Biochimica et Biophysica Acta 1861 (2016) 1170–1179

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Integrin-mediated cell migration is blocked by inhibitors of human neuraminidase

Feng Jia, Md. Amran Howlader, Christopher W. Cairo *

Alberta Glycomics Centre, Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form 16 June 2016
Accepted 20 June 2016
Available online 22 June 2016

Keywords:
Glycolipid
Ganglioside
Neuraminidase
Sialidase
Integrin
Cell migration

ABSTRACT

Integrins are critical receptors in cell migration and adhesion. A number of mechanisms are known to regulate the function of integrins, including phosphorylation, conformational change, and cytoskeletal anchoring. We investigated whether native neuraminidase (Neu, or sialidase) enzymes which modify glycolipids could play a role in regulating integrin-mediated cell migration. Using a scratch assay, we found that exogenously added Neu3 and Neu4 activity altered rates of cell migration. We observed that Neu4 increased the rate of migration in two cell lines (HeLa, A549); while Neu3 only increased migration in HeLa cells. A bacterial neuraminidase was able to increase the rate of migration in HeLa, but not in A549 cells. Treatment of cells with complex gangliosides (GM1, GD1a, GD1b, and GT1b) resulted in decreased cell migration rates, while LacCer was able to increase rates of migration in both lines. Importantly, our results show that treatment of cells with inhibitors of native Neu enzymes had a dramatic effect on the rates of cell migration. The most potent compound tested targeted the human Neu4 isoenzyme, and was able to substantially reduce the rate of cell migration. We found that the lateral mobility of integrins was reduced by treatment of cells with Neu3, suggesting that Neu3 enzyme activity resulted in changes to integrin–co-receptor or integrin–cytoskeleton interactions. Finally, our results support the hypothesis that inhibitors of human Neu can be used to investigate mechanisms of cell migration and for the development of *anti*-adhesive therapies.

1. Introduction

Cell migration is critical for normal development and plays an important role in many disease states. The seeding of tumors by cell metastasis is a target for cancer therapies; therefore, a more detailed understanding of mechanisms which contribute to cell migration are needed [1]. The extracellular matrix (ECM) surrounds tissues and organs, providing a substrate which adhesion receptors bind to. Cells then use this matrix as an anchor through association of receptors to the cytoskeleton. Integrins are a major class of adhesion receptors that interact with the ECM, and which participate in transmembrane signaling [2,3]. The integrins are heterodimeric proteins which vary by cell type, with α4β1 and α5β1 being the most prevalent on epithelial cells [4,5], making them potential targets for cancer therapies [6].

The β1 integrins are regulated through a variety of biochemical mechanisms, including phosphorylation, clustering, conformational change, and lateral association with proteins and glycolipids [7,8]. Glycolipids are known to participate in integrin regulation. For example, GT1b negatively regulates cell motility, spreading, and adhesion on fibronectin (FN) through direct molecular interactions with the α5 subunit of the α5β1 integrin [9–12]. GM3 has been proposed to associate with the β1 integrin [13]. GM3 also affects cell migration and invasion by altering the expression of matrix metalloproteinase-9 (MMP-9), a co-receptor which directly interacts with the α5β1 integrin [14]. Other integrin-associated proteins, including EGFR and the urokinase-type plasminogen activator receptor (uPAR) interact with specific glycolipids which regulate their activity [15,16]. Thus, glycolipid composition of the membrane may directly or indirectly modulate integrin function.

Glycolipid composition of the plasma membrane is regulated through biosynthesis, catabolism, exocytosis, and endocytosis [17,18]. Sialic acid residues are typically at terminal positions within glycolipids, and their content is regulated by sialidase (also called neuraminidase, Neu) and sialyltransferase enzymes (SiaT). Most SiaT are found in intracellular compartments; [19] although, some SiaT activity has been found in the extracellular space [20–22]. The GM3 synthase (GM3S), a SiaT, has been shown to modulate the migration of cells in diabetes [23]. Human Neu enzymes (hNeu) have been linked to the regulation of cancer cell

Abbreviations: CER, ceramide; C9-4HMT-DANA, 9-[4-hydroxymethyl-[1,2,3]triazol-1-yl]-2,3-didehydro-*N*-acetyl-neuraminic acid; DANA, 2,3-didehydro-*N*-acetyl-neuraminic acid; ECM, extracellular matrix; FN, fibronectin; hNeu, human neuraminidase enzyme; HPTLC, high performance thin layer chromatography; LN, laminin; 4MU-NANA, 4-methylumbelliferyl-*N*-acetyl-neuraminic acid; pfNeu, *C. perfringens* neuraminidase; SPT, single particle tracking; TIRF, total internal reflection fluorescence.

* Corresponding author.
E-mail address: ccairo@ualberta.ca (C.W. Cairo).

http://dx.doi.org/10.1016/j.bbalip.2016.06.013
1388-1981/

proliferation and motility [24]. Two isoenzymes of hNeu, Neu3 and Neu4, are known to modify the glycolipid composition of cells [25,26].

Glycolipid processing enzymes are known to have a variety of roles in the regulation of cell motility, adhesion, and signaling through integrins. Neu3 expression alters the proliferation of cancer cells [27], and the enzyme has been shown be a positive regulator of cell migration in renal and prostate cancers [28,29]. Lactosyl ceramide (LacCer), a product of Neu activity on GM3 substrates, is known to mediate neutrophil adhesion, phagocytosis, and superoxide generation [30–32]. Importantly, studies in animals have found that prostate cancer cells with suppressed Neu3 activity have reduced metastasis and growth [33,34]. Neu4 has been shown to negatively regulate cell motility in matrigel [35]. Therefore, evidence suggests that Neu3 and Neu4 can play an important regulatory role in integrin-mediated cell migration. Specific inhibitors of hNeu isoenzymes would then be useful tools for the development of therapeutics. However, few selective and potent inhibitors of hNeu have been reported or tested for this purpose.

Our group has been working to develop specific inhibitors of hNeu in order to investigate their role in adhesion and cancer [36]. The general sialidase inhibitor, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA), has activity against human, bacterial, and viral neuraminidase enzymes [37,38]. Previous studies have shown that DANA has a low micromolar IC_{50} against all isoenymes of hNeu [39]. We have reported inhibitors with specificity for Neu2, Neu3 [37,39], and Neu4 [40]. A selective inhibitor of Neu4 has been shown to alter stem-cell marker expression and suppress proliferation of glioblastoma cells [41]. However, neither DANA nor these newly identified compounds have been used to investigate the role of native sialidase enzymes in the regulation of integrin adhesion. Evidence of the ability of Neu inhibitors to alter integrin-mediated cell migration would validate this family of enzymes as potential targets for *anti*-adhesive therapeutics [6,42].

Herein we report our investigation of the role of human neuraminidase enzymes in the regulation of β1 integrin-mediated cell migration of transformed cells on a FN substrate. We implemented a scratch assay to measure changes in the rate of cell migration [43]. Additionally, we employed immunofluorescence and single-particle tracking (SPT) to observe changes in the subcellular location and diffusion of integrin receptors within the membrane. To gain insight into the role of specific glycolipids in this process, we tested the effect of recombinant glycolipid processing enzymes (Neu3 and Neu4), a general neuraminidase inhibitor (DANA), and exogenous gangliosides (LacCer, GM1, GM3, GD1a, GD1b and GT1b) on cell migration. Critically, we tested the only known nanomolar-active inhibitor of a single Neu isoenzyme (Neu4) and found that the compound was an excellent inhibitor of cell migration.

2. Results

2.1. Glycolipid composition of cells is altered by Neu treatment and inhibition

Cellular gangliosides can be qualitatively analyzed by high-performance thin layer chromatography (HPTLC) [44]. Treated cell extracts were analyzed to determine changes in sialo- and asialo-glycolipids. Individual glycolipid bands attributed to LacCer or GM3 were integrated by densitometry, and a ratio was determined by comparison with that of GM3 (LacCer:GM3; asialo:sialo) (Fig. S1). Examination of lipids extracted from cells treated with the NanI sialidase from *Clostridium perfringens* (pfNeu) showed an increase in asialo glycolipids. Treatment with the sialidase inhibitor, DANA, showed an opposite effect with a decrease in the asialo:sialo ratio of glycolipids, confirming inhibition of native Neu activity. A small increase in asialo glycolipids was observed when cells were treated with recombinant human Neu3 enzyme [39]. These results confirmed that exogenous sialidase enzymes can alter membrane glycolipid composition, and that inhibition of native sialidase enzymes resulted in an increase of sialic acid-containing glycolipids.

2.2. Human Neu3 and Neu4 have α(2,3)- and α(2,8)-sialidase activity for glycolipids

To provide insight into the specificity of these enzymes for complex ganglioside substrates, we tested the activity of recombinant Neu3 and Neu4 in vitro [39,45,46]. Lipid substrates were incubated with the enzyme and the products were detected using HPTLC. Substrates were used at identical concentrations and were incubated with enzyme samples calibrated for similar specific activity using a 4MU-NANA assay [46]. Gangliosides GM1, GM3, GD1a, GD1b, and GT1b were tested with three neuraminidase enzymes: Neu3, Neu4, and pfNeu (Fig. S2). The data confirmed that Neu3, Neu4, and pfNeu were able to cleave the α(2,3) linked sialic acid of GM3; however, none of the enzymes were able to cleave the same linkage in GM1 at an appreciable rate – not even the promiscuous pfNeu enzyme [47]. We suspect that the resistance of the internal α(2,3) linkage of GM1 may indicate that the recognition of the substrate requires a free C4-hydroxyl of the internal galactose residue [46]. We observed that GT1b, which contains both α(2,3) and α(2,8) linkages was a substrate for all three enzymes. In this experiment, a new band was observed for all three enzymes at the R_f of GM1, as well as an intermediate band which was ascribed to GD1b. Further testing of both GD1a and GD1b confirmed that GD1a was a substrate for all three enzymes, while GD1b showed only trace cleavage by Neu3 (Table S1). These data are consistent with an α(2,8) activity for both Neu3 and Neu4; however, the absence of a GD1a intermediate in the GT1b cleavage experiments implies that the α(2,3) activity is much greater than that of the α(2,8) of both enzymes. The observed specificity of all three enzymes is summarized in Fig. 1.

2.3. Cell migration on fibronectin is modulated by Neu3 and Neu4

We proceeded to test the role of hNeu in cell-matrix interactions using a cellular migration assay. A variety of assays can be used to evaluate changes in cell adhesion and migration [48]. We selected a scratch assay as it is easy to implement, and could be used to measure small changes in cell migration rates using microscopy [49]. We used two human epithelial cell lines: HeLa and A549. Lung epithelia has been shown to express Neu3 [50], where they play a role in cellular migration [51]. In order to provide a controlled surface with known integrin-matrix interactions, substrates were coated with fibronectin (FN), which primarily interacts with the α4β1 and α5β1 integrins [52]. Control experiments confirmed that treatment of the substrate with FN was required for cell attachment, and blocking with an *anti*-α5 integrin antibody resulted in cell detachment. Thus, while these cells express other integrins, the matrix used in the experiment restricts interactions with the substrate to those between FN and α5β1 integrin.

Cells were grown to a monolayer on FN-coated plates, and a wound was generated using a pipette tip [43]. Representative raw data are shown for the control (buffer treated) condition in Fig. S3. We found that the data for both cell lines were well fit as a line (r^2 values 0.92–0.99). To avoid enzyme degradation treatment with Neu3 and pfNeu were conducted over a 3 h period; Neu4 experiments were conducted over 1 h period, (all with four time points including $t = 0$). In control experiments, FN-coated plates were pre-treated with Neu3 and pfNeu enzymes. No effect on cell migration was observed for Neu-treated FN (Fig. S4), allowing us to conclude that effects from the enzyme are the result of changes in cellular targets rather than the ECM substrate. With our assay established, we examined conditions that could contribute to changes in cell migration.

Among the neuraminidase enzymes tested with HeLa cells, Neu3, Neu4, and pfNeu were all found to increase the rate of cell migration (Fig. 2a). The effect of Neu3 was confirmed to be a result of enzyme activity, as an inactive Neu3(Y370F) mutant showed no effect on