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Control of cell motility by interaction of gangliosides, tetraspanins, and epidermal growth factor receptor in A431 versus KB epidermoid tumor cells

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Dedicated to Professor Hans Kamerling on the occasion of his retirement from Utrecht University, The Netherlands

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ABSTRACT

Growth of epidermoid carcinoma cell lines, A431 and KB, has been known to be controlled by the interaction of epidermal growth factor (EGF) and its receptor (EGFR) with tyrosine kinase. Ganglioside GM3 was previously found to interact with EGFR and to inhibit EGFR tyrosine kinase. However, motility of these cells, controlled by EGFR and ganglioside, was not studied. The present study is focused on the control mechanism of the motility of these cells through interaction of ganglioside, tetraspanin (TSP), and EGFR. Key results are as follows: (i) The level of EGFR expressed in A431 cells is ~6 times higher than that expressed in KB cells, and motility of A431 cells is also much higher than that of KB cells, yet growth of A431 cells is either not affected or is inhibited by EGF. In contrast, growth of KB cells is enhanced by EGF. (ii) Levels of TSPs (CD9, CD82, and CD81) expressed in A431 cells are much higher than those expressed in KB cells, and TSPs expressed in A431 cells are reduced by treatment of cells with EtDO-P4, which inhibits the synthesis of glycosphingolipids (GSLs) and gangliosides. (iii) These TSPs are co-immunoprecipitated with EGFR in both A431 and KB cells, indicating that TSPs are closely associated with EGFR. (iv) High motility of A431 cells is greatly reduced, while low motility of KB cells is not affected, by treatment of cells with EtDO-P4. These results, taken together, suggest that there is a close correlation between high motility of A431 cells and high expression of EGFR and TSPs, and between ganglioside GM3/GM2 and TSP. A similar correlation was suggested between the low motility of KB cells and low levels of EGFR and TSP. The correlation between high motility and high level of EGFR with the ganglioside–TSP complex in A431 cells is unique. This is in contrast to our previous studies that indicate that motility of many types of tumor cells is inhibited by a high level of CD9 or CD82, together with growth factor receptors and integrins.

1. Introduction

Human epidermoid carcinoma cell lines A431 and KB are characterized by a clear expression of epidermal growth factor receptor (EGFR) tyrosine kinase activity.[1–3] Both A431 and KB cells stimulated with epidermal growth factor (EGF) display enhanced tyrosine kinase activity, which is inhibited by ganglioside GM3 but not by ganglioside GM1 or sialyl-nLc4.[4] Such an inhibitory effect by ganglioside GM3 was found to be due to the interaction of GM3 ganglioside with the extracellular EGFR domain.[5] Recent studies on the mechanism of such interaction in A431 cells indicated that GM3 interacts with N-linked glycan having multiple GlcNAc termini, expressed in EGFR, and such interaction was assumed to inhibit EGF-induced EGFR tyrosine kinase.[6]

Previous studies on A431[7] and KB cells[8] were focused on cell growth based on EGF-stimulated EGFR tyrosine kinase, and the effect of gangliosides on cell growth through modulation of EGFR kinase activity. No studies have been conducted so far on the regulation of EGFR-dependent cell motility. The present study is based on our recent, incidental observation that A431 cells express much higher levels of TSPs CD9, CD81, and CD82 than do KB cells. These TSPs were shown to interact with ganglioside GM3 or GM2,[9,10] and such ganglioside–TSP complexes were found to inhibit

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; EtDO-P4, D-*theo*-1-(3′,4′-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM3, NeuAcα3Galβ4Glcβ1Cer; GM2, GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer; GSL, glycosphingolipid; HRP, horseradish peroxidase; ITS, insulin–transferrin–selenium medium; TLC, thin-layer chromatography; T–TBS, TBS (140 mM NaCl–10 mM Tris–HCl, pH 8.0) with addition of 0.05% Tween 20.

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motility, in addition to growth, of various tumor cells, possibly through integrin-dependent pathways,[9–12] although the exact mechanism still remains unclear. In the present study, we therefore explore how the level of TSPs is correlated with motility of A431 versus KB cells, in association with the level of EGFR expressed in A431 versus KB cells. A novel GSL–TSP–EGFR association in A431 cells, which mediates high cell motility, is clearly demonstrated.

2. Materials and methods

2.1. Cell lines

Two types of human epidermoid carcinoma cell lines, A431 and KB, were purchased from ATCC and were originally described in Refs. 7 and 8, respectively. These cells were grown and maintained in DMEM supplemented with 10% FCS, 100 IU/mL penicillin, and 100 unit/mL streptomycin at 37 °C in 5% CO_2–95% air.

2.2. Antibodies and other materials

Antibodies used were as follows: mouse anti-GM2 IgM mAb MK1-8[13] was donated by Reiji Kannagi (Aichi Cancer Center, Nagoya). Mouse anti-GM3 IgG3 mAb DH2 was established in this laboratory.[14] Mouse anti-EGFR mAb, rabbit polyclonal anti-CD9 IgG, rabbit polyclonal anti-CD82 IgG, mouse monoclonal anti-integrin α3 IgG_1, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, and bovine anti-goat IgG-HRP (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA); mAb anti-phospho-Tyr (PY20, BD Biosciences); mouse anti-CD81 mouse IgG2a (Beckman–Coulter, Brea, CA); and mouse anti-γ-tubulin mAb (Sigma, St. Louis, MO). PVDF membrane was obtained from Millipore Corp. (Billerica, MA). A cell proliferation assay kit including WST-1–ECS solution was obtained from Chemicon (Temecula, CA). Lipid stain reagent, Primulin, was obtained from Sigma–Aldrich Chemical Co. (Milwaukee, WI). EtDO-P4, the potent inhibitor of GlcCer synthase,[15] was donated by James A. Shayman (Dept. of Internal Medicine, Univ. of Michigan).

2.3. Expression pattern of epidermal growth factor receptor in A431 and KB cells

Levels of epidermal growth factor receptor (EGFR) expressed in A431 and KB cells were determined by Western blot analysis, followed by densitometry of the EGFR band by the SCION IMAGE program, relative to γ-tubulin as the loading control, which was blotted in a separate membrane.

Cells (7×10^5) were grown in a 100-mm round culture plate until 80% confluency. Cultured cells, harvested by a rubber scraper, were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM tetrasodium pyrophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 5 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 2 mM phenylmethanesulfonyl fluoride, and 0.076 IU/mL aprotinin), and centrifuged at 13,000 rpm for 10 min. A defined amount of the lysate protein (20 μg) determined by the Micro BCA™ Protein Assay Kit, was subjected to SDS–PAGE, followed by transfer to PVDF membrane. After blocking with 5% skim milk for 2 h, the membrane was rinsed with T–TBS, and bands for EGFR and γ-tubulin in each separate membrane were blotted with respective mAbs overnight at 4 °C. Membranes were rinsed, incubated with goat anti-mouse IgG-HRP, and developed by the Supersignal Chemiluminescence Substrate Kit (Pierce).

2.4. Determination of A431 and KB cell growth with or without various concentrations of EGF

The effect of EGF on cell growth was studied by dose-dependent and time-dependent changes of EGF (i) an increase in cell number determined under a microscope; (ii) ^{3}H-thymidine incorporation as described below.

A431 or KB cells (2×10^4) in 400 μL of 10% FCS–DMEM were plated in 24-well plates, incubated in a CO_2 incubator at 37 °C for 24 h, washed twice with 5% FCS–DMEM, and further cultured in 200 μL of 5% FCS–DMEM, without addition of EGF (0 pM), or with addition of various concentrations of EGF (1.7 pM, 3.4 pM, or 3.4 nM) for various durations (1, 3, or 5 days). Fresh 5% FCS–DMEM containing EGF was changed every 24 h. At the indicated time, the wells were washed three times with 0.5 mL of 5% FCS–DMEM and subjected to two procedures: (i) Cells were stained with Trypan Blue, and cell numbers were counted in all nine sections of the hemocytometer under a microscope. (ii) Cells were further cultured in 200 μL 5% FCS–DMEM containing ^{3}H-thymidine (10 μCi/mL) in a 37 °C CO_2 incubator for 2 h, detached by treatment with 200 μL of trypsin–EDTA, and transferred into a scintillation counting vial. After recovering all cells by washing with 5×0.5 mL of PBS, ^{3}H was counted.

2.5. Colorimetric determination of EGF-induced cell proliferation

Cell proliferation, based on activity of mitochondrial dehydrogenases, was colorimetrically determined using Chemicon's WST-1 reagent, according to the manufacturer's instructions. Briefly, A431 or KB cells (1×10^5) suspended in 100 μL culture medium (serum-free DMEM, F12–DMEM + insulin–transferrin–selenium medium [ITS], and 5% FCS–DMEM) were plated into each well of a 96-well plate, incubated for 24 h, and washed twice with 100 μL of each medium. The cells were further incubated in 100 μL of each culture medium containing EGF (0, 1.7 pM, 3.3 pM, 6.6 pM, 13.3 pM, 26.6 pM, 53.1 pM, 106 pM, 213 pM, 425 pM, 850 pM, 1.7 nM, 3.4 nM, 6.8 nM, and 13.6 nM). After incubation for 44 h, 10 μL WST-1–ECS solution from the Cell Proliferation Assay Kit was added to each well, cells were incubated for 3 h, shaken thoroughly for 1 min on a shaker, and the absorbance was measured using a microplate reader ($\gamma 1$ = 405 nm, $\gamma 1$ = 630 nm). This assay is based on reductive cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases.

2.6. Flow cytometric analysis of expression of gangliosides GM3 and GM2, and tetraspanins in A431 and KB cells

Cells were cultured in a T-75 flask containing DMEM with 10% FCS until 80% confluency, detached by 2 mL of 1 mM EDTA–PBS, added to 2 mL of 10% FCS–DMEM, and centrifuged at 1000 rpm for 5 min. After counting the cell number, cells (1×10^5) were placed in a 1.5-mL Eppendorf tube, centrifuged at 1000 rpm for 5 min, washed with 1% BSA–PBS, and incubated with (i) 100 μL of mouse IgG (10 μg/mL in 1% BSA–1% goat serum–0.05% azide), (ii) mouse IgM (10 μg/mL in 1% BSA–1% goat serum–0.05% azide), (iii) anti-GM3 DH2, and (iv) anti-GM2 MK1-8, on ice for 1.5 h. After washing twice with 0.5 mL of 1% BSA–PBS, the cells were mixed with 50 μL of goat anti-mouse Ig (G+M)–FITC (25 μg/mL) and incubated on ice for 45 min in the dark. After centrifugation and washing twice with 0.5 mL of PBS, cells were fixed with 100 μL of 1% paraformaldehyde–PBS overnight. Stained cells were subjected to flow cytometric analysis.

2.7. Effect of inhibition of the GSL synthesis by EtDO-P4 on the phosphorylation of EGFR

To explore the functional role of GM3, GM2, and other GSLs on the growth and motility of A431 and KB cells, EtDO-P4, the potent inhibitor of GlcCer synthase, was used. This treatment inhibits the synthesis of all GSLs derived from GlcCer, without significant