘properly clustered membrane cholesterol only’, seem useful to investigate the eventual role of cholesterol in the process of HIV-1 entry, as well as may serve as a molecular basis for the development of new, combined lipid raft-oriented approaches in HIV-1 therapy. Beside this inhibitory effect, IgG ACHAs may modulate other immune functions as well, which needs further investigation.

In case of GL7 antibody, recognizing a Neu5Ac-containing epitope (GL7), the presented data show that the expression profile of GL7 is different in human and rodents. Although, the presence of the epitope in human is also lymphocyte-restricted but it is constitutively expressed, while in mouse GL7 is a late activation antigen. The specific expression of the GL7 epitope and its high degree of raft-association on lymphocytes allow us to suggest a functional role for GL7 epitope and/or some supposed counterparts.

Publications connected to the thesis:


   Cytometry A 2008. 73:220-229.  impact factor: 3.259

3. Endre Kiss, Péter Nagy, Andrea Balogh, János Szöllősi, János Matkó: “Cytometry of raft and caveola membrane microdomains: from flow and imaging techniques to high throughput screening assays”
   Cytometry A. 2008. 73:599-614.  impact factor: 3.259


impact factor: 2.858

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Other publication:

1. Andrea Balogh, Judit Pozsgay, János Matkó, Tibor Várkonyi, János Rigó Jr, Zoltán Papp, Roberto Romero, Hamutal Meiri, and Nándor Gábor Than: “Placental Protein 13 (PP13) / galectin-13 undergoes subcellular redistribution in preeclampsia and HELLP syndrome: an analysis with immunohistochemistry-based confocal microscopy” (manuscript prepared for submission to Cytometry A)

Conference abstracts:


