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## Could membrane lipids influence the receptor tyrosine kinase activity? Epidermal growth factor receptor and its interactions with gangliosides\*\*

### Czy lipidy błonowe modyfikują aktywność receptorowych kinaz białkowych? Receptor epidermalnego czynnika wzrostu i jego oddziaływania z gangliozydami

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#### Summary

Cell membrane is a complex structure. Recently great attention is devoted to specialized membrane fragments responsible for compartmentalization of cellular processes called lipid rafts. Lipid rafts are made of a combination of cholesterol, sphingolipids, including glycosphingolipids, and proteins among them a number of receptors. The glycosphingolipids, especially gangliosides, have been proven to play crucial roles in the regulation of biological functions of raft localized proteins under both physiological and pathological conditions. Gangliosides modulate activity of protein receptors activated by fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin, and epidermal growth factor (EGF). One of the most interesting mechanism of regulation of receptor activity by gangliosides has been shown for epidermal growth factor receptor (EGFR). EGFR activity is dependent on ganglioside composition in its vicinity. Thus GM1 or GD1a gangliosides enhance EGFR activity while GM3 is a well-known EGFR inhibitor. Probably the different ways by which gangliosides influence the EGFR activity is associated with specific structures of both the ganglioside molecule and EGFR. Numerous studies proved direct interaction between these two types of molecules. This interaction could possibly prove to be crucial in the treatment of invasive cancers.

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Key words: epidermal growth factor receptor, lipid rafts, gangliosides

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#### Streszczenie

Błona komórkowa jest niezwykle skomplikowaną strukturą, w obrębie której ostatnio wyróżniono wyspecjalizowane fragmenty, zwane tratwami lipidowymi, odpowiedzialne za regionalizację procesów życiowych komórki. Tratwy lipidowe są kombinacją szeregu lipidów: cholesterolu, sfingolipidów, w tym glikosfingolipidów i szczególnych białek, wśród których wyróżniono liczne białka receptorowe. Wyniki wielu badań wykazały, że aktywność białek receptorowych jest zależna od glikosfingolipidów, a szczególnie od gangliozydów zarówno w warunkach fizjologicznych, jak i patologicznych. Wykazano, że białkami receptorowymi, których aktywność jest regulowana przez gangliozydy, są receptory fibroblastowego czynnika wzrostu (FGF), płytko-pochodnego czynnika wzrostu (PDGF), nerwowego czynnika wzrostu (NGF), insulinowego, a także epidermalnego czynnika wzrostu (EGF). Najciekawszy mechanizm regulacji aktywności białka receptorowego przez gangliozydy przedstawiono dla receptora epidermalnego czynnika wzrostu (EGFR). Wykazano, że aktywność EGFR jest uzależniona od składu gangliozydów występujących w jego otoczeniu. Zaobserwowano wzrost aktywności EGFR jeśli w jego otoczeniu znajdują się takie gangliozydy jak GM1 lub GD1a. Jednocześnie akumulacja gangliozydu GM3 w sąsiedztwie EGFR przyczynia się do spadku aktywności receptora. Wyjaśnienia wielorakość efektów oddziaływań między gangliozydami i EGFR szuka się w zależnościach w budowie cząsteczek gangliozydów i białka receptorowego. Na podstawie analizy struktury obu molekuł wykazano możliwość tworzenia bezpośrednich wiązań między domeną zewnątrz błonową EGFR a hydrofilowym fragmentem cząsteczki gangliozydu. Obecność tej bezpośredniej interakcji próbuje się wykorzystać w terapii inwazyjnych nowotworów.

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Słowa kluczowe: receptor epidermalnego czynnika wzrostu, tratwy lipidowe, gangliozydy

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INTRODUCTION

Glycosphingolipids (GSLs) are ubiquitous components of all vertebrate cell membranes. They represent from less than 5% (erythrocytes) to more than 20% (myelin) of membrane lipids (1). Although GSLs have common schematic structure, they are highly heterogeneous group of lipids. With regard to their headgroups, the GSLs were divided into two groups: galactosphingolipids and glucosphingolipids (2). The sialic acid containing glucosphingolipids are the gangliosides (3).

It was observed that GSLs are not uniformly distributed in the membrane. Instead GSLs are clustered in structures called "lipid rafts", which are defined as "areas in the membrane different in lipid composition from other membrane regions and characterized by a lateral organization dictated by the properties of their lipid composition. This has significant influence on large number of biological events" (4-10).

GANGLIOSIDES AND MEMBRANE PROTEIN FUNCTION

As it was already mentioned GSLs, including gangliosides, are lipid rafts components. Uneven distribution of gangliosides is probably the result of their specific structure. They have a hydrophobic long carbon chain residue (ceramide) and a hydrophilic oligosaccharide moiety (hydrophilic headgroup) (fig. 1A). The long saturated carbon chains of ceramide have unique biophysical properties, which allow self-aggregation and formation of "clusters" (1). The gangliosides hydrophilic oligosaccharides chains provide sites for interaction with extracellular molecules such as toxins, extracellular matrix components, adhesion molecules,

receptors and enzymes on the surface of adjacent cells (trans interaction). Moreover gangliosides hydrophilic headgroups take part in lateral interaction (cis interaction) with receptors and enzymes in the same membrane (11).

The interaction between gangliosides and proteins in lipid rafts are still not fully understood. It was proven that "cluster" of gangliosides is a less fluid part of the membrane, where membrane associated proteins could be confined. Within the lipid rafts there are lateral interactions between lipids and proteins. But also there are limited interactions between proteins associated with different lipid rafts (12).

It should be mentioned that only some proteins classes are highly concentrated in lipids rafts (13). The most popular motifs present in lipid rafts proteins are a glycoposphatidylinositol anchor or a lipid modifications of protein such as myristoylation or palmitoylation. Also transmembrane proteins are present in lipid rafts despite the modeling of transmembrane peptides that indicated in general that targeting to liquid-ordered phases is disfavored (12). These transmembrane proteins localized in the lipid rafts are growth factor receptors, including receptors for fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin and epidermal growth factor (EGF) (13). It has been assumed that the trapping of certain proteins in lipid rafts might be somehow crucial to their biological role (14).

This hypothesis was confirmed by a wide-range of studies on the role of gangliosides in function of membrane protein receptors. The negative (inhibitory) and positive (activatory) effect of gangliosides on receptor proteins were observed. For example GM1, GM2,

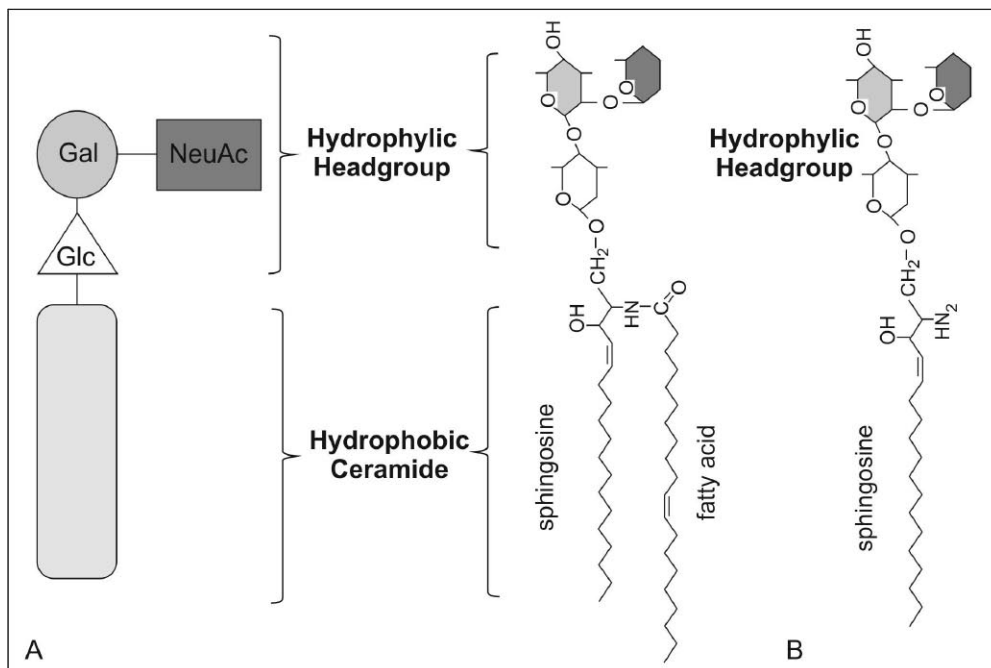


Fig. 1. Gangliosides and lyso-gangliosides structures: A: GM3 – schematic and structural formula; B: lyso-GM3 structural formula; (Glc – glucose, Gal – galactose, NeuAc – neuraminic acid).

GD1a and GT1b cause inhibition of PDGFR phosphorylation (15-16) and GM3 reduces EGFR phosphorylation. In contrast enhancement of growth factor receptor phosphorylation has been observed with GD1a and GM1 for EGFR (17-19) and FGFR (20). The opposite effects were detected as a result of interactions between receptor and gangliosides, thus suggesting that the mechanism of these interactions is complex.

Probably, this situation results from several possibilities of binding between ganglioside molecules and proteins. The first option is the binding between ganglioside and proteins amino acid residues in the extracellular loops. The second is the binding between ganglioside headgroup and sugar residues in glycans of a glycosylated proteins. The last option is the hydrophilic binding with the oligosaccharide part of an anchor of GPI-anchored proteins (12). These three types of binding could induce different conformation changes in membrane protein, which probably would influence their biological functions.

### EGFR FUNCTION DEPENDS ON GANGLIOSIDES

The epidermal growth factor receptor (EGFR) belongs to a EGFR family that consists of four receptors. EGFR is a 170 kDa transmembrane glycoprotein that comprises of 1186 amino acids (21). EGFR resides as a monomer in lipid rafts. In the monomeric structure one can distinguish three domains: an extracellular domain, a transmembrane domain, and a tyrosine kinase

cytoplasmic domain. The extracellular domain is responsible for epidermal growth factor (ligand) binding whereas the cytoplasmic domain plays a crucial role in signal transduction. The EGFR monomers could form predimers in ligand independent (fig. 2A) or dependent way (fig. 2B). The consequence of ligand binding, dimerisation, and exit from the lipid rafts is the EGFR autophosphorylation and subsequently downstream signaling (14).

The EGFR activity could be modified in each step that occurs before, during, and after ligand binding. Gangliosides were proposed to act as modulators of EGFR activity. It was mentioned that ganglioside GM1 enhances the EGFR activity whereas GM3 was defined as a EGFR inhibitor. The mechanism of regulation of the EGFR activity by gangliosides was illustrated in previous sections.

### GM3 AND EGFR ACTIVITY

In 1986 ganglioside GM3 was identified as an inhibitor of epidermal growth factor receptor (EGFR) (22). The mechanism of EGFR activity regulation is still investigated. It is undeniable that GM3 binds to EGFR because it is possible to co-immunoprecipitate a complex GM3-EGFR (23). This specific interaction was also confirmed by ELISA technique (24). Furthermore, lipid rafts disruption by the methyl- $\beta$ -cyclodextrin dependent cholesterol depletion leads to dissociation of GM3-EGFR complex and causes constitutive EGFR activation (25).

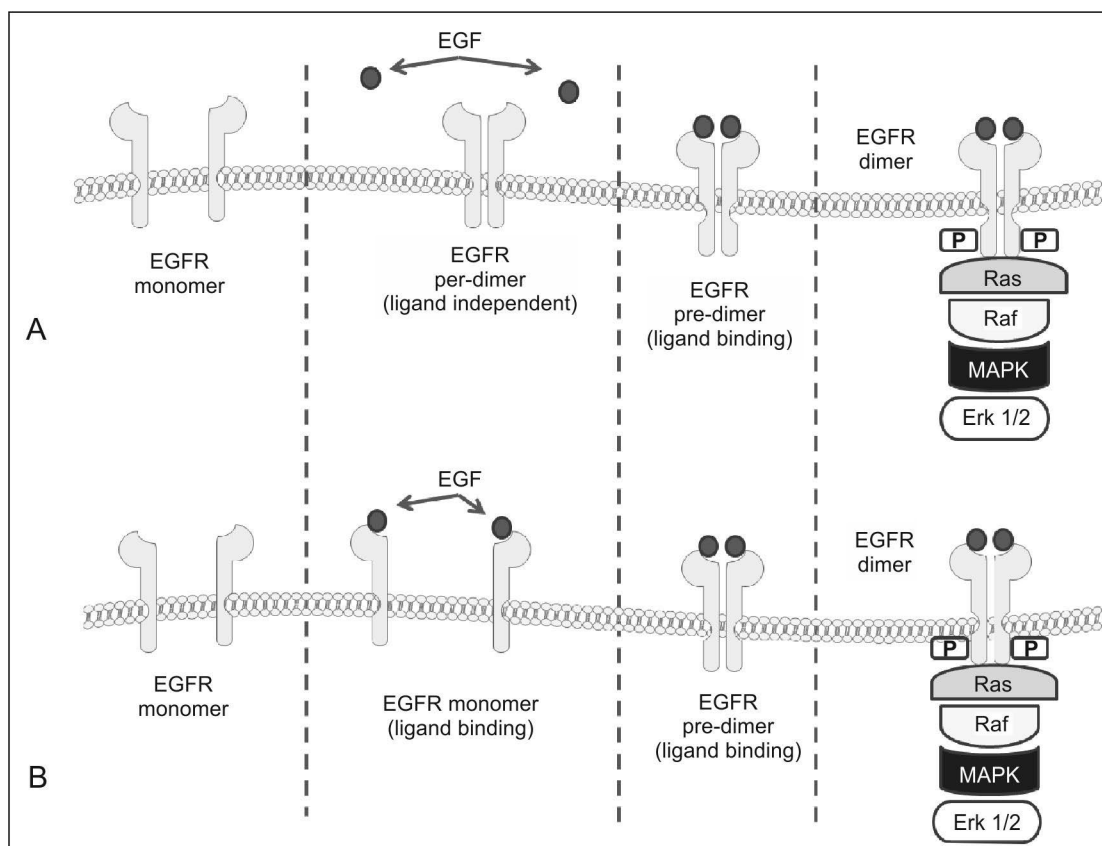


Fig. 2. The epidermal growth factor receptor (EGFR) activation. A: ligand independent dimer formation and B: ligand dependent dimer formation; (EGF – epidermal growth factor).

This statement poses the question about each step of EGFR activation and the regulation by GM3. Zhou and co-workers (26) proved that GM3 could interact equally well with both EGFR monomers and EGFR dimers. The next question concerns the possible ways of binding between GM3 and EGFR receptor. The answer is probably hidden in the EGFR and GM3 structures. It is well-known that in the EGFR extracellular domains have from eleven to twelve potential glycosylation sites, which are crucial for both its ligand binding and ability to transduce the signal (27-28). It should be emphasized that the headgroup of GM3 is relatively simple. It consists of one glucose, one galactose, and one sialic acid residue. The analysis of the EGFR and the GM3 structures suggest that between these molecules there could be carbohydrate-to-carbohydrate interactions. This hypothesis was examined in different cell cultures (29-32). The most interesting result was presented by Guan and co-workers (31). They used a UDP-Gal/UDP-GalNAc 4-epimerase deficient variant of Chinese Hamster Ovary cell line (IdID4) transfected with EGFR gene (IdID4/EGFR). The IdID4 cell line is incapable of synthesizing galactose containing glycans, including gangliosides and glycoproteins, unless galactose is provided to the culture media (33). These defects are completely reversible by addition of galactose and N-acetylglucosamine. That is why Guan and co-workers cultured the IdID/EGFR cells in media with galactose (+Gal) or without galactose (-Gal) and also with exogenous GM3. The results received confirm, in agreement with previous observation by Bremer and co-workers (22), the inhibitory effect of the exogenous GM3 on the activation of the EGFR tyrosine kinase only in +Gal media. At the same time the biological consequence of adding the exogenous GM3 resulted in the inhibition of cell growth (34). The conclusion is that carbohydrate-to-carbohydrate interaction between GM3 and N-acetylglucosamine termini of N-linked oligosaccharide chains of the receptor, plays a crucial role in GM3 dependent EGFR regulation of activity.

It should be mentioned that the effect of this action of the GM3 does not result in the increased phosphatase activity as a mechanism to decrease EGFR activation (35) but GM3 decreased the EGF binding for receptor. Wang and co-workers have shown this dependence using keratinocyte-derived SCC12 cell line with reduced GM3 ganglioside level. SCC12 cell line was treated with <sup>125</sup>I-labeled EGF. In the cells, which have lower gangliosides concentration, increased <sup>125</sup>I-labeled EGF binding to the EGFR was observed (36). Few years later it was found that the GM3-induced decrease in EGF binding was highly correlated with decreased availability of the EGFR in the membrane. The lower EGFR availability in higher GM3 concentration conditions was caused by increased endocytosis, which was not correlated with EGFR degradation (37).

#### GM1 AND EGFR ACTIVITY

The gangliosides have not only negative but also positive effect on EGFR activity. EGFR activity enhanc-

ers are GD1a and GM1 gangliosides. These gangliosides stimulate EGFR phosphorylation in response to EGF (38-40). In this section correlation between GM1 and EGFR will be discussed.

Hofman and co-workers (41), using fluorescence microscopy and Forster resonance energy transfer, confirmed the colocalization of EGFR with GM1. They also detected that colocalization was independent of the presence of cholesterol in cell membrane. These results suggest the direct interaction between GM1 and EGFR (41).

The interaction between GM1 and recombinant EGFR extracellular domain, purified from insect cells, has been shown in 2002 (29). Another group of scientists suggested that GM1 and EGFR interaction depends upon the presence of cysteine-rich region in the extracellular domain. This cysteine-rich region is not responsible for EGFR ligand binding but is necessary to EGFR lipid rafts localization (42). The possible role of post-translational modifications, especially glycosylation of extracellular EGFR GM1 binding site, has not been proven (41). Nevertheless the direct interaction between EGFR and GM1 resulted in lipid shell formation around EGFR transmembrane domain. Furthermore, large concentration of GM1 near EGRF could possibly affect the conformation of the receptor extracellular domain. The plausible consequence of this conformation changes might be a higher efficiency of ligand binding, receptor dimerization or even predimer formation (43). Despite such a possible molecular mechanism, interaction between GM1 and EGFR still remains to be clarified.

#### CONCLUSIONS

Cell surface gangliosides occur in glycosphingolipid-enriched domains (38) and play a key role in the regulation of cellular processes such as proliferation and differentiation (44). The biological effects of gangliosides result from their ability to modulate transmembrane signaling.

The epidermal growth factor receptor (EGFR) is one of many receptors regulated by gangliosides. It has been shown that gangliosides could regulate EGFR activity in positive or negative way. For example over-expression of GM1 generates enzymes that improve EGF-dependent cell proliferation (45). On the other hand, an inhibitory effects of GM3 have been described for EGFR signaling (13, 41).

The negative regulation of the EGFR activity by GM3 could be useful for treatment of invasive cancers, because their cells usually express higher level of EGFR or have significantly elevated EGFR activity. This possibility have opened new areas of studies. Many GM3 mimetics have been synthesized and tested. One of them was GM3 polymer, which has inhibitory effect on proliferation of NIH3T3 cells (46) and have shown a very specific binding to EGFR (47). Another group of potential drugs is lyso-GM3 (fig. 1B) and it's derivatives such as lyso-GM3 dimers, trimers, and tetramers. The lyso-GM1 and its derivatives have demonstrated

different inhibitory effect on EGFR activity. The strongest inhibitor was lyso-GM1 but it was shown to have also high cytotoxicity in comparison to not modified GM3. The second tested molecule was a dimer of lyso-GM1. It has been proven that lyso-GM1 dimer has been less toxic and also caused stronger inhibitory ef-

fect on EGFR activity than GM3 (48). The mechanism of interaction between EGFR and GM3 analogues is still unclear. Nevertheless some GM3 analogues, for example lyso-GM3 dimer, may be a good candidate for pharmacological inhibition of epidermal tumor growth.

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