Membrane-bound sialidase NEU3, often referred to as the “ganglioside sialidase,” has a critical regulatory function on the sialoglycosphingolipid pattern of the cell membrane, with an anti-apoptotic function, especially in cancer cells. Although other sialidases have been shown to be involved in skeletal muscle differentiation, the role of NEU3 had yet to be disclosed. Herein we report that NEU3 plays a key role in skeletal muscle differentiation by strictly modulating the ganglioside content of adjacent cells, with special regard to GM3. Induced down-regulation of NEU3 in murine C2C12 myoblasts, even when partial, totally inhibits their capability to differentiate by increasing the GM3 level above a critical point, which causes epidermal growth factor receptor inhibition (and ultimately its down-regulation) and an higher responsiveness of myoblasts to the apoptotic stimuli.

Skeletal muscle differentiation is a multistep process in which myoblasts, upon exit from the cell cycle, differentiate into myocytes and eventually fuse into multinucleated myotubes (1, 2). Muscle cell commitment to differentiation is strictly regulated by a group of transcription factors, referred to as the myogenic regulatory factors (3, 4). During differentiation, a profound remodeling of both cell plasma membrane and cytoskeleton takes place, which ultimately leads to the formation of multinucleated syncytia (myotubes) (5). These events have also been shown to be associated with modifications of the cell surface lipid composition, with a key role being played particularly by sialylated glycolipids (gangliosides) (6–8). Along this line, sialidases (9), the enzymes that specifically remove sialic acid from sialylated glycoconjugates, have been shown to participate in the regulation of the myogenic event (10–12). These findings further corroborate the evidence that sialidases, and their sialylated substrates, are fundamental in many physiological processes and that their de-regulation may lead to different pathologies, including cancer (13–16). Mammals possess four different sialidases (NEU1, NEU2, NEU3, NEU4) with different subcellular localization and substrate specificity, suggesting that each of them may possess a characteristic role. Actually, the cytosolic sialidase NEU2 and the lysosomal sialidase NEU1 seem to have different functions in skeletal muscle differentiation. In fact, the cytosolic sialidase gradually increases during muscle differentiation (10), and an induced down-regulation of the enzyme completely inhibits muscle differentiation, suggesting that NEU2 exerts its activity by desialylating key glycoconjugates involved in the process. On the other hand, lysosomal sialidase NEU1 shows an increase of both enzyme expression and activity only during the first stages of muscle differentiation, followed by their decrease, suggesting a possible regulatory role of NEU1 in the early stages of myogenesis (12). Moreover, the NEU1 promoter was proven to be highly up-regulated by MyoD and repressed by activated MEK3 kinase, further supporting NEU1 strong association with the differentiation process (12). Surprisingly, no data are available on a possible involvement of the plasma membrane-bound sialidase NEU3 (17, 18) in muscle differentiation. Nevertheless, the NEU3 role seems quite plausible, as the enzyme has a critical regulatory function on the sialoglycosphingolipid pattern of the cell plasma membrane (19). For instance, NEU3 of COS-7 cells is able to modify the sialoglycosphingolipid pattern of adjacent cells (20), supporting its involvement in cell–cell interactions (see Fig. 1A). On these bases, we decided to investigate the effects of NEU3 on muscle differentiation by constitutively silencing NEU3 with small hairpin RNA (shRNA) using murine C2C12 myoblasts as the cell model. Our results show that (a)

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the induced down-regulation of the enzyme in murine C2C12 myoblasts completely inhibited their ability to enter the differentiation process; 

(b) upon induction of differentiation or when grown to confluence, NEU3-silenced myoblasts underwent a massive apoptotic cell death; 

(c) NEU3 silencing caused epidermal growth factor receptor (EGFR) inhibition and down-regulation because of the increased levels of endogenous ganglioside GM3; 

(d) supplementation of GM3 in the culture medium of wild-type C2C12 strongly reduced their differentiation capability; and 

(e) NEU3-silenced myoblasts, when co-cultured with wild-type C2C12 cells, re-acquired the capability to differentiate and fused to form major histocompatibility complex (MHC)-expressing myotubes.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Stable Silencing of NEU3 in C2C12 Myoblasts**—Mouse C2C12 myoblasts (from American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (proliferation medium). Muscle differentiation was induced when cells were at 70–80% confluence by replacing 10% fetal bovine serum with 2% horse serum in the culture medium (differentiation medium). All cell culture reagents were purchased from Sigma. Silencing of NEU3 in C2C12 cells was achieved by designing a short hairpin targeting the mouse NEU3 gene sequence 894–914 (5’-GCACATGGCAAGTTCATTGA-3’), whereas the shRNA scrambled sequence used was 5’-GGATGATACCGTAGTTGTTACG-3’ (GenBank™ accession no. NM_016720) with the Block-iT RNA iDesigner Software (Invitrogen). Viral particles were formed by transfecting 3 μg of pLent4/BLOCK-iT-shNEU3 vector and 9 μg of packaging vector mix (Invitrogen) in 293FT cells by Lipofectamine™ 2000 reagent (Invitrogen). After 48 h, the culture medium was collected and used to infect proliferating C2C12 cells (multiplicity of infection of 5) according to the manufacturer’s procedure. Infected clones were isolated after selection with Zeocin (600 μg/ml). NEU3-silenced cells were named “iNEU3” cells, whereas control cells transduced with the scrambled sequence were named “SCR.”

**Metabolic Labeling of Cell Sphingolipids**—[3-3H]Sphingosine (19.8 Ci/mmol) (PerkinElmer Life Sciences) dissolved in ethanol was transferred into a glass sterile tube and dried under a nitrogen stream; the residue was then dissolved in an appropriate volume of pre-warmed (37 °C) Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum to obtain a final concentration of 0.25 μCi/100-mm dish (corresponding to 2.53 × 10^-9 m). After a 2-h incubation (pulse), the medium was removed, and cells were incubated for 44 h with Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum not containing radioactive sphingosine (chase). At the end of the chase, cells were washed and harvested in ice-cold phosphate-buffered saline by scraping. The cell suspensions were frozen and lyophilized.

**Extraction and Analysis of Radioactive Lipids**—Total lipids from lyophilized cells were extracted twice with 20:10:1 (v/v) chloroform/methanol/water. Lipid extracts, dried under a nitrogen stream, were dissolved in 2:1 (v/v) chloroform/methanol and subjected to a two-phase partitioning 20% (v/v) water. The aqueous and organic phases obtained were counted for radioactivity, and the contained [3H]sphingolipids were analyzed by high performance thin layer chromatography (Kieselgel 60, 20 × 10 cm, Merck GmbH), the solvent system 55:20:3 (v/v) chloroform/methanol/water and 60:40:9 (v/v) chloroform/methanol/aqueous CaCl₂ (0.2%) for the organic and aqueous phases, respectively. Radioactive lipids were visualized with a Beta-Imager 2000 (Biospace) and identified by comparison with radiolabeled standards. The radioactivity associated with individual lipids was determined with the specific β-Vision software (Biospace).

**EGFR Phosphorylation**—C2C12 and iNEU3 cells in proliferation and grown to confluence were starved overnight in serum-free medium and then stimulated for 15 min with 100 ng/ml epidermal growth factor (EGF) (Sigma). After stimulation, cells were washed with phosphate-buffered saline two times and then lysed for 15 min at 4 °C in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1 mM Na₃VO₄, 0.5% (v/v) Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A). Insoluble material was removed by centrifugation at 13,000 × g for 10 min, and supernatants were collected and assayed for protein concentration with Coomassie Protein Assay (Pierce). Samples were analyzed by immunoblotting with anti-phospho-EGFR (Tyr1148) (Calbiochem). Gene expression, cell morphology, growth curve, proliferation and sialidase activity assays, immunofluorescences, Hoechst 33342 staining, caspase-3 activation, DNA ladder, treatment of C2C12 cells with GM3, treatment of iNEU3 cells with 1-phenyl-2-palmitoyl-3-morpholino-1-propanol (PPMP), Western blot analyses, and co-culture experiments of C2C12 and GFP-iNEU3 myoblasts are described in the supplemental “Experimental Procedures.”

**RESULTS**

**Silencing of NEU3 Sialidase in C2C12 Cells**—C2C12 cells were transduced with a lentiviral vector engineered with a shRNA targeting the coding region of NEU3. Zeocin-resistant clones were expanded and tested to evaluate the ability of the designed shRNA to specifically inhibit NEU3 gene translation. For this purpose, cells were cultured at subconfluence levels in normal growth medium, and NEU3 expression levels were assayed by reverse transcription-PCR (RT-PCR) and real-time PCR. Among eight different clones tested (Supplemental Fig. 1A), clone “4-4,” which from now on will be referred to as “iNEU3,” showed the lowest sialidase expression levels analyzed by real-time PCR and quantified as about 40% of that observed in normal proliferating C2C12 cells or in C2C12 cells transduced with a scrambled shRNA sequence (Fig. 1, B and C, and Supplemental Fig. S1). It is noteworthy that we could not detect any significant difference in the expression levels of the lysosomal sialidase NEU1, whereas sialidase NEU2 and NEU4 mRNAs were undetectable (data not shown) in both wild-type C2C12 and iNEU3 clones, as expected (10, 11). Successively, NEU3 activity was determined on the artificial substrate 4-MU-NeuAc, displaying a 3-fold decrease compared with proliferating wild-type C2C12 cells (Fig. 1E).
**Effects of NEU3 Silencing on C2C12—iNEU3 myoblasts revealed no significant macroscopic morphological differences compared with normal C2C12 cells (Fig. 1, F and G), but they showed a reduced proliferation rate, as confirmed by cell count (Fig. 1D) and tetrazolium hydroxide test (60% reduction, 48 h after plating) (data not shown). Successively, iNEU3 and control C2C12 cells were cultured to 75–80% confluence in growth medium and then cultured in differentiation medium (containing 2% horse serum). Upon differentiation stimulus, control C2C12 cells gradually fused to form multinucleated myotubes (Fig. 1H), as expected (4), whereas iNEU3 cells did not fuse, as essentially no myotube could be detected in the culture plate, whereas a substantial cell death occurred as soon as 1 day after the differentiation stimulus (Fig. 1I). At the end of differentiation, control C2C12 cells showed a 7-fold increase in sialidase expression by real-time PCR (Fig. 1C) and a 10-fold increase in enzymatic activity toward 4′-MU-Neu5Ac (Fig. 1E) compared with proliferating myoblasts. Instead, iNEU3 myoblasts showed low sialidase expression and activity (Fig. 1, C and E), comparable with those observed for proliferating myoblasts. Moreover, all myotubes originated from control C2C12 cells stained positive for differentiation markers MYOG and MHC (Fig. 2A, left panels), whereas iNEU3 cells remained mononucleated and did not stain at all for MYOG nor MHC (right panels). These results were confirmed by RT-PCR and real-time PCR, revealing an increasing expression of MYOG and MHC during differentiation in control C2C12 cells, whereas barely detectable levels of MYOG and MHC were found in iNEU3 cells (Fig. 2, B–D).

**Evaluation of Apoptosis after Induction of Muscle Differentiation—**C2C12 and iNEU3 myoblasts were cultured to confluence and stained with Hoechst 33342 dye. The results indicated the presence of DNA condensation and fragmentation only in iNEU3 cells (Fig. 3A). This result was also supported by DNA laddering, which was only observed in iNEU3 cells grown to confluence or when they were induced to differentiate (Fig. 3D). Analogous results were obtained when subconfluent cultures (70–80%) of iNEU3 myoblasts were induced to differentiate. Western blot analysis with an antibody specific for the 17-kDa caspase-3 active fragment revealed high levels of the protein only in iNEU3 cells 24 and 48 h after differentiation induction, whereas only low amounts of the same fragment could be detected in control
NEU3 and GM3 Involvement in Muscle Differentiation

C2C12 cells (Fig. 3B). Caspase-Glo 3/7 luminescence assay in control C2C12 showed the occurrence of an activation of caspase-3/7 at the beginning of differentiation (first 2 days), followed by its decrease later on (Fig. 3C). Instead, iNEU3 cells showed a steady and continuous caspase-3/7 increase during the differentiation process (Fig. 3C).

Ganglioside Pattern Modifications after NEU3 Silencing and GM3 Effects on EGFR—The glycosphingolipid profile of both control and iNEU3 myoblasts was determined by administration of [3-3H]sphingosine, which led to an extensive and stable labeling of the gangliosides namely gangliosides, neutral glycosphingolipids, sphingomyelin, and ceramide, at steady-state conditions (20). The ganglioside pattern analysis showed a 7–8-fold increase of GM3 absolute concentration, becoming 95% of total gangliosides, whereas no significant variation could be detected for the other major gangliosides, as shown in Fig. 4 (A and B). Conversely, no statistically significant differences were observed between the radioactive organic phase sphingolipids, sphingomyelin, and ceramide, at steady-state conditions (20). The ganglioside pattern analysis showed that NEU3 silencing caused a marked decrease of total EGFR expression (Fig. 4D), which became almost undetectable in the membrane fraction. Moreover, barely detectable levels of EGFR mRNA could be found in iNEU3 cells by RT-PCR and real-time PCR (Fig. 4, E and F).

As GM3 is known to inhibit EGFR activation (21), wild-type C2C12 cells were preincubated for 24 h with 100 μM GM3 and then induced to differentiate for 7 days. At the end of the differentiation treatment, only a few myotubes could be detected in the culture plate, compared with control C2C12 cells without GM3 (Fig. 5, A and B), accompanied by low MHC expression levels, suggesting that GM3 is involved in the inhibition of C2C12 cells differentiation (Fig. 5C). Moreover, inhibition of ganglioside synthesis in iNEU3 cells with PPMP, a potent inhibitor of glucosylceramide synthase, accompanied by concurrent stimulation of EGFR with EGF, partially restored their capability to undergo the differentiation process, as several MHC-expressing myotubes could be detected after 7 days in differentiation medium (Fig. 5D). Instead, in the same iNEU3 cells, PPMP treatment or EGF stimulation alone gave unsuccessful results (data not shown).

NEU3-silenced Myoblast Differentiation by Co-culture with Wild-type C2C12 Cells—Initial attempts to induce iNEU3 cells differentiation with a pre-conditioned differentiation medium (obtained from cultures of wild-type C2C12 cells at the end of the differentiation treatment) were unsuccessful. Moreover, indirect co-cultures of iNEU3 and wild-type C2C12 myoblasts with a Transwell system, which would have had the advantage of allowing soluble molecules released by C2C12 cells to quickly reach and stimulate iNEU3 myoblasts to differentiate, were also ineffective. Finally, direct co-culture of iNEU3 cells and wild-type C2C12 cells was performed to verify if direct cell contact with C2C12 cells (expressing normal levels of NEU3) would induce iNEU3 cells to differentiate. To this end, iNEU3 cells were stably transfected with pEGFP-N1 vector (GFP-iNEU3) to discriminate them from wild-type C2C12 cells. The selected clone “EL2,” which expressed the highest levels of GM3, could not be induced to differentiate under normal differentiation conditions. On the other hand, when GFP-iNEU3 cells were directly co-cultured with C2C12 cells (Fig. 5E) and induced to differentiate, the formation of GFP- and MHC-positive myotubes was observed (Fig. 5F), supporting the co-differentiation of both GFP-iNEU3 and wild-type C2C12 cells. MHC-expressing myotubes that did not stain for GFP (Fig. 5F, single white arrowhead) were found in the culture plate, ruling out the possibility of an aspecific GFP staining. Moreover, few GFP-positive cells that did not stain for MHC (Fig. 5F, double white arrowhead) could also be detected, indicating that most, but not all, iNEU3 cells were induced to differentiate by co-culture with differentiating wild-type C2C12 cells. Moreover, few mononucleated GFP-iNEU3 myoblasts that stained positive for MHC (Fig. 5G) could also be detected, indicating that iNEU3 cells differentiated to myocytes before fusing into multinucleated myotubes.
FIGURE 4. GM3 increase and consequent EGFR inhibition after iNEU3 silencing. Sphingolipid profile of proliferating C2C12 and iNEU3 cells treated for 2 h with [3-3H]sphingosine is shown. After a 24-h chase, which warrants reaching a steady-state condition, cells were harvested and treated for lipid analysis. A, HPTLC separation of gangliosides. Multiple spots reflect the heterogeneity of the ceramide moiety. The solvent system used was 60:40:9 (v/v) chloroform/methanol/aqueous CaCl2 (0.2%). The HPTLC image was acquired by radiochromatoscanning. B, ganglioside content of C2C12 and iNEU3. Data are the means ± S.D. of four experiments. The significance of difference between wild-type C2C12 and iNEU3 cells is according to Student’s test, for GM3 p < 0.001. C, Western blot analysis of total cell lysates of C2C12 and iNEU3 cells stained with anti-phospho-EGFR antibody. D, Western blot analysis of total cell, soluble, and membranes extracts of C2C12 and iNEU3 cells stained with anti-EGFR antibody. E and F, RT-PCR and real-time PCR quantification, respectively, of EGFR mRNA levels in proliferating iNEU3 and C2C12 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

DISCUSSION

Mammalian sialidases have been shown to be implicated in many physiological processes, including skeletal muscle differentiation. Although some studies described the involvement of the lysosomal sialidase NEU1 (12) and the cytosolic sialidase NEU2 (10, 11) in the differentiation process, the role of the “ganglioside sialidase” NEU3, if any, had yet to be disclosed. The key role played in apoptosis suppression, especially in cancer cells, has been clearly demonstrated (22, 23). Actually, it is known that apoptosis has connections with cell differentiation (24). For instance, caspase-3 activation has been shown to be critically related to the differentiation of myoblasts (25), osteoclasts (26), bone marrow stromal cells (27), neurons (28), and neural and glial progenitor cells (29, 30), suggesting the notion that the caspase apoptotic pathway regulates not only cell death but also cell differentiation. However, it is still a standing question what causes differentiation versus death phenotype, as caspases seem to target the same substrates in both processes. One possibility is that timing and intensity of the signal may be crucial to discriminate the two outcomes (24, 31). In this context, the observed increase of membrane sialidase activity, occurring in L6 myoblasts during differentiation (10), was the stimulus to investigate in more details the involvement of NEU3 and its physiological substrate ganglioside GM3, in myoblast transition from proliferation to differentiation. To this purpose, the murine myoblast cell line C2C12, with normal expression of NEU3, was chosen for this study and compared with partially, but stably NEU3-silenced C2C12 clones the use of shRNA targeting the coding region of NEU3. Remarkably, control C2C12 cells gradually fused to form multinucleated myotubes, a clear sign of differentiation (Fig. 1H), whereas iNEU3 myoblasts not only failed to exhibit myotube formation, but extensively died (Fig. 1A). These results are consistent with the notion that a well defined “level” of NEU3 activity is required for C2C12 to enter the differentiation process and that NEU3 silencing elicits a massive process of cell death by apoptosis.

Finally, efforts were made to investigate the possible molecular mechanisms that underlies the effects of NEU3 down-regulation. Because NEU3 possesses a high specificity toward gangliosides, we compared the glycosphingolipid profile of iNEU3 myoblasts and wild-type C2C12 cells by steady-state metabolic labeling with [3-3H]sphingosine. Remarkably, in iNEU3 myoblasts, ganglioside GM3 underwent a 7–8-fold increase of absolute concentration, covering about 95% of the total gangliosides, possibly as the result of a biosynthetic flux not counterbalanced by adequate depletion. Thus, we hypothesized that the high levels of this ganglioside could be the basis of the new observed phenotype. Following the studies by Hakomori and coworkers (21, 32–34) and Paller and coworkers (34), showing that GM3 specifically inhibits activation of EGFR, which is known to play a central role in cell growth control (35), we verified the effects of NEU3 silencing on the functional state of EGFR. Previous studies have shown that GM3 affects EGFR activity through a direct interaction that requires receptor glycosylation, providing a hypothetical model of carbohydrate-carbohydrate interaction between EGFR and gangliosides in what is considered the “ganglioside signaling domain” (34). In our experiments, we not only observed a marked decrease of the active, phosphorylated form of EGFR in iNEU3 myoblasts, but a substantial down-regulation of the EGFR itself. Furthermore, analysis of cell membranes alone revealed almost undetectable levels of EGFR, suggesting a possible internalization of the residual receptor present (36–38). Thus, we propose that prolonged EGFR inhibition by persistent high levels of GM3, because of NEU3 silencing, may lead to internalization of the inhibited receptor (36–38) and ultimately to a down-regulation of EGFR at the transcription level. This line of interpretation is supported by the observation that (a) the addition of GM3 in the culture medium strongly inhibited wild-type C2C12 capability to differentiate, and (b) the stimulation of the residual EGFR present in iNEU3 cells by direct administration of EGF in the culture medium, combined with inhibition of ganglioside biosynthesis with PPMP, caused a partial restoration of their capability to differentiate. Moreover, it has been shown that NEU3 co-immunoprecipitates with EGFR in cancer cells and that high levels
of NEU3 in cancer cells suppress apoptosis by promoting EGFR phosphorylation probably through its association with EGFR and activation of Ras cascades (39). Finally, as NEU3 has been shown to exert its catalytic activity on adjacent cells (20), we tested if iNEU3 myoblasts could be rescued and induced to differentiate by direct co-culture with wild-type C2C12 cells, in which NEU3 is expressed at normal levels. To this purpose, iNEU3 cells were marked by transfection with a GFP-expressing plasmid and co-cultured with differentiating wild-type C2C12 myoblasts (Fig. 5E). At the end of the differentiation process, we observed the formation of several GFP-expressing myotubes, indicating that wild-type C2C12 cells at least partially rescued iNEU3 cells, which differentiated and co-participated in myotube formation. Instead, the pre-conditioned medium of differentiating C2C12 myoblasts, or indirect co-cultures with Transwell systems, did not induce iNEU3 differentiation, suggesting that potential soluble factors released from wild-type C2C12 cells alone were not able, or sufficient, to restore the lost phenotype.

At this point, we can envisage a plausible role of NEU3 in skeletal muscle differentiation. The gradual increase of NEU3 during differentiation may be responsible of directing cells toward the differentiating phenotype while protecting them from apoptosis. According to our view, because of its peculiar position on the outer cell membrane, NEU3 modulates the ganglioside content of adjacent cells with special regard to GM3 concentration, thus governing the cell-to-cell signaling program required for skeletal muscle differentiation. Decrease of NEU3 activity, even when only partial, causes a rise of GM3 concentration over a critical point, resulting in the inhibition of the EGFR activation, the down-regulation of the same receptor, and a higher responsiveness of myoblasts to the apoptotic stimuli. On the other hand, although expected, we did not observe any significant variation in lactosylceramide content after NEU3 silencing. This was presumably due to the fact that GM3 accumulation, in our stably NEU3-silenced cells, may result in a reduced biosynthesis of the ganglioside, thus explaining no appreciable variations in lactosylceramide content; therefore,
we tend to exclude its involvement in the observed phenomena. On the other hand, it has been recently reported that pathological human fibroblasts in which GM3 synthase was mutated and not functional showed a complete depletion of gangliosides on the cell membrane and an inhibition of EGFR activation (40). This may be the result of the reduced levels of other key gangliosides such as GD1a, which is known to enhance autophosphorylation of EGFR (41). Thus, whereas it is clear that each cell line may display a different ganglioside pattern and may respond differently to ganglioside pattern variations, evidences clearly indicate that individual gangliosides are directly and specifically involved in an array of physiological and pathological processes. In our cell model, after NEU3 silencing, GM3 becomes by far the most abundant ganglioside on the cell membrane, but we cannot exclude that it may also interact with different receptors, other than EGFR, as gangliosides are known to interact with functional membrane proteins, such as integrins, growth factor receptors, tetraspanins, and non-receptor cytoplasmatic protein kinases (42). Nevertheless, to further verify our hypotheses, it would be interesting to study the effects of NEU3 overexpression in C2C12 cells. This issue is currently under investigation in our laboratories.

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