Membrane Regulation of EGFR Signaling by Gangliosides

Xiao-qi Wang and Amy S. Paller[*]

Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA; Department of Dermatology and Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Abstract: Signal transduction is initiated at the cell membrane by the interaction of membrane-anchored receptors (such as growth factors or integrin) with extracellular stimuli (ligands). The availability of these receptors for their ligands and how the activated receptors triggers intracellular signaling cascades are regulated, at least in part, by cholesterol and sphingolipids of the plasma membrane. Gangliosides, sialylated glycosphingolipids, were first shown to regulate epidermal growth factor receptor (EGFR) signal transduction at the membrane level of epithelial cells in the mid-1980's. Since that time gangliosides have been shown to influence the signaling of a variety of other growth factor receptors. Regulation of signaling by gangliosides involves ganglioside-induced shifts of signaling components at the membrane level that either promote or disrupt signaling complexes. To decrease phosphorylation of the EGFR, ganglioside GM3 has been shown to promote the formation of a complex in which the caveolin-1 and tetraspanin CD82-dependent association of PKC-α with EGFR leads to increased threonine phosphorylation of the EGFR and EGFR internalization, making it unavailable for ligand stimulation. The lack of available EGFR at the membrane may also impact EGFR crosstalk with other signaling pathways, such as those triggered by activation of integrins and the urokinase-like plasminogen activator receptor.

As an interface between the extracellular environment and living cells, glycosylated proteins and lipids of the outer leaflet of the cell membrane serve as sensors for extracellular stimuli, initiating a cascade of cellular responses to stimuli *via* membrane-anchored receptors. One of the important classes of receptors is epidermal growth factor receptor (EGFR[1]). EGFR is a 170 kDa transmembrane glycoprotein comprised an 1186 amino acid polypeptide chain. EGFR is composed of three domains: an extracellular ligand-binding domain, a single transmembrane lipophilic region, and an intracellular domain that exhibits intrinsic tyrosine kinase activity [1-3]. The EGFR family has four members (EGFR or ErbB1; ErbB2; ErbB3; and ErbB4), and the family of EGFR ligands includes at least 13 members, among them EGF, transforming growth factor-α (TGF-α), amphiregulin, and heparin-binding EGF-like growth factor (HB-EGF) (for reviews, [4, 5]). The EGFR contains 11-12 potential glycosylation sites in its extracellular domain which are critical for both its ligand binding and ability to signal. Binding of EGFR with its ligands activates EGFR signaling by phosphorylation of its intracellular tyrosine kinase sites [1, 6]. EGFR and its ligand binding induces receptor dimerization, activation, and internalization with recycling [7]. EGF ligand induced cell proliferation involves a signaling cascade that requires downstream activation of Ras, Raf, and MAP kinase, and activation of phosphatidylinositol (PI) 3 kinase promotes cell survival. The EGFR also actively crosstalks with several other membrane-based receptors (e.g. integrins) and promotes keratinocyte motility through focal adhesion kinase (FAK) activation.

Gangliosides are sialylated membrane glycosphingolipids. Their classification is based on their number of sialic acid residues, carbohydrate composition, and their chromatographic mobility [8]. First discovered in 1942 in ganglion cells [9], gangliosides are now known to be ubiquitous membrane components that are important plasma membrane receptors for toxins [10-13] as well as modulators of signaling that leads to cell growth, motility, differentiation, and survival [14-20]. Each cell type has a "signature" of ganglioside content that is altered with neoplastic transformation [21, 22]. Lipids comprise 10% of the dry weight of epidermis and gangliosides 0.1% of the total epidermal lipids, far less than that of acylglucosylceramide (3.4%), acylceramide (1.0%), other ceramides (11.3%) and other glycosphingolipids (4.1%) [23]. Ganglioside GM3 is the predominant ganglioside of cultured keratinocytes and epidermis (approximately 64% of epidermal gangliosides) and is the precursor for more complex gangliosides of epidermis [23, 24].

GM3 was first implicated as a regulator of EGFR signaling in epithelial cells by Bremer *et al.* in 1986 based on studies with the A431 and KB epithelial cell lines [14]. The expression of GM3 prevents the tyrosine phosphorylation of the EGFR and decreases cell mitogenesis [14]. Since the mid-1980's, gangliosides have also been shown to regulate the signaling of several other membrane-based receptors, such as platelet-derived growth factor receptor, fibroblast growth factor receptor, insulin receptor, urokinase-like

*Address correspondence to this author at the Department of Dermatology, Northwestern University Feinberg School of Medicine, 676 N St. Clair, Suite 1600, Chicago, IL 60611-2997, USA; Tel: 312-695-3721; Fax: 312-695-0664; E-mail: apaller@northwestern.edu

[1]EGFR, epidermal growth factor receptor; GM3, NeuAcα2-3Gal β1- 4Glcβ1-Cer; GT1b, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8Neu Acα2-3)Gal β1-4Glcβ1-Cer

plasminogen activator, hepatocyte growth factor receptor (c-Met), and integrins [25-30]. This review will focus on the effects of gangliosides and ganglioside depletion on the EGFR specifically in keratinocytes and keratinocyte-derived cell lines and the mechanisms that lead to the observed biologic effects.

A potential role for GM3 in the physiologic regulation of keratinocyte proliferation *in vivo* was first suggested by epidermal GM3 depletion, based on immunohistochemical staining with anti-GM3 antibody, in a variety of disorders of epidermal hyperproliferation, among them squamous cell carcinoma, psoriasis, bullous congenital ichthyosiform erythroderma, and the cornoid lamellae of porokeratosis [16]. Consistent with these results, supplementation with purified GM3 (10-100 μM), and to a much lesser extent for other gangliosides of the "b" pathway of ganglioside biosynthesis (such as GD3 and GT1b), was shown to inhibit proliferation of normal keratinocytes, as well as psoriatic and ichthyotic keratinocytes [15]. This inhibition of proliferation was not accompanied by increased differentiation or demonstration of cell toxicity [15].

GM3 BINDS DIRECTLY TO THE EGFR AND DECREASES ITS AVAILABILITY FOR LIGAND BINDING AT THE MEMBRANE

The mechanism by which gangliosides suppress EGFR signaling has been the focus of investigations during the past decade. GM3 is able to co-immunoprecipitate with the EGFR [31] and binds to the EGFR by ELISA assays [32]. Based on bead binding assays with EGFR immunoprecipitated from SCC12 and A-431 cell lines, GM3 is known to directly bind to the EGFR through carbohydrate-to-carbohydrate interactions that require both glycosylation of the EGFR and sialylation of GM3 [33, 34]. Increases in GM3 content induced by antisense-induced blockade of GM3 metabolism prevent EGFR dimerization, concurrent with inhibition of EGFR and ERK activation [35]. GM3 does not increase phosphatase activity as a mechanism to decrease EGFR activation [19]. Scatchard and displacement plots have shown that GM3 decreases the binding of ^{125}I-labeled EGF for its receptor in the keratinocyte-derived SCC12 cell line, whereas depletion of gangliosides, including GM3, through cleavage of their terminal sialic acid residue by stable overexpression of plasma membrane-based ganglioside-specific sialidase (Neu3) increases ligand binding to the EGFR [19]. The GM3-induced decrease in ligand binding is associated with decreased availability of the EGFR on the membrane, which has more recently been shown to result from increased endocytosis, but not degradation of the EGFR, in the presence of increased GM3 [36].

GANGLIOSIDES AS ORGANIZERS OF MEMBRANE MICRODOMAINS

The current model of the cellular plasma membrane postulates that different classes of membrane associated proteins reside in specific plasma membrane microdomains, namely lipid rafts [37-39]. Lipid rafts are highly mobile, cholesterol-dependent domains in which receptors and signaling molecules are able to associate (see other papers in this supplement). Gangliosides are components of lipid raft domains within the membrane, and each ganglioside sorts into separate but overlapping domains by Triton X-100 extraction of membranes and sucrose gradient separation (Wang and Paller, unpublished data). Increasing evidence suggests that gangliosides are part of a team of organizers within these membrane microdomains, often working jointly with caveolin-1 and/or tetraspanins to promote or disrupt signaling in a manner that is ganglioside- , receptor- and cell type-specific [35, 36, 40]. Caveolin-1 is predominantly located in caveolae, a particular form of functional lipid raft. Caveolae have been distinguished ultrastructurally by their invaginated shape and by the presence of caveolin-1. As a key component of caveolae, caveolin-1 is thought to facilitate the interaction of receptors and signaling components by which to either stimulate or inhibit signaling activation in caveolae. EGFR is largely localized in flat noncaveolar membrane domains, and is thought to interact directly with caveolin-1 through the "scaffolding domain" of caveolin-1 (for review, [41]). Binding of caveolin-1 to the EGFR suppresses EGF-induced proliferation and migration, while dissociation from caveolin-1 promotes EGFR activation [36].

GM3 has been localized to carbonate- and detergent-insoluble low density glycosphingolipid-enriched membrane regions that biochemically match the distribution of caveolae in SCC12 cells [35]. However, when GM3 is increased, either through biochemical supplementation or by genetic blockade of GM3 metabolism, clustering of GM3 can be found by confocal microscopy at the epithelial cell membrane, suggestive of shifts of GM3 out of caveolar domains [35]. This clustering of GM3 is accompanied by increased distribution of GM3 into noncaveolar regions that correspond to the fractionated region in which EGFR predominates [35]. The increase in membrane content of GM3 concurrently shifts caveolin-1 from caveolar domains into the EGFR-enriched noncaveolar domains and stimulates caveolin-1 tyrosine phosphorylation; GM3 and tyrosine phosphorylated caveolin-1 co-immunoprecipitate with the EGFR under these conditions. When GM3 is depleted by sialidase overexpression, or by incubation with a small molecule inhibitor PPPP, caveolin-1 shifts in its entirety into caveolar regions where it no longer co-immunoprecipitates with EGFR and, as a result, EGFR signaling is increased [35].

PKC-α plays an important role for keratinocyte differentiation in the presence of calcium, concurrently inhibiting cell proliferation and preventing EGFR signaling [42, 43]. The association of caveolin-1 with GM3 and EGFR is now known to enable EGFR to be part of a complex that also includes tetraspanin CD82 and PKC-α [36]. The inhibition by GM3 or CD82 of EGFR signaling requires PKC-α activation by translocating PKC-α to the membrane and phosphorylating PKC-α at its serine/threonine sites. Activation of PKC-α triggers EGFR Thr^{654} phosphorylation, induces the internalization of EGFR, and makes EGFR unavailable for ligand binding-induced activation or crosstalk with other membrane molecules, such as integrin [36, 44]. All components of this complex are required for PKC-α to down-regulate EGFR activation. Furthermore, cholesterol depletion by methyl-β-cyclodextrin to disrupt the lipid rafts and ganglioside depletion both dissociates the complex and leads to activation of the EGFR. These observations are consistent with the demonstration by

Lambert *et al.* that EGFR is constitutively activated, even in the absence of EGFR ligand, by lipid raft disruption *via* cholesterol depletion [45] (see Cholesterol and Lipid Rafts as Regulators of Signaling through the EGFR in Epidermal Cells in this journal).

GANGLIOSIDES IMPACT THE ABILITY OF EGFR TO CROSSTALK

GM3 has also been shown to inhibit ligand-independent integrin-EGFR crosstalk [44]. Endogenous accumulation of GM3 disrupts the association of the integrin β1 subunit with EGFR, thereby inhibiting ligand-independent, matrix-dependent cell proliferation and concurrently inactivating ERK signaling [44]. Depletion of ganglioside, in contrast, increases the association of EGFR with tyrosine phosphorylated integrin β1, activates EGFR, and promotes the downstream Tyr[397] phosphorylation of FAK as well as cell proliferation [44]. The blockade of integrin-induced EGFR activation through elevating GM3 expression suppresses Src family kinase, PI3 kinase, ERK, and involves inhibition of tyrosine phosphorylation at residues 845, 1068, and 1148 on the EGFR [44]. Recent studies have shown that GM3 also cosegregates with uPAR in lipid rafts [46], suggesting that GM3 may regulate cell behavior impacted by uPAR signaling in specific membrane microdomains. In fact, GM3 also inhibits uPA-induced EGFR phosphorylation through blocking the association of uPAR with EGFR, but not the association of uPAR with α5β1 integrin [29]. Inhibition of EGFR signaling with AG1478 decreases the migration of epithelial cells induced by uPA to the same extent as down-regulation of the expression of FAK. Another example of the inhibitory effect of GM3 on crosstalk of the EGFR and integrin is the impact of GM3 elevation on matrix metalloproteinase-9 (MMP-9) activation [20]. Depleting GM3 specifically promotes the association of MMP-9 with α5β1 integrin, which requires activation of EGFR [20]. Depletion of GM3 in the presence of EGF results in a 15-fold increase in SCC12 cell invasiveness as a result of the MMP-9 activation [20]. CD9 (a tetraspanin that is frequently found in epithelial cells) associates with integrin in bladder epithelial cells and facilitates cell motility. However, in the presence of increased GM3, it inhibits the migration of these cells [47]. It is possible that tetraspanin is required in the association of integrin with the EGFR that activates MMP-9 in keratinocytes and keratinocyte-derived cell lines.

In summary, several investigations position ganglioside GM3 as a regulator of EGFR function, not only in ligand-stimulated EGFR activation, but also through suppression of EGFR crosstalk with integrin. In this way, membrane expression of GM3 is able to impact not only cell proliferation, but also other ligand-independent EGFR functions that require crosstalk, such as cell migration and invasiveness. Investigations to dissect the molecular mechanisms that lead to the membrane-based events of ganglioside modulation will be critical for our understanding of the function of lipid rafts and our ability to translate these mechanisms to the cure of human disease.

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