Plasma-membrane-associated sialidase (NEU3) differentially regulates integrin-mediated cell proliferation through laminin- and fibronectin-derived signalling

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We have found previously that human plasma-membrane-associated sialidase (NEU3), a key glycosidase for ganglioside degradation, was markedly up-regulated in human colon cancers, with an involvement in suppression of apoptosis. To elucidate the molecular mechanisms underlying increased NEU3 expression, in the present study we investigated its role in cell adhesion of human colon cancer cells. DLD-1 cells transfected with NEU3 exhibited increased adhesion to laminins and consequent cell proliferation, but decreased cell adhesion to fibronectin and collagens I and IV, compared with control cells. When triggered by laminins, NEU3 clearly stimulated phosphorylation of FAK (focal adhesion kinase) and ERK (extracellular-signal-regulated kinase), whereas there was no activation on fibronectin. NEU3 markedly enhanced tyrosine phosphorylation of integrin $\beta 4$ with recruitment of Shc and Grb-2 only on laminin-5, and NEU3 was co-immunoprecipitated by an anti-(integrin $\beta 4$) antibody, suggesting that association

of NEU3 with integrin $\beta 4$ might facilitate promotion of the integrin-derived signalling on laminin-5. In addition, the promotion of phosphorylation of integrin $\beta 1$ and ILK (integrin-linked kinase) was also observed on laminins. G_{M3} depletion as the result of NEU3 overexpression, assessed by TLC, appeared to be one of the causes of the increased adhesion on laminins and, in contrast, of the decreased adhesion on fibronectin – NEU3 probably having bimodal effects. These results indicate that NEU3 differentially regulates cell proliferation through integrin-mediated signalling depending on the extracellular matrix and, on laminins, NEU3 did indeed activate molecules often up-regulated in carcinogenesis, which may cause an acceleration of the malignant phenotype in cancer cells.

Key words: fibronectin, ganglioside, integrin, laminin-5, NEU3, sialidase.

INTRODUCTION

Cell interactions with the ECM (extracellular matrix) is crucial for essential biological processes such as adherence, migration, proliferation and differentiation, as well as survival [1,2]. Alterations in ECM components and the integrin family of receptors have been observed in various cancers [3,4], but their specific roles and regulatory mechanisms remain largely unclear. Ganglioside sialic-acid-containing glycosphingolipids present in cell-surface membranes are thought to make important contributions to cell-surface interactions and transmembrane signalling [5]. Altered sialylation of glycosphingolipids is observed in cancer as a ubiquitous phenotype, leading to the appearance of tumour-associated antigens, aberrant adhesion and blocking of transmembrane signalling [6]. Several reports have provided evidence that gangliosides are involved in cell adhesion to ECM through modulation of integrin functions. Ganglioside G_{M3} is required for adhesion of FUA169 cells to fibronectin through integrin $\alpha 5\beta 1$ functions [7], promoting interactions of integrin $\alpha 3$ with tetraspanin CD9 in microdomains [8], and inhibiting the association of EGFR (epidermal growth factor receptor) with integrin $\beta 1$ [9] and of integrin $\alpha 5\beta 1$ with MMP-9 (matrix metalloproteinase-9) [10].

Gangliosides are metabolically regulated by the functional balance between sialidases and sialyltransferases that are responsible for their degradation and synthesis respectively. To understand the pathological significance of aberrant alterations of gangliosides in cancer, we have been focusing on sialidases of mammalian origin. The four forms, abbreviated to Neu1, Neu2, Neu3 and Neu4 [11,12], differ in their major subcellular localizations and substrate specificities, although their functional roles are not fully understood. Plasma-membrane-associated sialidase (Neu3) is a key enzyme for ganglioside hydrolysis. To obtain functional evidence regarding Neu3, we cloned and characterized sialidase cDNAs of mammalian origin previously [13–15]. In the present study we have employed a human orthologue *NEU3* cDNA [14].

Consistent with the frequent aberrant expression of gangliosides in cancer, we have demonstrated previously [16] a remarkable up-regulation of the human plasma-membrane-associated sialidase (NEU3) in colon cancers. Because of its unique character in specifically hydrolysing gangliosides at plasma membranes, it is likely to participate in cell-surface events through modulation of gangliosides. To shed light on the molecular mechanisms underlying the increased expression of NEU3 in colon cancer, in the present study we investigated the influence of NEU3 on

Abbreviations used: BrdU, bromodeoxyuridine; ECM, extracellular matrix; EHS, Engelbreth–Holm–Swarm tumour; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; HA, haemagglutinin; HGF, hepatocyte growth factor; HRP, horseradish peroxidase; ILK, integrin-linked kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.

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integrin-mediated signalling in colon cancer cells and found promotion of cell adhesion and integrin signalling on laminins, but opposite effects on fibronectin, which could be of advantage to the progression of colon carcinoma cells.

EXPERIMENTAL

ECMs and antibodies

Laminin from EHS (Engelbreth–Holm–Swarm tumour) and fibronectin from human plasma were purchased from Asahi Techno Glass. Laminin from human placenta was obtained from Sigma. Human recombinant laminin-5 was prepared and purified as described previously [17]. Neutralizing antibodies to integrins $\alpha 3$ (ASC-1), $\alpha 6$ (GoH3), $\beta 1$ (6S6) and $\beta 4$ (ASC-8; Chemicon) were used for adhesion inhibition assays and flow cytometric analyses. An antibody to integrin $\beta 4$ (3E1) for immunoprecipitation and stimulation was also obtained from Chemicon. HRP (horseradish peroxidase)-conjugated anti-(mouse IgG₁) antibodies, antibodies to integrin $\beta 1$ for immunoprecipitation (MAR4) and immunoblotting (clone18) respectively, and antibodies to phospho-tyrosine (PY20) and Shc, were obtained from BD Biosciences. Antibodies to FAK (focal adhesion kinase), integrin $\beta 4$ and the transferrin receptor were obtained from Santa Cruz Biotechnology. The anti-phosphoserine antibody was from Sigma. Antibodies to phospho-threonine, phospho-ERK (Thr²⁰²/Tyr²⁰⁴; where ERK is extracellular-signal-regulated kinase), ERK, phospho-FAK (Tyr⁹²⁵) and phospho-Shc (Tyr³¹⁷) were from Cell Signaling Technology. Antibodies to phospho-FAK (Tyr³⁹⁷) and ILK (integrin-linked kinase) were purchased from Upstate. The HRP-conjugated anti-(rat IgG) antibody was from Jackson ImmunoResearch Laboratories. FITC-conjugated anti-(mouse Ig) and anti-(rat Ig) antibodies were obtained from Biosource; anti-HA (haemagglutinin) antibodies were from Roche Diagnostics; and monoclonal anti-G_{M3} antibodies (M2590) were from Nippon Biotech Laboratory. A monoclonal anti-NEU3 antibody, prepared as described previously [18], was subjected to HRP conjugation and was used for detection of endogenous NEU3.

Cell culture and NEU3 transfection

Human colon adenocarcinoma-derived DLD-1 cells (Health Science Research Sources Bank, Osaka, Japan), HCT-116 cells (A.T.C.C.) and Colo 205 cells (Cancer Cell Repository, Tohoku University, Sendai, Japan) were maintained at 37 °C with 5 % CO₂ in RPMI 1640 containing 10 % (v/v) FBS (fetal bovine serum). Cell-culture dishes and plates were coated with fibronectin (10 µg/ml), EHS-laminin (20 µg/ml), human placenta laminin (1 µg/ml), human recombinant laminin-5 (0.5 µg/ml) or poly-D-lysine (30 µg/ml), incubated at 37 °C for 1 h or at 4 °C overnight, washed with PBS (pH 7.4) and overlaid with 1 % (w/v) heat-denatured BSA at 37 °C for 1 h. Collagen I- and collagen IV-coated plates were purchased from BD Biosciences and were overlaid with BSA as described above. To obtain NEU3 stable transfectants, a NEU3 expression vector was constructed by subcloning the ORF (open reading frame) of human NEU3 cDNA into the pCEP4 expression plasmid vector (Invitrogen). The vector was then transfected into DLD-1 cells by Effectene^{TM} (Qiagen). Positive clones were selected under hygromycin (250 µg/ml). For transient transfection, an expression plasmid, constructed by inserting HA-tagged NEU3 cDNA into the pCAGGS expression vector [19], was transfected transiently as described above. To determine sialidase activity, cells were sonicated in 9 vol. of PBS containing 1 mM EDTA and 0.5 mM PMSF, leupeptin and pepstatin, and centrifuged at 1000 g for 10 min. The supernatant (crude extract) was then used for the measurement of sialidase

activity at pH 4.6 with mixed gangliosides from bovine brain (Sigma) as a substrate in the presence of Triton X-100. The released sialic acid was determined by the modified thiobarbituric acid method as described previously [13]. Protein concentrations were determined by dye-binding assay (Bio-Rad Laboratories). A unit of activity was defined as the amount of enzyme that cleaved 1 nmol of sialic acid.

Flow-cytometric analyses

Subconfluent cells were harvested by trypsinization, washed with PBS, resuspended in PBS containing 0.5 % BSA and incubated with monoclonal anti-integrin antibodies or control mouse IgG (Santa Cruz Biotechnology) in 0.5 % BSA for 30 min on ice. After washing twice with PBS, cells were incubated with FITC-conjugated secondary antibodies in 0.5 % BSA for 30 min on ice. Cells were then washed twice with PBS and cell-surface integrins were determined using a flow cytometer (BD Biosciences).

Cell-adhesion assays

For cell-adhesion assays, a Crystal Violet colorimetric method was employed [20]. Briefly, subconfluent cells were harvested by trypsinization, washed and suspended in serum-free medium containing 0.1 % BSA at 1×10^6 cells/ml, then plated on 96-well cell-culture plates coated with ECM and incubated at 37 °C for 15–60 min. Cell-culture plates were washed with PBS to remove unbound cells, fixed with 70 % ethanol at 4 °C for 15 min, stained with 0.1 % Crystal Violet (Sigma) at room temperature for 25 min and washed twice with distilled water. Crystal Violet in adherent cells was eluted with 10 % (v/v) acetic acid, and cell adhesion was determined by A₅₅₀ on a microplate reader (Corona Electric). In cell-adhesion-inhibition assays, suspended cells were incubated with 5 µg/ml neutralizing anti-integrin antibodies at 37 °C for 30 min before plating. In some experiments, cell-adhesion assays were performed at 4 °C to determine integrin $\alpha 6\beta 4$ -mediated cell adhesion [21]. To examine the effects of ganglioside G_{M3} on cell adhesion, cells were untreated or pretreated with G_{M3} (Alexis Biochemicals) at 50 µM in conditioned medium for 24 h at 37 °C and were assayed for cell adhesion.

Cell-proliferation and DNA-synthesis assays

Cell growth rate was measured using a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Subconfluent cells were harvested, washed and suspended in serum-free medium at 3×10^4 cells/ml. Cells were seeded on to 96-well ECM-coated cell-culture plates at 100 µl/well and incubated at 37 °C. After 0–96 h, WST-1 reagent was added, incubated for 1 h at 37 °C, and formazan produced by live cells was measured with a microplate reader at A₄₅₀ (reference A₆₃₀). DNA synthesis was determined by BrdU (bromodeoxyuridine) incorporation using a BrdU Labeling and Detection kit III (Roche Diagnostics). Cells, seeded on 96-well plates as above, were incubated at 37 °C for 16 h, and BrdU was then added to the cell culture. After incubation for a further 4 h, incorporated BrdU was detected according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Subconfluent cells maintained in serum-free medium for 20–24 h were harvested and suspended in serum-free medium under rotation at 37 °C for 45 min. Cells were allowed to adhere for 15–60 min to cell-culture dishes coated with ECM as described above. After incubation, dishes were rinsed with PBS to remove

Figure 1 Sialidase activity level of *NEU3*-transfected DLD-1 cells and their flow-cytometric analyses of integrin subunits

Sialidase activity was assayed with ganglioside as substrate in *NEU3*-transfected DLD-1 cells (black bars) and mock transfectants (white bars) (a), and the cells were labelled with monoclonal antibodies specific for the respective integrins and stained with FITC-conjugated second antibodies (b). Broken lines, cells treated with control mouse IgG; solid lines, cells treated with specific antibodies.

unattached cells and the adherent cells were extracted with modified RIPA buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 % Nonidet P40, 2 mM EDTA, 7.5 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 mM NaF, 2 mM orthovanadate, 0.25 % sodium deoxycholate and 2 mM PMSF] for 30 min on ice. Suspended cells without adhesion were used as the controls. Lysates were briefly sonicated and precleared with centrifugation at 1000 *g* for 10 min before use. Protein-matched samples were added to anti-(integrin $\beta 1$) or -(integrin $\beta 4$) antibodies and incubated at 4°C for 30 min (integrin $\beta 4$) or 2 h (integrin $\beta 1$). Protein G-Sepharose (Amersham Biosciences) was then added and immunoprecipitation was continued for another 3 h (integrin $\beta 4$) or overnight (integrin $\beta 1$) at 4°C. The immunoprecipitates were separated by SDS/PAGE and were transferred on to PVDF membranes (Amersham Biosciences). Transferred proteins were detected with specific antibodies by ECL® (enhanced chemiluminescence) (Amersham Biosciences) and a CCD (charged coupled device) imaging system (Bio-Rad Laboratories). Densitometric analyses were performed with Scion Image and Quantity One software (Bio-Rad Laboratories). In the immunoprecipitation of *NEU3* with the anti-(integrin $\beta 4$) antibody, 24 h after transient transfection with HA-tagged *NEU3*, cells were cultured for a further 24 h under serum depletion and then plated on to laminin-5. The cells harvested were lysed and subjected to immunoprecipitation with the anti-(integrin $\beta 4$) antibody. *NEU3* and Grb2 were detected in the immunoprecipitates with anti-HA and anti-Grb2 antibodies respectively. Endogenous *NEU3* was detected with an HRP-conjugated anti-*NEU3* antibody.

TLC

The glycolipid pattern was analysed by TLC. From 10^7 cells, glycolipids were extracted with 2 ml of propan-2-ol/hexane/water

(11:5:4, by vol.) and hydrolysed phospholipids with 0.1 M NaOH/methanol. After desalting with a SEP-PAK C₁₈ cartridge, total lipid extracts were applied to DEAE-Sephadex A25 minicolumns. Neutral glycolipids were eluted with chloroform/methanol/water (15:30:4, by vol.) and acidic glycolipids with chloroform/methanol/2 M sodium acetate (15:30:4, by vol.). After dialysis, equal amounts of each sample were applied on to HPLC plates (Baker). Neutral glycolipids were separated by chromatography in a solvent system of chloroform/methanol/0.02 % aqueous CaCl₂ (60:40:9, by vol.), and acidic glycolipids by chromatography in chloroform/methanol/0.02 % aqueous CaCl₂ (60:35:8, by vol.). Both were visualized with orcinol/H₂SO₄. TLC immunostaining was performed using monoclonal anti-G_{M3} antibodies and an avidin-biotin immunoperoxidase staining kit (Vector).

RESULTS

NEU3 promotes cell adhesion to laminins

Human colon cancer DLD-1 cells were stably transfected with *NEU3* cDNA (Figure 1a), and the clones with the highest (*NEU3*-8) and intermediate (*NEU3*-4) expression levels were examined routinely for cell adhesion and growth. When integrin expression was compared between *NEU3*-transfected and control cells by flow-cytometric analysis with their respective antibodies, the cells exhibited no significant difference in the levels of integrins $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$ and $\beta 4$ (Figure 1b), irrespective of the introduction of the *NEU3* gene. Examination of adhesion on four ECM proteins showed that stably *NEU3*-transfected cells exhibited an increase by 214 % when plated on EHS-laminin, but a decrease with fibronectin, collagen I and collagen IV, compared with control cells (Figure 2a). Adhesion to the matrix proteins reached a maximum at 30 min and remained almost constant until 60 min (results not

Figure 2 Effects of NEU3 on cell adhesion on laminins, fibronectin and collagens I and IV

(a) Cell adhesion on various ECM proteins in stable *NEU3* transfectants (shaded and closed bars) and mock transfectants (open bars). Cells were seeded on 96-well plates coated with EHS-laminin (EHS Ln), fibronectin (Fn) or collagens (Col.) I and IV and, after 30 min of incubation, adhesion activity was measured using a Crystal Violet colorimetric method. (b) Cell adhesion on different types of laminins in stable *NEU3* transfectants and control cells. Adhesion activity was determined with cells grown on plates coated with EHS-laminin, human placenta laminin (hLn) or laminin-5 (Ln5). (c) Cell adhesion activity on laminin-5, EHS-laminin and fibronectin in the transiently transfected DLD-1 and HCT-116 cells. Values are means $\pm$ S.D. ** $P < 0.01$ compared with mock transfectants, as determined by Student's *t* test. n.s., no significant difference.

shown). As it is known that EHS-laminin purified from the mouse tumour contains only laminin 1, we tested two other laminin isoforms, human placenta laminin, composed mainly of laminin-10 and -11, and laminin-5, purified from cells expressing the recombinant protein [17]. The latter laminin has often been reported to be highly expressed in carcinomas [3]. The attachment to all of the three isoforms was increased in *NEU3*-overexpressing cells compared with control cells (Figure 2b). When DLD-1 cells and another colon carcinoma cell line, HCT-116 cells, were transiently transfected with the pCAGGS vector containing *NEU3* and the cells had increased sialidase activity (4.0- and 5.7-fold respectively), adhesion was largely similar to that of the stable *NEU3*-transfected DLD-1 cells (Figure 2c), indicating that the

Figure 3 Effects of anti-integrin antibodies on cell adhesion

NEU3 (closed bars) and mock (open bars) transfectants were pre-incubated with various neutralizing anti-integrin antibodies at 37 °C for 30 min before plating on EHS-laminin (a) and laminin-5 (b). Adhesion activity was then determined. (c) *NEU3* (closed bars) and mock (open bars) transfectants were pre-incubated without (none) or with anti-(integrin β 4) antibody (anti- β ; ASC-8) at 4 °C for 30 min, plated on to laminin-5 at 4 °C for 30 min, and the inhibitory activity was evaluated. Values are means $\pm$ S.D. *P* values indicate a significant difference compared with the mock transfectants, as determined by Student's *t* test. n.s., no significant difference.

changes in adhesion activity were due to *NEU3* overexpression. To check which integrin was involved in the attachment of each laminin form, cell-adhesion-inhibition assays were performed using antibodies inhibiting integrin adhesion. Adhesion to EHS-laminin was dramatically decreased by anti-(integrin β 1) and anti-(integrin α 6) antibodies, but only slightly by antibodies to integrins α 3 and β 4 (Figure 3a). Adhesion to laminin-5 was inhibited by antibodies against integrins α 3, β 1, β 4 and α 6 (Figure 3b). Although the levels of inhibition were generally not remarkable, almost all of the activity was decreased with the anti-(integrin β 4) antibody at 4 °C (Figure 3c), as the cold conditions excluded the adhesion activity due to integrin β 1 [21]. Increased adhesion due to *NEU3* was almost prevented by treatment with the adhesion-inhibitory antibodies. These data indicate that cell attachment on EHS-laminin and laminin-5 is essentially mediated by integrin α 6 β 1 and by integrins α 3 β 1, α 6 β 1 and α 6 β 4 respectively, consistent with the observations on integrin

Figure 4 Differential effects of NEU3 on cell proliferation of cells on laminins and fibronectin

(a) Cell proliferation on EHS-laminin (EHS Ln), laminin-5 (Ln5) and fibronectin (Fn). BSA was used as a control. Cell-growth curves were determined by MTT assays. Cell growth was compared between *NEU3* transfectants (●) and mock transfectants (○). *P < 0.05 and **P < 0.01 compared with mock transfectants, as determined by Student's *t* test. (b) BrdU incorporation of cells on EHS-laminin, laminin-5 and fibronectin. BrdU incorporation into *NEU3* transfectants (closed bars) and the control cells (open bars) was determined using a BrdU Labeling and Detection kit III (Roche Diagnostics), according to the manufacturer's instructions. The effects of function-inhibitory (c) and -stimulatory (d) anti-integrin antibodies on cell proliferation were examined in the *NEU3* transfectants (closed bars) and the control cells (open bars). Values are means ± S.D. *P* values indicate a significant difference compared with the mock transfectants, as determined by Student's *t* test. n.s., no significant difference.

receptors specific for these laminins [22,23], and that the effects of *NEU3* overexpression on adhesion are dependent on matrix substrates.

NEU3 promotes cell proliferation on laminins

Results for cell growth on matrix proteins, as examined by MTT assay, are shown in Figure 4(a). Similar to the pattern of adhesion, cell proliferation was enhanced in the transfectants on EHS-laminin and laminin-5, but was decreased in cells on fibronectin. To confirm this observation, BrdU incorporation into DNA was measured (Figure 4b). Uptake in the cells on laminins was stimulated by *NEU3* overexpression, whereas a decrease was again

apparent with fibronectin. When the cells were treated with antibodies against specific integrins, the anti-(integrin $\beta 1$) antibody clearly inhibited cell proliferation on laminin-5 (Figure 4c), whereas the anti-(integrin $\beta 4$) antibody (ASC-8) affected this to a lesser extent. Since there is a possibility that the anti-(integrin $\beta 4$) antibody may be inhibitory only for cell adhesion, we used the function-activating anti-(integrin $\beta 4$) antibody (3E1) (Figure 4d). Proliferation of the treated mock transfectants was 144% of that of control-antibody-treated cells and the value of *NEU3* transfectants was 178% of the control, indicating that integrin $\beta 4$ was actually involved in the promotion of proliferation by *NEU3* transfection.

NEU3 promotes integrin-mediated signalling on laminins and facilitates the formation of a Shc-Grb2 complex

To investigate the molecular mechanisms underlying the differential effects of *NEU3* on laminins and fibronectin, the phosphorylation levels of molecules involved in integrin-mediated signalling were evaluated. As shown in Figure 5(a), tyrosine phosphorylation of FAK, which is a mediator of integrin signalling that is overexpressed in tumour cells [24,25], was increased by attachment to EHS-laminin, having a maximal stimulation at approx. 15–30 min. In *NEU3* transfectants, adhesion caused further stimulation of phosphorylation compared with that in mock transfectants. This phenomenon was observed in transfectants expressing different levels of *NEU3* (Figure 5b). At 15 min after adhesion to laminin-5 or EHS-laminin, the level of phosphorylation was also much higher in *NEU3* transfectants than in control cells. In contrast, no significant stimulation was found with fibronectin, even when accompanied by *NEU3* overexpression (Figure 5c). Human placenta laminin, the predominant ligand for $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins [26], exhibited an activation pattern similar to EHS-laminin (results not shown).

We then evaluated the tyrosine phosphorylation of Shc, an adaptor protein in another major tyrosine-kinase-dependent pathway activated by integrins which is often constitutively activated in tumours [27]. The level was relatively high, even without adhesion, and was increased by *NEU3* transfection in the cells on laminin-5, but the cells on EHS-laminin and fibronectin had decreased Shc phosphorylation (Figure 5d). As it has been proposed that integrins activate ERK through FAK or Shc, the phosphorylation of ERK was examined in cells kept in suspension or plated on to EHS-laminin. As shown in Figure 5(e), ERK was phosphorylated by adhesion to laminin in mock transfectants and to a larger extent in the two independent *NEU3*-transfected clones, with the extent of activation being enhanced in the transfectants with higher *NEU3* activity. The cells on laminin-5 had ERK phosphorylation similar to that on EHS-laminin, whereas adhesion to fibronectin did not cause significant phosphorylation (Figure 5f). These findings indicate that *NEU3* overexpression stimulates ERK phosphorylation through the FAK and Shc pathways by adhesion to laminin-5; a pathway different from that induced by EHS-laminin which had hardly any Shc phosphorylation. Furthermore, adhesion to fibronectin resulted in no significant phosphorylation, but, instead, inhibited the phosphorylation of these molecules. It should be noted that ERK phosphorylation in the cells on collagen IV was unaffected, which was different from cells on fibronectin having decreased phosphorylation. Considering the differential effects of the two laminins, we then examined how the integrin $\beta 1$ and $\beta 4$ receptors for these matrix proteins contributed to the integrin-mediated signalling, since integrins $\alpha 3\beta 1$, $\alpha 6\beta 4$ and $\alpha 6\beta 1$ have been shown to be specific receptors for laminin-5 and integrin $\alpha 6\beta 1$ for EHS-laminin [22,23]. As shown in Figure 6(a), serine/threonine phosphorylation of

Figure 5 Effects of NEU3 on the phosphorylation of FAK and ERK upon adhesion of cells on laminins and fibronectin

(a) Tyrosine phosphorylation of FAK was determined in cells attached to EHS-laminin (EHS Ln) for 30 or 60 min and in suspended (susp) cells. Cell lysates were immunoprecipitated (IP) with an anti-FAK antibody and tyrosine phosphorylation of the immunoprecipitates was detected by immunoblotting (IB) with anti-phosphotyrosine antibodies (p-Tyr). Levels of immunoprecipitated FAK are shown in the lower immunoblot. (b) Two different *NEU3*-transfected clones (*NEU3-4* and *NEU3-8*) were tested for FAK phosphorylation on Tyr³⁹⁷ with an anti-[phospho-FAK (Tyr³⁹⁷)] antibody. Total FAK is shown in the lower immunoblot. (c) Tyrosine phosphorylation of FAK on Tyr³⁹⁷ and Tyr⁹²⁵ was assessed in cells attached to EHS-laminin, laminin-5 (Ln5) or fibronectin (Fn) by immunoblotting the cell lysates with anti-(phospho-FAK) antibodies. Total FAK is shown in the bottom immunoblot. (d) Tyrosine phosphorylation of Shc in cells plated on to various ECMs. Cell lysates were analysed by immunoblotting with an anti-(phospho-Shc) antibody. Total Shc is shown in the lower immunoblot. (e) Threonine/tyrosine phosphorylation of ERK in two *NEU3*-transfected clones (*NEU3-4* and *NEU3-8*) attached to EHS-laminin or in suspended (susp) cells. Total ERK (p42 and p44 ERK) is shown in the lower immunoblot. (f) Threonine/tyrosine phosphorylation of ERK in the cells attached to EHS-laminin, laminin-5 or fibronectin. Total ERK (p42 and p44 ERK) is shown in the lower immunoblot. Representative immunoblots are shown, and quantification of the phosphorylation levels of FAK (c), Shc (d) and ERK (f) are shown relative to those observed in the mock transfectants without adhesion.

integrin $\beta 1$ was stimulated in the *NEU3* transfectants grown on the two laminins compared with the control cells, but again the phosphorylation was not increased by adhesion to fibronectin, although elevated phosphorylation was observed even without cell attachment in all of the cases. ILK, an intracellular serine/threonine kinase interacting with integrin $\beta 1$ [28], was then immunoprecipitated with an anti-ILK antibody, and the level of threonine phosphorylation was estimated. Consistent with integrin $\beta 1$ phosphorylation, an increase in phosphorylation of ILK was observed with *NEU3* overexpression in cells attached to the laminins, whereas this was decreased in cells on fibronectin

(Figure 6b). Next, integrin $\beta 4$ phosphorylation was analysed by immunoprecipitation of cell lysates with an anti-(integrin $\beta 4$) antibody, followed by immunoblotting with an anti-phosphotyrosine antibody. Integrin $\beta 4$ was found to be phosphorylated effectively on adhesion to laminin-5, and the level was much higher after *NEU3* transfection; however, phosphorylation was inhibited on EHS-laminin (Figure 6c). To verify the differential effects of *NEU3* on integrin $\beta 4$ phosphorylation between the two laminins, the immunoprecipitates were analysed for recruitment of the related adaptors, Shc and Grb2, in response to adhesion. Integrin $\beta 4$ activation was accompanied by recruitment of Shc and its

Figure 6 Effects of NEU3 on the phosphorylation of integrin $\beta 1$, ILK and integrin $\beta 4$ upon adhesion of cells on laminins and fibronectin

(a) Threonine/serine phosphorylation of integrin $\beta 1$ in cells attached to various ECM elements and in suspended (susp) cells. The phosphorylation level was assessed by immunoprecipitation (IP) with an anti-(integrin $\beta 1$) antibody, followed by immunoblotting (IB) with anti-phosphothreonine (p-Thr) and anti-phosphoserine (p-Ser) antibodies. Levels of integrin $\beta 1$ immunoprecipitated are shown in the bottom immunoblot. (b) Threonine phosphorylation of ILK on various ECM elements. To detect phosphorylation, cell lysates were immunoprecipitated (IP) with an anti-ILK antibody, followed by immunoblotting (IB) with an anti-phosphothreonine (p-Thr) antibody. Levels of ILK immunoprecipitated are shown in the lower immunoblot. (c) Tyrosine phosphorylation of integrin $\beta 4$ and recruitment of Shc and Grb2. Cell lysates were immunoprecipitated (IP) with an anti-(integrin $\beta 4$) antibody and the immunoprecipitates were immunoblotted (IB) with anti-phosphotyrosine (p-Tyr), anti-Shc and anti-Grb2 antibodies. Levels of integrin $\beta 4$ immunoprecipitated are shown in the bottom immunoblot. (d) Co-immunoprecipitation of NEU3 with integrin $\beta 4$. Cells transiently transfected with HA-tagged NEU3, either in suspension or attached to laminin-5, were used for immunoprecipitation (IP) with an anti-(integrin $\beta 4$) antibody and NEU3 and Grb2 in the immunoprecipitates were determined by immunoblotting (IB) with anti-HA and anti-Grb2 antibodies respectively. Levels of integrin $\beta 4$ immunoprecipitated are shown in the second immunoblot. This experiment was repeated three times with similar results. (e) Co-immunoprecipitation of endogenous NEU3 with integrin $\beta 4$. Colo 205 cells attached to laminin-5 or in suspension were collected for immunoprecipitation (IP) with an anti-(integrin $\beta 4$) antibody, and endogenous NEU3 in the immunoprecipitates was determined with an anti-NEU3 antibody. This experiment was repeated twice with similar results. Representative immunoblots are shown, and quantification of the phosphorylation levels of integrin $\beta 1$ (a), ILK (b) and integrin $\beta 4$ (c) and recruitment of Shc (c) relative to those observed in the mock transfectants without adhesion was determined. EHS Ln, EHS-laminin; Ln5, laminin-5; Fn, fibronectin.

association with Grb2, as reported previously [29], and activation was prominent in NEU3 transfectants (Figure 6c), consistent with the level of Shc phosphorylation (Figure 5d). Additionally, exogenous NEU3 was detected in the immunoprecipitates with the anti-(integrin $\beta 4$) antibody (Figure 6d) under conditions where neither the NEU3 protein band in the cells treated with control IgG nor the transferrin receptor, expressed abundantly (results not shown), was detected, suggesting that the presence of NEU3 is possibly specific for this event with integrin $\beta 4$. To strengthen

the effect of NEU3, an attempt to detect endogenous NEU3 in the immunoprecipitates with the anti-(integrin $\beta 4$) antibody was determined using Colo 205 cells, which have relatively high NEU3 expression. Endogenous NEU3 was also detected as a faint band, but reproducibly in the immunoprecipitates (Figure 6e). Although the co-immunoprecipitation of NEU3, expressed either endogenously or exogenously, with integrin $\beta 4$ was not adhesion-dependent, recruitment of NEU3 may create an environment in which it is readily accessible to integrin $\beta 4$ to activate signalling.

Figure 7 Effect on the level of G_{M3} (a and b) and G_{M3} -mediated cell adhesion (c) in *NEU3*-transfected cells

(a) Glycolipids extracted from *NEU3*-transfected DLD-1 cells and mock transfectants were analysed by TLC. Ganglioside fractions were developed in chloroform/methanol/0.02 % aqueous $CaCl_2$ (60:35:8, by vol.) and were visualized with orcinol/ H_2SO_4 . (b) Gangliosides were analysed further by immunostaining using a monoclonal anti- G_{M3} antibody and an avidin–biotin staining kit. (c) Effects of G_{M3} on adhesion activity were examined by pretreating cells with G_{M3} , followed by plating on to EHS-laminin, laminin-5 or fibronectin. Values are means $\pm$ S.D. P values indicate a significant difference compared with its pair, as determined by Student's *t* test. n.s., no significant difference.

Taken together, the data indicate that *NEU3* overexpression promoted integrin- $\beta 4$ -mediated signalling on laminin-5, in addition to integrin $\beta 1$ activation, leading to greater activation of FAK and Shc pathways and subsequent ERK phosphorylation than observed on EHS-laminin only. Unlike these laminins, fibronectin negatively influenced integrin activation due to *NEU3*.

Possible involvement of gangliosides in cell adhesion

Finally, we examined the possible mechanisms of an alteration in integrin-mediated signalling caused by *NEU3* overexpression. To determine whether glycolipids produced as the result of *NEU3* enzyme activity actually affect cell adhesion, glycolipids from DLD-1 cells employed in the experiments were analysed by TLC. In addition to a slight increase in lactosylceramide in neutral

glycolipid fractions (results not shown), *NEU3* transfectants had a significant decrease in G_{M3} gangliosides compared with control cells in the acidic glycolipid fractions (Figure 7a), which was confirmed by TLC immunostaining using an monoclonal anti- G_{M3} antibody (M2590; Figure 7b). Therefore we incubated cells with G_{M3} in culture medium containing 10 % (v/v) FBS for 24 h and measured cell adhesion on EHS-laminin, laminin-5 and fibronectin. As shown in Figure 7(c), G_{M3} significantly inhibited adhesion of cells on EHS-laminin, but essentially had no effect on laminin-5. However, addition of G_{M3} had a tendency to increase adhesion on fibronectin. The results indicate that a decrease in G_{M3} is a possible mechanism for *NEU3*-induced stimulation of adhesion to EHS-laminin and subsequent activation of integrin-mediated signalling. However, a change in the level of G_{M3} was not effective for adhesion to laminin-5, whereas G_{M3} had the opposite effect in cells on fibronectin by increasing adhesion. In cells on laminin-5, acceleration of adhesion and the subsequent signalling might be partially due to positive regulation by interaction of *NEU3* with the Shc–Grb2 complex as shown in Figure 6(c), although the participation of a small amount of an unidentified glycolipid product(s) cannot be excluded.

DISCUSSION

The present study provides the first evidence that plasma-membrane-associated sialidase (*NEU3*) promotes cell adhesion to laminins, integrin-mediated signalling to ERK and subsequent activation of cell proliferation, but attenuates adhesion to fibronectin and its related signalling. Among the three types of laminins tested, only laminin-5, a major ligand for integrin $\alpha 6 \beta 4$, contributed to Shc phosphorylation in addition to FAK phosphorylation; *NEU3* enhancing both adhesion-dependent and -independent tyrosine phosphorylation of integrin $\beta 4$ and Shc, recruitment of Grb2 and ERK phosphorylation. Although the distinct roles of Shc and FAK in ERK activation are not fully understood, they have been suggested to participate in early and persistent events respectively [30]. *NEU3* overexpression may thus contribute to enhance and sustained ERK activation through Shc and FAK signalling when cells are attached to laminin-5, leading to adhesion-dependent cell proliferation. ERK phosphorylation on EHS-laminin appeared to be relatively modest (Figure 5f) compared with the magnitude of enhancement of cell adhesion and proliferation, but tyrosine phosphorylation of integrin $\beta 1$ was more evident than on laminin-5 (Figure 6) and this may lead to the enhanced adhesion and proliferation via not only ERK, but also other pathways, including PI3K (phosphoinositide 3-kinase)/Akt, that are responsible for regulation of cell proliferation and survival. In fact, Akt phosphorylation was activated by *NEU3*-overexpressing cells (results not shown). In the present study, attachment to laminin-5 was also found to increase cell motility, which was largely inhibited by anti-(integrin $\beta 1$) antibodies, but with little effects when an anti-(integrin $\beta 4$) antibody (ASC-8) was used (results not shown). It is still possible that the antibody used for the latter is inhibitory only for adhesion, but not for cell proliferation and cell motility in this cell system. Whatever the case, the results indicate that *NEU3* differentially regulates cell adhesion and consequent signalling dependent on the individual matrix substrate, mediated by integrin $\alpha 6 \beta 1$ for EHS-laminin, by integrins $\alpha 3 \beta 1$, $\alpha 6 \beta 1$ and $\alpha 6 \beta 4$ for laminin-5 and possibly by integrin $\alpha 5 \beta 1$ for fibronectin. A schematic model for the hypothetical role of *NEU3* in integrin-mediated signalling is shown in Figure 8.

Our present results suggest further that G_{M3} depletion by *NEU3* may be one of the causes of stimulation of cell adhesion and proliferation mediated by integrins $\alpha 3 \beta 1$ and $\alpha 6 \beta 1$ through increased

Figure 8 Model of the hypothetical role of NEU3 in integrin-mediated signalling

A decrease in GM_3 levels or glycolipid accumulation, as a result of the reaction catalysed by highly expressed NEU3, promotes cell adhesion to laminins, integrin-mediated signalling to ERK and subsequent activation of cell proliferation, but attenuates adhesion to fibronectin and its related signalling pathway. On laminins, including laminin-1, laminin-10/11 and laminin-5, stimulation of cell adhesion and proliferation could be mediated by integrins $\alpha_6\beta_1$ and $\alpha_3\beta_1$ via increased integrin β_1 phosphorylation, whereas suppression of integrin $\alpha_5\beta_1$ -mediated signalling would occur on fibronectin. Furthermore, in the absence or presence of NEU3 product(s), the NEU3 molecule itself could interact with integrin $\alpha_6\beta_4$ and promote Shc phosphorylation and recruitment of Grb2 through integrin β_4 phosphorylation.

integrin β_1 phosphorylation, but suppression of integrin $\alpha_5\beta_1$ -mediated signalling (Figure 6). With or without yet unidentified NEU3 product(s), the NEU3 molecule itself could interact with integrin $\alpha_6\beta_4$ and promote Shc phosphorylation and recruitment of Grb2 through integrin β_4 phosphorylation (Figure 6). This is in line with our previous finding that NEU3 is localized in membrane microdomains, caveolae or rafts and interacts with signalling molecules such as caveolin-1 [18] and Grb2 [31] in insulin signalling. Furthermore, the compartmentalization of integrin $\alpha_6\beta_4$ in lipid rafts has been shown to be required for efficient signalling to ERK in keratinocytes [32]; NEU3 possibly being accessible to integrin $\alpha_6\beta_4$ and then promoting Shc phosphorylation and recruitment of Grb2. In this context, it is intriguing to know whether NEU3 affects HGF (hepatocyte growth factor)-induced signal transduction via the Met receptor, since the signalling has been proposed to be associated with integrin $\alpha_6\beta_4$, leading to invasion of carcinoma cells [33]. Our preliminary results have shown that NEU3 activated HGF-dependent tyrosine phosphorylation of Met receptor in DLD-1 cells (results not shown).

In direct contrast with our present results, there have been several studies reporting that adhesion to fibronectin is promoted by GM_3 depletion in SSC cells [10] and Id1 cells [8]. However, other reports have described that GM_3 depletion inhibits cell adhesion to fibronectin in rat hepatoma cells [34] and mouse mammary carcinoma cells [7]. In the present study, we have shown a reduction in ILK phosphorylation by cells on fibronectin, in contrast with its activation by GM_3 depletion in SSC cells [35]. It is necessary to elucidate why and how NEU3 displays differential effects, and this presumably occurs through recognition of individual integrin receptors. In this context, it is of interest that increased adhesion on laminins and the decrease on fibronectin by NEU3 overexpression was also observed in oral squamous cell carcinoma cells HSC-2 (K. Kato, K. Shiga and T. Miyagi,

unpublished work). This indicates that NEU3 might induce these phenomena independently of cell type, via modulation of signalling by interacting with signalling molecules, such as integrin β_4 , in addition to changing ganglioside patterns.

We have found previously a remarkable increase in expression of NEU3 in colon cancer tissues compared with adjacent non-cancerous tissues at both the mRNA and enzyme activity levels [16]. Moreover, the present results clearly indicate that NEU3 is indeed involved in activation of signalling molecules, including FAK [24,25], ILK [28], Shc [27], integrin β_4 [3,36] and also Met [33], often up-regulated in carcinogenesis. Among matrix proteins, laminins have been implicated in an increase in the malignant phenotype of tumour cells. Neo-expression of laminin-5 is associated with proliferation of carcinoma cells and this ECM element often accumulates in invading edges of carcinomas [3]. Integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ are cell-surface receptors acting with laminin-5. By contrast, fibronectin has been shown to reduce progression of carcinomas [37,38]. Since there were no significant differences in expression level of integrin receptors between NEU3 transfectants and control cells in the present study, NEU3 might function distinctly with individual integrins. In fact, NEU3 does not activate, but rather attenuates, integrin- $\alpha_5\beta_1$ -mediated signalling. From the present results, elevated expression of NEU3 in colon cancer may cause tumour progression through further activation of molecules that are up-regulated on adhesion to laminins which usually accumulate in this type of cancer. In contrast, NEU3 might negatively regulate signalling on fibronectin, resulting in suppression of cell adhesion, proliferation and migration. It should be borne in mind that NEU3 elevation is characteristic in cancer and found in a wide spectrum of lesions, including tumours of the digestive system (K. Shiga, K. Kato, T. Wada and T. Miyagi, unpublished work). The molecular mechanisms and significance of increased expression of NEU3 in cancer could be targeted with the hope of providing valuable information for diagnosis and therapy.

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