Human plasma membrane-associated sialidase (NEU3), specifically hydrolyzing gangliosides, plays crucial roles in the regulation of cell surface functions. Here we demonstrate that NEU3 mRNA level are increased in renal cell carcinomas (RCCs) compared with adjacent non-tumor tissues, significantly correlating with elevation of interleukin-6 (IL-6), a pleiotropic cytokine that has been implicated in immune responses and pathogenesis of several cancers, including RCCs. In human RCC ACHN cells, IL-6 treatment enhanced NEU3 promoter luciferase activity 2.5-fold and the endogenous sialidase activity significantly. NEU3 transfection or IL-6 treatment resulted in both suppression of apoptosis and promotion of cell motility, and the combination had synergistic effects. NEU3 scarcely affected MAPK- or IL-6-induced STAT3 activation but promoted the phosphatidylinositol 3-kinase (PI3K)/Akt cascade in both IL-6-dependent and -independent ways. Consistent with these data, NEU3 markedly inhibited staurosporine-induced caspase-3 activity and enhanced IL-6-dependent inhibition, which was abolished by LY294002, a PI3K inhibitor. Furthermore, IL-6 promoted Rho activation, and the effect was potentiated by NEU3, leading to increased cell motility that was again affected by LY294002. NEU3 silencing by siRNA resulted in the opposite: decreased Akt phosphorylation and inhibition of Rho activation. Gycolipid analysis showed a decrease in ganglioside GM3 and increase in lactosylceramide after NEU3 transfection, with these lipids apparently affecting cell apoptosis and motility. The results indicate that NEU3 activated by IL-6 exerts IL-6-mediated signaling, largely via the PI3K/Akt cascade, in a positive feedback manner and contributes to expression of a malignant phenotype in RCCs. NEU3 thus may be a useful target for RCC diagnosis and therapy.

IL-6 is a pleiotropic cytokine involved in a variety of cellular functions in inflammation and immune responses (1, 2). In addition, IL-6 is implicated in the pathogenesis and prognosis of several cancers and is known to be an autocrine growth factor for RCCs, in which it is highly expressed (3). The level is positively correlated with tumor size and stage and thus might be one factor for a poor diagnosis (4, 5). On IL-6 binding to its receptor, composed of two subunits (gp80 and gp130), the gp130-associated tyrosine kinases Jak1, Jak2, and Tyk2 become activated and phosphorylate the gp130 cytoplasmic tail on specific tyrosine residues. These phosphotyrosines serve as docking sites for signal molecules, including STATs and molecules inducing activation of the Ras/MAPK and PI3K cascades (6). Recent observations have suggested that increased production of IL-6 in RCCs is caused partially by p53 mutations (7). Although IL-6-induced proliferation involves the STAT3 pathway in renal cancer ACHN cells (8), the mechanism and significance of IL-6-mediated signaling in RCCs is not fully understood.

Gangliosides, sialic acid-containing glycosphingolipids present in cell surface membranes, are thought to make important contributions to cell surface interactions and transmembrane signaling (9). Their alteration is ubiquitously observed in cancers, with the appearance of tumor-associated antigens, aberrant adhesion, and blocking of normal signaling (10, 11). To elucidate the associated molecular mechanisms we have focused on sialidase, which catalyzes the removal of sialic acids from glycoproteins and glycolipids in an initial step of degradation of these molecules (12). In line with the aberrant expression of gangliosides in cancer, we recently demonstrated remarkable up-regulation of the plasma membrane-associated sialidase (NEU3) in human colon cancers (13). Because of its unique character in specifically hydrolyzing gangliosides at plasma membranes (14, 15), this enzyme is likely to participate in cell surface events. In fact, recent reports have presented evidence that NEU3 participates in neuronal differentiation (16–19) and transmembrane signaling (20) as well as carcinogenesis (13).

In the present study, we investigated NEU3 in RCC in connection with IL-6 function using tumor tissues from RCC patients and renal cancer ACHN cells. Our results demonstrate: 1) that sialidase is up-regulated in tumor tissues and is strongly linked to the IL-6 expression level; and 2) that NEU3 in ACHN cells is activated by IL-6 and acts in a positive feedback manner on the cytokine function, mainly through PI3K/Akt pathway, resulting in suppression of apoptosis and promotion of migration. Consistent with our previous observations (20), NEU3 was suggested to play a role as a signal modulator.

**EXPERIMENTAL PROCEDURES**

Antibody and Reagents—Recombinant human IL-6 was purchased from PeproTech and Y294002 (a PI3K inhibitor), AG490 (a JAK family kinase inhibitor), and PD98059 (a MEK inhibitor) from Calbiochem. Staurosporine and a protein kinase C inhibitor were from Sigma.
siRNAs were obtained from Dharmacon RNA Technologies, Inc. Transfection.

Without hygromycin B and were used for some experiments after 48 h of transfection.

We constructed an expression plasmid (pCEP-hNeu3) by inserting the entire open reading frame (1.2 kb) of the human NEU3 gene (14) into an expression vector, pCEP4 (Invitrogen), and hygromycin B (Roche Applied Science)-resistant clones were isolated at a concentration of 100 μg/ml. The expression plasmid was transfected into ACHN cells (1×10^6/6-well dish) using Effectene (Qiagen) as recommended by the manufacturer. ACHN cells were transfected with NEU3 without hygromycin B and were used for some experiments after 48 h of transfection.

siRNA Transfection—siRNAs targeting NEU3 and control scrambled siRNAs were obtained from Dharmacon RNA Technologies, Inc. (Lafayette, CO). The sequence for targeting NEU3 was AAGGGAGTTGGTAAAGTTT (NEU3 siRNA) beginning at nucleotide 839 of the NEU3 open reading frame sequence, and the scrambled siRNA control was GCGATTAATGTAGGTTCGA. Transfection into ACHN cells was performed in 60-mm dishes with Lipofectamine 2000 (Invitrogen). At 24–48 h after transfection, cells were used for experimentation.

SiAlidase Activity Assay—Cells were washed with PBS, sonicated on ice in 9 volumes of ice-cold PBS containing 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10 μg/ml leupeptin and pepstatin, and centrifuged at 1,000 × g for 10 min. The supernatant (crude extract) was then used for measurement of sialidase activity at pH 4.6 with mixed gangliosides from bovine brain (Sigma) as the substrate in the presence of Triton X-100. The released sialic acid was determined by the modified thioarbituric acid method or by fluorometric high-performance liquid chromatography with 1,2-diamino-4,5-methylenedioxybenzene as described elsewhere (21). Protein concentrations were determined by dye-binding assay (Bio-Rad). One unit was defined as the amount of enzyme cleaving 1 nmol of sialic acid/h.

Quantitative Reverse Transcription-PCR Analysis—Human NEU3 and IL-6 mRNA levels were evaluated by quantitative reverse transcription-PCR (real-time PCR, Lightcycler®; Roche Diagnostics) as described previously (22). Total RNA was isolated from primary tumors and adjacent tissues and also from ACHN cells with an RNKeasy kit (Qiagen) as recommended by the manufacturer. First strand cDNAs were synthesized by reverse transcription and used as templates for PCR. Human NEU3 primers were sense (5′-AGGTCAGTCTCCAGTACCTTC-3′, nucleotides 187–208 from start codon) and antisense (5′-ACATCCGACATCTGGTAGT-3′, nucleotides 588–609).

Human IL-6 primers were sense (5′-TCCATGCCAGCGTAGTACCTAC-3′, nucleotides 237–256 from start codon) and antisense (5′-GCTAGATGGACAGGGAGG-3′, nucleotides 683–702). To normalize for sample variation in ACHN cells, expression of a housekeeping isofrom of human porphobilinogen deaminase (PBGD) was measured as an internal control as described previously (22). For RCC tissue samples, human 5-aminolevinolate synthase (ALAS1) mRNA level was also measured as an internal control. Primers were sense (5′-TCCATGCCAGCGTAGTACCTAC-3′, nucleotides 220–241 from start codon) and antisense (5′-CGTGGGTTCCTGGAGG-3′, nucleotides 569–590).

Luciferase Reporter Assay—A cosmid clone was isolated from a human placenta genomic library in pWE15 (BD Biosciences Clontech), using a 1.2-kb cDNA probe containing the entire open reading frame of the human NEU3 gene, and employed for the construction of reporter plasmids by inserting DNA fragments containing the 5′-flanking region of the gene into the Smal site of the promoter- and enhancer-less luciferase reporter vector, PGV-B (Wako, Osaka, Japan). Reporter plasmids containing 1.5- and 0.5-kb fragments upstream of the apparent transcription initiation site were designed and used for transfection of ACHN cells seeded on 6-well plates. At 24 h after transfection, IL-6 was added followed by further culture for 24 h, and luciferase activity was assayed using the Dual Luciferase reporter assay system (Promega).

IL-6 Measurement by ELISA—To determine the amount of IL-6 protein produced by ACHN, ELISA was performed. Cells were plated at 1×10^6/60-mm dish, and after 24 h cell culture supernatants were harvested, centrifuged, and stored at −20 °C. Quantikine®, a human-specific IL-6 ELISA (R&D Systems), was used for determination of the amount of IL-6 present under conditions of absorbance at 450 nm, with correction at 570 nm using a microplate reader.

Western Blotting—Cells were rinsed with ice-cold PBS and lysed with cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% nonionic detergent). The samples were clarified by centrifugation at 1,000 × g for 10 min and were subjected to electrophoresis in 7.5% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes and probed with specific antibodies against phospho-IL-6 (Thr202/Tyr204), and anti-cleaved caspase-3 (Asp175) from Cell Signaling Technology; anti-STAT3 and anti-phospho-STAT3 (Tyr 705) from Upstate Biotechnology; and anti-human IL-6 from R&D Systems.
Nonidet P-40, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). After extracts were clarified by centrifugation at 10,000 g for 5 min, cell lysates containing equal amounts of proteins were resolved on 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes, blocked with TBS-Tween containing 1% bovine serum albumin, and incubated with primary antibodies. Bound antibodies were detected by using the appropriate peroxidase-coupled secondary antibodies with ECL detection (Amersham Biosciences) and the Versa Doc imaging system (Bio-Rad). Densitometric analyses were performed with Quantity One quantitation software (Bio-Rad).

**Rho Activity Assay**—Cells were washed with ice-cold PBS and lysed in Mg2+-containing lysis buffer (MLB: 25 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 25 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Cell lysates were clarified by centrifugation at 14,000 g at 4 °C for 5 min, and equal volumes were incubated with 20 μg of rhotekin RBD (Rho-binding domain)-agarose beads (Upstate Biotechnology) for 45 min at 4 °C with gentle agitation. The agarose beads were then washed with MLB and resuspended in Laemmli reducing sample buffer. Bound Rho proteins were detected by Western blotting using anti-Rho rabbit IgG fraction antibodies (Sigma).

**Cell Motility Assays**—Chemokinesis assays (undirected migration) were performed by an established method with some modification (23). In brief, cells were suspended in serum-free medium supplemented with inhibitors and/or IL-6 and seeded on the upper surface membranes of cell culture inserts (Falcon). Lower chambers were filled with the same medium, and after 24 h cells were fixed and stained with Giemsa solution. After wiping the upper surface of the stained membranes with a cotton swab to remove non-migrating cells, all cells present on the lower surface of the membranes were counted under a microscope.

**Apoptosis Assays**—Apoptosis analysis was performed by annexin V staining (Annexin-V Fluos staining kit, Roche Molecular Biochemicals). In brief, at 24 h after seeding into 60-mm dishes in 10% fetal bovine serum containing minimal essential medium, cells were treated with staurosporine in the presence or absence of IL-6 in serum-free medium.
for the appropriate periods. The cells were then double-stained with annexin V followed by propidium iodide for 10 min, and apoptotic cells were analyzed by FACSscan (BD Biosciences).

**Thin Layer Chromatography (TLC)**—Glycolipids were extracted from cells (1 × 10⁷) in sequence with 2 ml of isopropanol/hexane/water (55:25:20, v/v/v) and hydrolyzed with 0.1M NaOH/methanol. After desalting with a Sep-Pak C₁₈ cartridge, total lipid extracts were applied to DEAE-Sephadex A-25 minicolumns. Neutral glycolipids were eluted with chloroform/methanol/water (30:60:8, v/v/v) and acidic glycolipids with chloroform/methanol/2M AcONa (30:60:8, v/v/v). After dialysis, equal amounts of each sample were applied to high-performance TLC plates (Baker). Neutral glycolipids were separated by chromatography in a solvent system of chloroform/methanol/0.02% aqueous CaCl₂ (60:40:9, v/v/v), and acidic glycolipids in chloroform/methanol/0.02% aqueous CaCl₂ (60:35:8, v/v/v). All glycolipids were visualized with orcinol-H₂SO₄. Densitometric analyses were performed with Scion Image (Scion Corp., Frederick, MD) and Quantity One software (Bio-Rad).

**RESULTS**

**Increased Expression of NEU3 in Primary Human RCCs and Its Activation by IL-6 in ACHN Cells**—RCC tumors were found to possess higher NEU3 mRNA levels than adjacent normal tissues by a mean...
5.6-fold, as shown in Fig. 1A. IL-6 mRNA levels in the same samples were found to be similarly increased by a mean 6.3-fold (Fig. 1B). The increase in these genes was significantly correlated (correlation coefficient, \( r = 0.5437, p = 0.002 \)). When NEU3 mRNA and sialidase activity were assessed in ACHN cells and its NEU3 stable transfectants, IL-6 treatment significantly increased endogenous NEU3 activity by 1.2–1.6-fold and the mRNA level by 1.2–2.5-fold in the control case (Fig. 2A). Even in the stably NEU3-transfected ACHN cells, a 1.2–1.8-fold increase was observed in the activity (137 ± 5 as compared with 95 ± 4 units/mg protein for the cells without IL-6). Considering a possible effect of increased NEU3 on IL-6 production in ACHN, basal IL-6 level in the conditioned medium was determined using ELISA. The IL-6 secretion level was, however, not different between NEU3 and mock transfectants (data not shown). To determine whether the NEU3 gene transcription is activated by IL-6, we transiently transfected the NEU3 promoter luciferase vector into ACHN cells. Different lengths of the 5’-upstream region were prepared by digestion with the appropriate restriction enzymes and inserted. After 24 h of IL-6 treatment, both 1.5- and 0.5-kb fragments exhibited higher luciferase activity by a maximal 2.5-fold as compared with nontreated cells (Fig. 2B), indicating that a IL-6-driven transcriptional factor binds to the NEU3 promoter and that NEU3 expression is transactivated by IL-6, even if not remarkably so. We could not find a typical STAT3 binding element, but five atypical Sp1 binding elements were located in the promoter region of NEU3 gene. Therefore there is still a possibility that the latter may be involved in IL-6-induced transactivation in cooperation with STAT3 (24).

**Suppression of Apoptosis and Promotion of Cell Motility by NEU3 Overexpression and Potentiation of IL-6 Effects**—To elucidate the significance of increased NEU3 expression in connection with IL-6, our observations on malignant phenotype were performed using two independent clones of NEU3 stable transfectants having a 15–25-fold increase in ganglioside sialidase activity over the control cells. Staurosporine-induced apoptosis was remarkably inhibited in the NEU3 transfectants as compared with that in mock transfectants, and this inhibition was further enhanced after the addition of exogenous IL-6, as assessed by annexin V and propidium iodide staining (Fig. 3A). Similar results were obtained for serum deprivation-induced apoptosis, although less markedly (data not shown). We also found an alteration of cell motility of ACHN cells by NEU3 overexpression and IL-6 treatment (Fig. 3B). The motility of mock transfectants with IL-6 treatment was increased about 2-fold. Moreover, the motility of NEU3-transfected cells was significantly higher than for mock-transfected cells either in the presence or absence of IL-6.

**Analyses of the Molecules Involved in IL-6 Signaling**—To cast light on the mechanism of how NEU3 overexpression suppresses apoptosis and enhances motility, molecular changes in IL-6 signaling were studied in...
ACHN cells. First, the effects of NEU3 overexpression and IL-6 treatment on activation of STAT3, ERK, and Akt in JAK/STAT3, ERK, PI3K pathways, respectively, were examined using phospho-specific antibodies (Fig. 4). Total ERK proteins were comparably expressed in mock and NEU3 transfectants, but the phosphorylation levels were not affected by IL-6 or NEU3 (Fig. 4A). ERKs were constitutively activated in ACHN cells, as reported previously (8), and NEU3 transfection was not further up-regulated by stimulation with exogenous IL-6. In addition, neutralization of IL-6 using an antibody did not reduce the activation level, indicating that endogenous IL-6 produced by the cells was not responsible for the constitutive activation of ERKs (data not shown). Using a phospho-specific antibody (STAT3 at Tyr705), activation of STAT3 by IL-6 could be shown to similar extents in both mock and NEU3 transfectants, under conditions where STAT3 protein expression levels were not affected (Fig. 4B). In contrast, activation of Akt (at Ser473) was induced by IL-6 in parental ACHN and mock transfectants but was more strongly activated in NEU3 transfectants. The activation was further enhanced by IL-6 when total Akt protein was at similar levels in mock and NEU3 transfectants. Thus, PI3K activation might be responsible for the suppression of apoptosis and increased motility caused by NEU3 overexpression and by IL-6 treatment.

To confirm this possibility, we examined activation of caspase-3 downstream of Akt (Fig. 5). Cleaved caspase-3 was detected in cells treated with 50 nM staurosporine in the presence or absence of 20 ng/ml IL-6 in serum-free medium. The level was decreased in both mock and NEU3 transfectants with IL-6, although NEU3 transfection itself caused a marked decrease (Fig. 5A). When NEU3 transfectants were treated with 25 μM LY294002, 25 μM PD98059, or 25 μM AG490 and staurosporine for 7 h, and cleaved caspase-3 levels were examined. The experiments were repeated three times with similar results. Representative photomicrographs are shown, and quantitative data for caspase-3 activity are presented as values relative to those observed in the mock transfectants without either IL-6 and staurosporine. 

FIGURE 5. Reduction of caspase-3 activity by NEU3 transfection and IL-6 treatment and the effects of inhibitors. A, decreased caspase-3 activity with NEU3 transfection and IL-6 treatment. B, effective abrogation of NEU3- and IL-6-induced reduction of caspase-3 activity by a PI3K inhibitor, LY294002. NEU3 transfectants were treated with 25 μM LY294002, 25 μM PD98059, or 25 μM AG490 in the presence or absence of 20 ng/ml IL-6 and 50 nM staurosporine for 7 h, and cleaved caspase-3 levels were examined. The experiments were repeated three times with similar results. Representative photomicrographs are shown, and quantitative data for caspase-3 activity are presented as values relative to those observed in the mock transfectants without either IL-6 and staurosporine.

Rho was also activated by stimulation with IL-6 in parental ACHN and mock-transfected cells, being already highly activated in NEU3 transfectants and enhanced further by addition of IL-6 (Fig. 6A). Inhibitory effects with LY294002 were again most evident both with and without IL-6 (Fig. 6B). Similar results were obtained in other clones of NEU3 stable transfectants and also in the transient transfectants (data not shown), although the effects were not in proportion to NEU3 expression level, probably because the level was already

FIGURE 6. Increased Rho activation by NEU3 transfection and IL-6 treatment and the effects of inhibitors. A, increased Rho activation by NEU3 and IL-6. Rho activity was examined by measuring GTP-Rho binding of glutathione S-transferase-rhotekin in a pull-down assay. Representative photomicrographs are shown, and quantitative data for Rho activity are presented as values relative to that observed in the ACHN parent cells without IL-6. B, effective reduction of cell motility by the PI3K inhibitor, LY294002. NEU3 transfectants were treated with 25 μM LY294002, 25 μM PD98059, or 25 μM AG490 in the presence or absence of 20 ng/ml IL-6 for 24 h, and the motility was examined as described in the legend for Fig. 3. p values were calculated by Student’s t test.

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high enough to show the effects. To confirm further whether NEU3 directly affects these phenomena, we employed siRNA-mediated silencing of NEU3 gene. The siRNA targeting NEU3 reduced mRNA level by 60% and the scrambled control showed no significant change of level, as estimated by real-time reverse transcription-PCR using glyceraldehyde-3-phosphate dehydrogenase as the internal control (Fig. 7A). The levels of Akt phosphorylation and Rho activity were evaluated in the cells at 48 h after transfection (Fig. 7, B and C). The results were entirely opposite of those obtained by NEU3 overexpression, as expected. Transfection with NEU3 siRNA reduced Akt and Rho activation in both IL-6-dependent and -independent ways. In the presence of IL-6, NEU3 siRNA lowered especially the Akt phosphorylation level to such an extent that IL-6-dependent activation of Akt and Rho without IL-6 was significantly potentiated by NEU3 (Fig. 8B). In contrast to the stimulating effects by NEU3 overexpression, NEU3 silencing showed significant reduction in activation of Akt and Rho in IL-6-dependent and -independent ways. This strongly suggests a crucial role of NEU3 in IL-6 signaling in RCC.

**DISCUSSION**

In this study we found that NEU3 is highly expressed in RCCs, demonstrating significant correlation with IL-6 expression, and that this sialidase in ACHN cells is up-regulated by IL-6. Furthermore NEU3 sensitizes IL-6 signaling mainly via the PI3K pathway in a positive feedback manner, leading to suppression of apoptosis accompanied by enhanced cell migration. Although the promoter region of the NEU3 gene does not appear to contain a typical STAT3 binding element, so that the mechanism of transactivation of the NEU3 gene by IL-6 in ACHN cells remains to be clarified, it is possible that the presence of the Sp1 binding element in the promoter region may be responsible for the transactivation, because interaction between STAT3 and Sp1 can drive IL-6-mediated transcriptional activation of the VEGF gene with a STAT3 binding element-free promoter (24).

ERK was constitutively activated in both mock- and NEU3-transfected ACHN cells, but NEU3 overexpression caused activation of Akt and Rho without IL-6 and further potentiated activation of these molecules after IL-6 treatment, indicating a contribution of NEU3 to the IL-6-induced effects. Although the latter potentiation by NEU3 was modest, siRNA-mediated silencing of the NEU3 gene significantly decreased Akt and Rho activation in both IL-6-dependent and -independent ways. As shown in Fig. 7, A and B, in contrast to the stimulating effects by NEU3 overexpression, NEU3 silencing showed significant reduction in activation of Akt and Rho in IL-6-dependent and -independent ways. This strongly suggests a crucial role of NEU3 in IL-6 signaling in RCC.

Although it is considered that IL-6 signaling is regulated mainly via JAK/STAT, ERK, and PI3K/Akt pathways, and the binding of IL-6 to the receptor induces activation of JAK resulting in activation of the downstream cascades, STAT3, ERK, and PI3K. The latter two have been suggested to be activated by Ras (25). The results with inhibitors of these cascades (Figs. 5 and 6) revealed that NEU3 stimulates IL-6-induced Akt and Rho activation to a significant extent and consequently inhibits caspase-3 and promotes cell motility. Because it is known that PI3K stimulates Rho via guanidine nucleotide exchange factors (26), in addition to activating Akt, these results indicate that NEU3 participates in regulation of the PI3K pathway, probably via a path not involving JAK. NEU3 thus may contribute to regulation of the PI3K cascade in two different ways, dependent on and independently of IL-6, which may be linked to signaling through growth factors including epidermal growth factor (EGF) and transforming growth factor-α (TGFα) via Ras activation, because these growth factors stimulate the growth of RCC cells in an autocrine manner (27). IL-6-dependent stimulation by NEU3 is possibly due to in part to the cooperation of IL-6 with receptors for these growth factors, leading to synergistic cellular responses mediated by gp130, as evidenced by previous reports (28, 29). This hypothesis is also supported by the observation that GM3 depletion by NEU3 overexpression activates epidermal growth factor receptor signaling (30, 31).

Considering the molecular mechanisms, it is feasible that glycolipid changes due to catalytic functions of NEU3 might contribute to activation of signaling pathways. As shown in Fig. 8, the finding that the cell...
**FIGURE 8.** Accumulation of Lac-Cer and reduction of GM3 by NEU3 overexpression and IL-6 treatment and the effects on cell motility. **A**, alterations of glycolipids by NEU3 overexpression and IL-6 treatment. Glycolipids were extracted from ACHN and mock- and NEU3-transfected cells and analyzed by TLC. Neutral glycolipids (upper part) and acidic glycolipids (lower part) were visualized with orcinol-H$_2$SO$_4$. NEU3 transfection and IL-6 treatment resulted in a slight increase of Lac-Cer and a decrease of GM3, as indicated by the arrows. Quantitative data for Lac-Cer (left panel) and GM3 (right panel) levels are presented as values relative to those observed in ACHN parent cells without IL-6. **B**, alteration of cell motility by treatment with GM3 or Lac-Cer. Mock and NEU3 transfectants were treated with 25 μM GM3 or Lac-Cer for 24 h, and the motility was examined using cell culture inserts in triplicates as described in the legend for Fig. 3. *p* values were calculated by Student’s *t* test.
motility was altered by the treatment with GM3 or Lac-Cer supports this possibility. In addition, NEU3 might act as a protein molecule, interacting with signaling molecules and consequently exerting stimulatory effects. We previously demonstrated findings that NEU3 interacts with caveolin-1 in caveolae in HeLa cells (32) and that sialidase also attenuates insulin receptor phosphorylation and consequent post-receptor signaling by association with Grb-2 as well as by changing ganglioside components as a result of enzyme reactions in NEU3 transgenic mice (20). Although it is uncertain at present whether NEU3 can interact with IL-6 receptors at plasma membranes, the results clearly suggest that glycolipid changes produced by NEU3 are likely to participate in this possibility. In addition, NEU3 might act as a protein molecule, interacting with signaling molecules and consequently exerting stimulatory effects. We previously demonstrated findings that NEU3 interacts with caveolin-1 in caveolae in HeLa cells (32) and that sialidase also attenuates insulin receptor phosphorylation and consequent post-receptor signaling by association with Grb-2 as well as by changing ganglioside components as a result of enzyme reactions in NEU3 transgenic mice (20). Although it is uncertain at present whether NEU3 can interact with IL-6 receptors at plasma membranes, the results clearly suggest that glycolipid changes produced by NEU3 are likely to participate in this possibility. In conclusion, the results obtained in this study indicate that NEU3 plays a crucial role in the pathogenesis of RCCs in cooperation with IL-6. Suppression of NEU3 expression could thus be a new and effective therapeutic approach for this tumor type.

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