Monocyte Differentiation Up-regulates the Expression of the Lysosomal Sialidase, Neu1, and Triggers Its Targeting to the Plasma Membrane via Major Histocompatibility Complex Class II-positive Compartments*5

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Human sialidase (neuraminidase) Neu1 catalyzes lysosomal catabolism of sialylated glycoconjugates. Here we show that during the differentiation of monocytes and the monocytic cell line, THP-1, into macrophages, the majority of Neu1 relocalizes from the lysosomes to the cell surface. In contrast to other cellular sialidases Neu2, Neu3, and Neu4, whose expression either remains unchanged or is down-regulated, Neu1 mRNA, protein and activity are specifically increased during the phorbol 12-myristate 13-acetate-induced differentiation, consistent with a significant induction of the transcriptional activity of the Neu1 gene promoter. The lysosomal carboxypeptidase, cathepsin A, which forms a complex with and activates Neu1 in the lysosome, is sorted to the plasma membrane of the differentiating cells similarly to Neu1. Both proteins are first targeted to the lysosome and then are sorted to the LAMP-2-negative, major histocompatibility complex II-positive vesicles, which later merge with the plasma membrane. Similar trafficking was observed for the internalized fluorescent dextran or horseradish peroxidase initially stored in the lysosomal/endoosomal compartment. The suppression of Neu1 expression in the THP-1-derived macrophages by small interfering RNA or with anti-Neu1 antibodies significantly reduced the ability of the cells to engulf bacteria or to produce cytokines. Altogether our data suggest that the up-regulation of the Neu1 expression is important for the primary function of macrophages and establish the link between Neu1 and the cellular immune response.

Human lysosomal sialidase (neuraminidase) Neu1, encoded by the NEU1 gene in the major histocompatibility complex III locus (1–3) catalyzes the hydrolytic cleavage of terminal sialic acid residues from oligosaccharides and glycoproteins. In the lysosome, Neu1 exists as a component of the multienzyme complex also containing the lysosomal carboxypeptidase A (cathepsin A/protective protein), β-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase (reviewed in Refs. 4 and 5). The dissociation of the complex in vitro results in the reversible inactivation of Neu1 (6). In vivo, inherited mutations in cathepsin A cause disruption of the complex and trigger galactosialidosis (OMIM 256540), an autosomal recessive disease characterized by combined deficiency of Neu1, β-galactosidase, and cathepsin A (reviewed in Ref. 4). Another autosomal recessive disease, sialidosis (OMIM 256550), is caused by mutations directly affecting the NEU1 gene (reviewed in Refs. 7 and 8).

We have previously shown that in addition to the lysosomes, Neu1 is also present on the surface of activated T cells (9), where it may influence the immune function (10–12). Recently (13), we have demonstrated that endogenous Neu1 sialidase activity of freshly isolated human monocytes is increased 20–30-fold per cell as they differentiate into macrophages. Monocyte-macrophages constitute an important cellular component of the mononuclear phagocytic system, playing an essential role in the recognition and activation of both innate and adaptive immune responses against pathogens. The differentiation of monocytes into macrophages in extravascular tissues is an essential event for the normal functioning of the system. Therefore, knowledge of the mechanism and the role of the Neu1 up-regulation during the monocyte differentiation should provide insights into the cellular immune response and may eventually lead to developing approaches for therapeutic intervention.

Here we have studied the regulatory mechanisms of the Neu1 expression and presented direct evidence that the NEU1 gene promoter is activated during the monocytic differentiation. We have also shown that the newly synthesized Neu1 is first targeted to the lysosomes and then, as a complex with cathepsin A, relocates to the cell surface, where it could participate in antigen presentation or affect intercellular interactions.
EXPERIMENTAL PROCEDURES

Isolation of Peripheral Blood Mononuclear Cells and Purification of Monocytes—Peripheral blood mononuclear cells were isolated by leukopheresis of blood from human immunodeficiency virus, type 1, and hepatitis B and C seronegative donors followed by centrifugation over Ficoll-Paque Plus (Amersham Biosciences) gradients using standard procedures. Monocytes were purified from peripheral blood mononuclear cells by an additional centrifugation over Percoll (Amersham Biosciences) gradients and then by negative selection using a monocyte separation kit (StemSep; Stem Cell Technologies, Vancouver, Canada) according to the manufacturer’s protocol. The purity of monocytes exceeded 95% as determined by flow cytometry after staining the cells with fluorochrome-labeled antibodies to CD3, CD14, CD19, and CD206 (all from BD PharMingen, San Diego, CA), and viability was greater than 97% as determined by trypan blue dye exclusion.

Culturing of Purified Monocytes—To obtain monocyte-derived macrophages, purified monocytes were suspended at 2 × 10⁶ cells/ml in RPMI medium 1640 (Invitrogen) containing 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA) and were maintained at 4 × 10⁶ cells/well in 6-well tissue culture plates (Costar; Corning Glass) at 37 °C in a 5% humidified, CO₂ incubator. At the indicated times, nonadherent cells were removed by two washes with phosphate-buffered saline (PBS),⁵ and the adherent, differentiating macrophages (larger and more granular than monocytes as seen by light microscopy) were harvested in PBS, pH 7.4, by gentle scraping with a polyethylene cell scraper (Nalge Nunc International, Rochester, NY). The harvested cells were confirmed to have characteristic macrophage cell surface phenotypic markers (CD14⁺, CD206⁺) by flow cytometry.

Culturing and Differentiation of THP-1 Cells—THP-1 cells, described in greater detail elsewhere (14), were cultured in the RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal calf serum, 5000 units of penicillin/streptomycin, activated fetal calf serum, 5000 units of penicillin/streptomycin, and Hepes buffer (Invitrogen) supplemented with 10% fetal calf serum, 5000 units of penicillin/streptomycin, and Fungizone. Cells seeded in 100-mm tissue culture plates at 70–80% confluence were cotransfected with the reporter constructs and pCMV-β-gal expression vector using Lipofectamine Plus reagent (Invitrogen) in accordance with the manufacturer’s protocol. THP-1 cells suspended in RPMI 1640 medium at a density of 5 × 10⁵ cells/ml medium were cotransfected with the reporter constructs and pCMV-β-gal expression vector using Effectene transfection kit (Qiagen) in accordance with the manufacturer’s protocol.

24 h after the transfection, the cells were washed with PBS and stimulated for 2–24 h with PMA (20 ng/ml), tumor necrosis factor-α (TNF-α) (40 ng per ml), or platelet-derived growth factor-AB (10 ng/ml) in the presence or absence of 20 μM N-acetylcysteine (NAC) or 0.5 μM curcumin (all from Sigma). In a separate experiment, curcumin and NAC were added in the same final concentration 1 h before the addition of PMA or TNF-α. After 24-h incubation, the cells were rinsed with PBS, harvested by scraping, and resuspended in 350 μl of TEN buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 150 mM NaCl). Cells were centrifuged at 12,000 rpm at 4 °C for 5 min and lysed by three freezing-thawing cycles in 0.25 μl Tris-HCl, pH 7.8. CAT and β-galactosidase activities were measured using [¹⁴C]choramphenicol and 4-methylumbelliferyl-β-D-galactopyranoside substrates as described (16). One unit of enzyme activity is defined as the conversion of 1 μmol of substrate/min. Proteins were assayed according to Bradford (17) with bovine serum albumin (Sigma) as a standard.

Enzyme Assays—Sialidase, β-galactosidase, and β-hexosaminidase activities in cellular homogenates and in culture medium were assayed using the corresponding fluorogenic 4-methylumbelliferyl-glycoside substrates (18, 19). Cathepsin A activity was determined with benzoylcarbonyl-Phe-Leu-Leu (20). Alkaline phosphatase, glutamate dehydrogenase, and GAPDH were measured as described elsewhere (21–23). Sialidase activity toward mixed bovine gangliosides was measured as described (24, 25) in the presence of 0.2% Triton X-100. The concentration of released sialic acid was measured by the thiobarbituric method (26). Enzyme activity is expressed as the conversion of 1 nmol of substrate/h.

To measure the enzyme activities on the outer cell surface, the cultured live cells (~10⁶ cells) were washed several times with Hanks’ balanced salt solution and overlaid with 500 μl of 20 mM acetylthioacetate buffer, pH 5.2, 0.25 mM sucrose, and either 0.4 mM sialidase substrate, cathepsin A substrate, β-galactosidase substrate, or N-acetyl β-hexosaminidase substrate. After 30 min of incubation, 200-μl aliquots of medium were collected, and the concentrations of the products were measured as described above. The integrity of plasma membrane was verified by West-
ern blot by measuring the amount of the cytosolic enzyme GAPDH released into the cell medium during the incubation. We found that the same amount of GAPDH was released from the cells incubated in 20 mM acetate buffer, pH 5.2, 0.25 M sucrose, and PBS. To measure the total amount of enzyme, the assay was performed in the presence of 0.3% Triton X-100.

Detection of CathA and Neu1 on the Macrophage Surface by Western Blot—Proliferating THP-1 cells and THP-1-derived macrophages (H11011/H11003 10^6 cells) were washed three times by ice-cold PBS and incubated at room temperature for 1 h in 5 ml of PBS containing a biotinamido hexanoic acid biotinylation reagent (Sigma) at a final concentration of 5 mg/ml with a gentle rotation. The cells were washed three times with TBS and homogenized in 0.5 ml of radiolmmune precipitation buffer. The cell lysate cleared by a 10-min centrifugation at 12,000 × g was loaded on a 0.2-ml Streptavidin-agarose column (Sigma), and the column was washed by PBS until the absorbance at 280 nm reached the base line. Biotinylated proteins were eluted with 0.2 ml of 0.1 M glycine-HCl (pH 2.5). The pH of the eluate was immediately adjusted to neutral with 1.5 M Tris buffer (pH 7.5). 20 μg of protein from the original cell lysate and the Streptavidin-agarose column flow-through fraction and 5 μg of protein from the eluate were separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. The membrane was hybridized with 32P-labeled Neu1 and β-actin cDNA probes and exposed to a BioMax film (left). Images were scanned, and relative intensities of the signals from Neu1 and β-actin probes were quantified using PDQuest software (Bio-Rad) (right). Values represent means ± S.D. of triplicate independent experiments. D, Western blotting of undifferentiated THP-1 cells (Day 0) and the cells treated for 1 (Day 1) and 4 days (Day 4) with 20 nM PMA. Each lane contains 20 μg of total cell lysates. C, positive control, Neu1 purified from human placenta. The immunoblotting was performed with anti-Neu1 and anti-Neu3 rabbit polyclonal antibodies as indicated.
Confocal Immunofluorescence Microscopy—Cells were washed twice with ice-cold PBS and fixed with 3.8% paraformaldehyde in PBS for 30 min and if necessary were permeabilized by 0.5% Triton X-100. Fixed cells were washed twice with PBS and stained with polyclonal rabbit anti-Neu1 or anti-cathepsin A antibodies and counterstained with Texas Red-labeled antibodies against rabbit IgG. Cells were also double-stained with rabbit antibodies against Neu1 or cathepsin A antibodies and monoclonal antibodies against LAMP-2 (Washington Biotechnology Inc., Baltimore, MD) or against MHC II (BD Biosciences, Mississauga, ON). The primary monoclonal antibodies were counterstained with Oregon Green 488-conjugated anti-mouse IgG antibodies (Molecular Probes). Cells were also double-stained with rabbit antibodies against Neu1 or cathepsin A antibodies and polyclonal antibodies against LAMP-2 (Washington Biotechnology Inc., Baltimore, MD) or against MHC II (BD Biosciences, Mississauga, ON). The primary monoclonal antibodies were counterstained with Oregon Green 488-conjugated anti-rabbit IgG antibodies and PE-conjugated monoclonal anti-mannose receptor antibodies or anti-CD14 antibodies (BD Pharmingen). Slides were examined on a Zeiss LSM510 inverted confocal microscope (Carl Zeiss Inc.).

Western Blot Analysis—Proteins were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane. Neu1 and Neu3 detection was performed with rabbit antibodies as described (13, 27), and LAMP-2 detection was performed with monoclonal anti-human LAMP-2 antibodies (Washington Biotechnology) using the BM Chemiluminescence kit (Roche Applied Science) in accordance with the manufacturer’s protocol.

RNA Isolation and Real-time RT-PCR—THP-1 cells and THP-1-derived macrophages were harvested as previously described, and total RNA was isolated using Trizol reagent (Invitrogen) following the protocol suggested by the manufacturer. The RNA preparation was treated with DNase I (Invitrogen) for 15 min at room temperature to remove the DNA contamination and then inactivated by adding 25 mM EDTA solution to the reaction. 1 μg of RNA was used to synthesize cDNA in the presence of 0.5 mM dNTPs, 50 ng of random hexamers (Invitrogen), and 200 units of SuperScript II reverse transcriptase (Invitrogen). Reverse transcription was allowed to proceed for 50 min at 42 °C.
PCR amplification was performed on a SmartCycler (Cepheid) using the SYBR Green PCR kit (Qiagen). The primer pairs, annealing temperatures, number of PCR cycles, and RNA amounts are shown in supplemental Table 1, which also shows the predicted sizes of the resulting PCR products. As positive controls for real-time PCR detection, we used mRNA coding for the ribosomal subunit 18 S (Ambion). All reactions were performed in triplicate. The accuracy of the reaction was monitored by the analysis of melting curves and product size on gel electrophoresis.

Northern Blot Analysis—Purified total RNA from THP-1 cells and THP-1-derived macrophages was resolved by agarose gel electrophoresis and blotted to Hybond-N⁺ membrane (Amersham Biosciences). The membrane was hybridized with the following probes: a 500-bp Neu4 cDNA fragment obtained from clone 4156395 by PvuII digestion, an entire Neu1 cDNA, a 700-bp Neu3 fragment obtained from a cDNA clone by PsiI digestion, and an entire cDNA of human β-actin. All probes were labeled with [32P]dCTP by random priming using MegaPrime labeling kit (Amersham Biosciences). Prehybridization of the blots was performed at 68 °C for 30 min in ExpressHybTM (Clontech). The denatured probes were added directly to the prehybridization solution and incubated at 68 °C for 1 h. The blots were washed twice for 30 min with 2× SSC, 0.05% SDS at room temperature and once for 40 min with 0.1× SSC, 0.1% SDS at 50 °C and exposed to a BioMax film overnight at −80 °C.

Neu1 Small Interfering RNA Gene Silencing in Human Skin Fibroblasts—Silencer™ predesigned siRNA ID:8386, ID:8481 and ID:8573 (CAT:16704) targeting exons 1, 2, and 3 of the human NEU1 gene, respectively, were obtained from Ambion (Austin, TX). THP-1 cells were transfected with siRNA using the Nucleofector™ Solution V reagent (Amaxa) according to the protocol provided by the manufacturer. GAPDH siRNA (Ambion) was used as a positive control for the optimization of transfection conditions. The siRNA efficacy was verified in preliminary experiments where THP-1 cells were harvested 24 and 48 h after transfection with three Neu1 siRNAs, and the sialidase activity was measured on the RNA and protein level by quantitative RT-PCR, Western blotting, and enzyme activity assay as described. On was measured on the RNA and protein level by quantitative RT-PCR, Western blotting, and enzyme activity assay as described. On average, sialidase activity in the differentiated Neu1 siRNA-transfected THP-1 cells was reduced more than 4-fold as compared with mock-transfected cells (not shown).

Phagocytosis Assay—Differentiation of THP-1 cells was induced by PMA in the presence of either anti-Neu1 rabbit antiserum or control preimmune serum (20 and 100 μg/ml of medium). THP-1 cells transfected with Neu1 siRNA were differentiated in the presence of PMA only. After 4 days, cells seeded on coverslips were incubated within 3 h with fluorescently labeled Escherichia coli (K-12 strain), bioparticles (Molecular Probes) in a ratio of 30 particles/cell, washed three times with cold PBS, fixed with 4% paraformaldehyde for 1 h, permeabilized with 0.5% Triton X-100, and washed twice with 0.05% Tween 20 in PBS. Cells were counterstained with 3 μM 4′,6-diamidino-2-phenylindole and washed again with 0.05% Tween 20 in PBS. Slides were studied on the Nikon Eclipse E6000 direct epifluorescence microscope.

Measurement of IL and TNF-α Production in Culture Supernatants by Enzyme-linked Immunosorbent Assay—In order to determine the potential effect of anti-Neu1 serum on the secretion of IL-6, IL-10, IL-12, IL-15, IL-1b, and TNFα, THP-1 cells cultured in a 6-well plate (3 × 10⁵ cells/well) were stimulated with PMA (20 ng/ml) in the absence and in the presence of rabbit preimmune serum (100 μg/ml) or rabbit antisemera raised against the human Neu1 (20 and 100 μg/ml of medium). After a 96-h incubation, the differentiated cells were further stimulated for 12 h by ionomycin (1 μg/ml; Sigma) and PMA (20 ng/ml) also in the absence and in the presence of rabbit preimmune serum or rabbit anti-Neu1 antisemera. The concentration of IL and TNF-α in the culture medium was measured by an enzyme-linked immunosorbent assay at Chemicon International Inc. (Temecula, CA).
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RESULTS

Neu1 Expression Is Increased during PMA-induced Differentiation of Monocytic Cell Line THP-1—Previous studies defined a monocytic cell line THP-1 from the peripheral blood of a patient with acute monocytic leukemia (14). Being induced by PMA, these cells differentiate into adherent cells having morphological, biochemical, and physiological characteristics of macrophages and expressing the characteristic macrophage cell surface phenotypic markers, such as CD14+ and CD206+ (28, 29). THP-1 cells have several advantages over primary human monocytes (higher transfection efficiency, homogeneity, accessibility) that make them a reliable model to study the mechanism of monocytic differentiation. We found that during the PMA-induced differentiation, the sialidase activity measured at acidic pH 4.5 with the synthetic fluorescent substrate 4MU-NANA was increased at least 4-fold, whereas the levels of other lysosomal enzymes (cathepsin A, B, C, and D) were not increased (Fig. 1A), we could exclude this enzyme. Three remaining enzymes, Neu1, Neu3, and Neu4, all have an acidic pH optimum, but they differ from each other in substrate specificity; Neu1 shows the highest activity against oligosaccharides and short glycopeptides (24), whereas Neu3 and Neu4 are equally active against α2–3-sialylated oligosaccharides, glycopeptides, and gangliosides (25, 31, 32). Sialidase activity of THP-1 cells measured against sialylated glycolipids (mixed bovine gangliosides) was slightly reduced during differentiation (1.1 ± 0.2 versus 1.7 ± 0.3 nmol/h/mg of total protein) (Fig. 1A), suggesting that the increase of sialidase activity is caused by the up-regulation of the lysosomal sialidase Neu1. In addition, both quantitative RT-PCR and Northern blots showed that during the differentiation, the Neu1 mRNA was increased at least 5-fold (Fig. 1, B and C), whereas Neu3 mRNA was decreased 3–4-fold according to the RT-PCR data (Fig. 1B), Neu2 and Neu4 expression could not be detected in the THP-1 monocytes as well as in the THP-1-derived macrophages, neither by RT-PCR nor by Northern blots. Western blots using the polyclonal anti-Neu1 and anti-Neu3 antibodies confirmed up-regulation of Neu1 and down-regulation of Neu3 at the protein level (Fig. 1D). Altogether, our data showed that the differentiation of THP-1 cells into macrophages results in significant and specific induction of Neu1.

Promoter Activity of the NEU1 Gene Is Increased during the Differentiation of THP-1 Cells—The transcriptional activity of the Neu1 gene promoter was studied by deletion mapping. Five restriction fragments of the NEU1 gene 5′-flanking region were cloned into a pBLCAT6 vector (15) in front of a reporter CAT gene and expressed in COS-7 cells (Fig. 2A). The transfection efficiency was measured using pCMV-β-gal vector containing the SV40 promoter-driven lacZ reporter gene. CAT activity in the homogenates of the cells transfected with the reporter constructs was compared with that of the cells transfected with the promoter-free pBLCAT6 vector (15). We found that the shortest fragment with a detectable promoter activity was extending 316 bp upstream of the ATG initiation codon (Fig. 2B). Truncation of this fragment to 176 bp almost completely abolished the promoter activity. The highest promoter activity was associated with a 542-bp fragment. Extension of this fragment to 936 bp and further to 1598 bp decreased the promoter activity, suggesting that potential elements for down-regulation of the gene expression could be present between the nucleotides −542 and −1598 (Fig. 2B).

FIGURE 4. Immunohistochemical localization of Neu1 during the differentiation of THP-1 cells (A and B) and primary monocytes (C and D). After 0, 1, 4, and 7 days of differentiation, the cells were fixed, stained with rabbit polyclonal anti-Neu1 antibodies, and counterstained with mouse monoclonal antibodies either against human LAMP-2 or against human mannose receptor (CD206) as indicated in the figure. A, C, and D show permeabilized cells; B shows nonpermeabilized cells. Panels represent randomly selected typical images obtained in triplicate experiments; 140–200 cells were studied for each condition in each experiment. Slides were examined on a Zeiss LSM510 inverted confocal microscope. Magnification was ×620.
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![Image](49x420 to 409x734)

**FIGURE 5. Immunohistochemical localization of cathepsin A (CathA) during the differentiation of THP-1 cells (A and B) and primary monocytes (C).** After 0, 1, 4, and 7 days of differentiation, the cells were fixed, stained with rabbit polyclonal anti-cathepsin A antibodies, and counterstained with mouse monoclonal antibodies against human LAMP-2 or against human mannose receptor (CD206) as indicated in the figure. A and C show permeabilized cells; B shows nonpermeabilized cells. Panels represent randomly selected typical images obtained in triplicate experiments; 140–200 cells were studied for each condition in each experiment. Slides were examined on a Zeiss LSM510 inverted confocal microscope. Magnification was ×620.

We further tested whether the promoter activity of the *NEU1* gene was induced during the differentiation of THP-1 cells into macrophages. THP-1 cells were transfected with the above reporter constructs, and 24 h later, their differentiation was induced by adding PMA to the culture medium. We found that 24 h after the induction of cells with PMA, the CAT activity was significantly increased for all constructs, the highest effect (8-fold increase) being achieved for the reporter construct, pBL-NEU1CAT3 containing the 542-bp promoter fragment (Fig. 2C). For this construct, the effect was observed as early as 4 h after the induction and lasted for at least 4 days (Fig. 2D). PMA also induced the expression of the reporter gene in COS-7 cells, but the effect was significantly lower than that in THP-1 cells (Fig. 2B).

Interestingly, the analysis of the sequence of the Neu1 promoter with a TRANSFAC 4.0 software that predicts regulatory elements (33) revealed that the fragment between the nucleotides −316 and −542 contains several conserved sequence motifs for binding promoter-selective transcription factor (Sp1), activator proteins 1 and 2 (AP-1 and AP-2), hematopoietic transcription factor (E2F-1), cAMP-response element-binding protein (CREB-2), and erythroid transcription factors (GATA-1 and NF-E2) (supplemental Fig. 1), suggesting that some of these factors may be involved in the regulation of the *NEU1* gene transcription.

Indeed, when THP-1 or COS-7 cells transfected with the pBL-NEU1CAT3 reporter construct were treated with a potent AP-1 activator, TNF-α, which increases the synthesis of both c-Fos and c-Jun proteins in many cell types (34–36), we found a significant induction of the CAT activity (Fig. 3). A detectable increase of the CAT activity was also caused by another AP-1 activator, platelet-derived growth factor-AB (not shown). In all cells, the effects of TNF-α, platelet-derived growth factor-AB, or PMA could be blocked by pretreatment of the cells with curcumin and a thiol antioxidant NAC, which inhibit c-Fos or c-Jun expression (34–36) (Fig. 3).

*Neu1* and Cathepsin A Are Targeted to the Cell Surface during the Differentiation of THP-1 Cells and Primary Human Monocytes—Intracellular localization of Neu1 in the proliferating THP-1 cells and the THP-1-derived macrophages was studied by confocal immunomicroscopy. In the nondifferentiated cells, Neu1 showed a cytoplasmic punctate staining pattern co-localizing with the lysosomal marker LAMP-2 (Fig. 4A, Day 0). 24 h after the induction with PMA, the intensity of anti-Neu1 staining was significantly increased, and its pattern changed. The cells clearly showed two pools of Neu1, one intracellular co-localizing with LAMP-2 and the other peripheral (Fig. 4A, Day 1). Three days later, when THP-1 cells showed morphological characteristics of macrophages, the majority of Neu1 was found on the periphery of the cell, whereas LAMP-2 still had the intracellular localization (Fig. 4A, Day 4).

To confirm the presence of Neu1 on the surface of the differentiating cells, we performed a double staining of the nonpermeabilized cells with anti-Neu1 antibodies and antibodies against the cell surface mannose receptor, CD206. The proliferating cells did not show any staining for both antigens (Fig. 4B, Day 0), whereas on the 1st and the 4th days of differentiation, the cells showed high expression of both Neu1 and mannose receptor on their surface (Fig. 4B). Similar results were also obtained for another surface marker, CD14 (not shown) and in the isolated human primary monocytes and the monococyte-derived macrophages (Fig. 4, C and D).

In the lysosome, Neu1 is a component of a multienzyme complex, which also contains the lysosomal carboxypeptidase A (also called cathepsin A or protective protein), β-galactosidase, and N-acetylgalactosamine-6-sulfate sulfatase (reviewed in Refs. 4 and 5). If the complex is disrupted, Neu1 loses its enzymatic activity and is rapidly degraded by the lysosomal pro-
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cathepsin A are activated only in the lysosome (27, 37), both though enzymatically nonactive precursors of Neu1 and lysosomal enzyme, generate increased 3.1-fold, whereas the sialidase activity on the outer cell surface, because the activity was increased only by differentiated THP-1 cells, the majority of Neu1 was present on the outer cell surface, because the activity was increased only by 25% after lysing the cells with Triton X-100. In the nondifferentiated THP-1 cells, the majority of Neu1 was found inside the cells, since the activity was ~5-fold higher for the lysed than for the intact cells (Fig. 6A, Neu1). In contrast, in the THP-1-derived macrophages, the majority of Neu1 was present on the outer cell surface, because the activity was increased only by 25% after lysing the cells with Triton X-100. During the differentiation, the total sialidase activity assayed in the cell homogenate increased 3.1-fold, whereas the sialidase activity on the surface increased 13-fold. In contrast, the activity of a control lysosomal enzyme, N-acetyl-β-hexosaminidase, was similar in the differentiated and nondifferentiated cells, and in both cases ~77% of the enzyme was not accessible for the substrate in the absence of detergent (Fig. 6A), showing that the ratio of the disrupted and intact cells was similar for macrophages and monocytes. We did not observe an increase of the total cathepsin A activity during the differentiation; however, 70% of the activity in macrophages was detected on the cell surface as opposed to only 25% of the activity in the nondifferentiated THP-1 cells (Fig. 6A). Interestingly, neither total activity nor the distribution of the third component of the multienzyme lysosomal complex, β-galactosidase, were changed during the differentiation.

Finally, proteins on the surface of THP-1 cells and the THP-1-derived macrophages were biotinylated and affinity-purified on a Streptavidin-agarose column. Then 20 µg of protein from the original cell lysate and the Streptavidin-agarose column flow-through and 5 µg of the biotinylated proteins from the column eluate were analyzed by Western blot using the polyclonal anti-Neu1 antibodies (Fig. 6B). In the nondifferentiated THP-1 cells, both Neu1 and cathepsin A were found almost exclusively in the flow-through fraction containing unlabeled intracellular proteins, whereas in the THP-1-derived macrophages, a significant percentage of these enzymes was biotinylated, suggesting that they are present on the cell surface (Fig. 6B).

The Differentiation of THP-1 Cells Induces Fusion of the Lysosome (Endosome)-Derived Vesicles with the Cell Surface—Although enzymatically nonactive precursors of Neu1 and cathepsin A are activated only in the lysosome (27, 37), both enzymes found on the cell surface in macrophages have catalytic activity suggesting that they are first targeted to the lysosome and later to the cell surface, possibly as a result of a fusion of the lysosomal membrane with the plasma membrane. To prove that such fusion happens during the differentiation, we have loaded the lysosomes of THP-1 cells with fluorescent dextran and followed its distribution in the cell. In the nondifferentiated THP-1 cells, the labeled internalized dextran was retained in the endosomal-lysosomal compartment for at least 7 days, showing co-localization with the lysosomal marker LAMP-2 (Fig. 7, Day 0). In contrast, 12 h after the induction of the cells with PMA as well as 4 days after, in the fully differentiated THP-1-derived macrophages, the internalized dextran was partially found in the LAMP-2-negative vesicles (which appeared to be migrating to the plasma membrane), on the cell surface, and outside the cells, whereas some dextran was still localized in the lysosomes (Fig. 7, Day 1 and Day 4). On the other hand, the differentiation did not increase the secretion of soluble lysosomal enzymes (not shown), suggesting that the
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Neu-1 Colocalizes with MHC II and with the Internalized Antigens during the Differentiation of THP-1—In the differentiated macrophages, the internalized antigens are proteolytically digested in the late endosomal-lysosomal compartment, and the resulting peptides bind MHC II proteins to be further targeted to the cell surface as the antigenic peptide-MHC II complexes, pMHC (reviewed in Refs. 38 and 39). The majority of MHC II is found in the late endosomes, lysosomes, and multivesicular bodies, collectively called MHC II-enriched compartments, although distinct pMHC loading compartments were also isolated from the antigen-presenting cells and called the class II-containing vesicles (40, 41). We found that during the differentiation of THP-1 cells and in the THP-1-derived macrophages, Neu1 co-localizes with MHC II-enriched compartments. In the nondifferentiated cells, the anti-MHC II antibodies only partially colocalized with the anti-Neu1 or the anti-cathepsin A antibodies as well as with the antibodies against the internalized antigen, HRP (Fig. 8, Day 0). However, 12 h after the PMA induction, MHC II-positive organelles, which also contained the majority of Neu1 and HRP, were clearly distinct from the LAMP-2-positive lysosomes (Fig. 8, Day 1). In the fully differentiated macrophages, the majority of Neu1, MHC II, and anti-HRP immunoreactivity was found on the cell surface (Fig. 8, Day 4).

Suppression of the Neu1 Expression in the THP-1 Cells Alters Their Functional Capacity—In order to understand whether up-regulation of the Neu1 expression and its targeting to the cell surface is important for the primary function of macrophages, we have used siRNA to inhibit the expression of Neu1 in THP-1 cells. The transfected cells, which had an ~4-fold reduced level of sialidase activity, were still capable of PMA-induced differentiation but showed a significantly reduced ability to ingest the bacteria. When the normal THP-1-derived macrophages were incubated for 3 h with the fluorescein-labeled E. coli bioparticles, their ingestion index (percentage of macrophages with phagocytized particles multiplied on the average number of particles phagocytized per ingested macrophage) was ~15.5. For the siRNA-transfected cells, this number was reduced to ~3.1 (Fig. 9). A similar effect on the ingestion capacity was observed when differentiated THP-1 cells were incubated in the presence of anti-Neu1 antibodies (ingestion index ~2.9), whereas the ingestion index of 12.2 for the cells differentiated in the presence of a similar amount of preimmune rabbit serum did not statistically differ from the control (Fig. 9).

In a similar fashion, we have observed that the treatment of THP-1-derived macrophages with the anti-Neu1 antibodies significantly reduced their ability to produce certain cytokines. As before, the cells were differentiated in the presence of the anti-Neu1 antiserum or the preimmune rabbit serum and then stimulated for 12 h by ionomycin also in the presence of the anti-Neu1 antiserum or the preimmune rabbit serum. We found that the anti-Neu1 antibodies but not the preimmune serum have inhibited ionomycin-induced production of IL-1β, IL-6, and TNF-α, whereas the changes in the production of IL-10 and IL-12 (p40) were not statistically significant (Fig. 10).

DISCUSSION

The endogenous sialidase activity significantly increases during activation in majority of immune cells, including T cells, B cells, and monocytes, whereas sialylation of some of their surface molecules decreases (10, 42–45). In particular, the reduction of sialylation was reported for MHC class I, required to render T cells responsive to antigen-presenting cells (43); Gαs3,Gαt5 ganglioside, which modulates Ca²⁺ immobilization and regulates IL-4 production in T cells (11); and monocyte receptor for hyaluronic acid,
CD44 (44, 45). In the activated B cells, the induction of the sialidase activity is important to convert so-called, vitamin D3-binding protein into a macrophage-activating factor (46). Indirect evidence suggested the involvement in these processes of Neu1, a ubiquitously expressed lysosomal sialidase responsible for the catabolism of glycoconjugates. In vivo, SM/J or SM/B10 mice strains with the missense mutation in the \( \text{NEU1} \) gene, which reduces the enzyme activity to \(~30\%\) of normal (47), have an impaired activation of macrophages, whereas their lymphocytes fail to synthesize IL-4, show low response for antigen-presenting cells, and do not produce macrophage-activating factor (10, 42, 46). In human sialidosis patients, Neu1 deficiency may result in reduced capacity of immune cells to produce cytokines and antibodies leading to partial immunodeficiency and may account for frequent pulmonary infections.6 We have provided direct evidence that Neu1 message and protein are induced severalfold during the differentiation of circulating human monocytes into professional antigen-presenting cells, such as macrophages (13) or dendritic cells.7 In this study, we investigated the mechanisms controlling the expression of the \( \text{NEU1} \) gene and found that the transcriptional activity of the \( \text{NEU1} \) gene promoter was significantly increased during the PMA-induced differentiation of monocytic cells into macrophages. The highest level of induction was observed for a 550-bp fragment of the \( \text{NEU1} \) gene promoter containing several potential sequence motifs for binding AP-1, a group of homodimeric and heterodimeric complexes composed of various \( \text{fos} \) and \( \text{jun} \) gene products, such as c-Jun, JunB, JunD, c-Fos, and FosB as well as the Fos-related antigens Fra-1 and Fra-2 (48–54). All of these complexes (also called protooncoproteins) interact with the common binding sites called PMA-responsive elements and mediate gene induction in response to growth factors, cytokines, phorbol esters, and a variety of other stimuli, including oxidative stress (34–36). In particular, one of the functions of AP-1 is to mediate inflammatory responses in lymphocytes and macrophages by stimulating cell activation and the expression of cytokines (55). Previously, it was shown that proinflammatory factors, lipopolysaccharide and TNF-\( \alpha \), caused 16-h MAPK-dependent induction of the endogenous acidic sialidase activity in monocytes (44, 45), resulting in desialylation of CD44. In turn, this increased the affinity of monocytes to hyaluronic acid, their adhesion, and the TNF-\( \alpha \) production (45). Since in both cases the 1.4–1.6-fold increase of the sialidase activity was detected only with 4MU-NANA and not with mixed bovine gangliosides as a substrate, it is conceivable that the effect was caused by the up-regulation of Neu1. Our experiments directly showed that the promoter activity of Neu1 was potently induced by TNF-\( \alpha \) that activates AP-1 expression and reduced by AP-1 inhibitors curcumin and N-acetylcysteine. Although we do not have direct evidence of the interaction between the \( \text{NEU1} \) gene promoter and AP-1, it is tempting to speculate that this protein may be involved in the regulation of the Neu1 expression during the monocyte activation and differentiation.

The fact that Neu1 is specifically induced in professional antigen-presenting cells suggests that this enzyme may be

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6 G. Mitchell, personal communication.
7 N. Stamatos, manuscript in preparation.
implicated in the antigen presentation. However, both MHC class II and I molecules are expressed at the cell surface, whereas Neu1 was found predominantly in the endosomal/lysosomal compartment. Here we show that during the differentiation of monocytes into macrophages, the Neu1 is constitutively transported to the plasma membrane along with class II molecules rather than being actively retained in the lysosomes. Using immunocytochemistry, we demonstrated that in the differentiating THP-1 cells and THP-1-derived macrophages as well as in the mature primary macrophages, a vast majority of the Neu1 was found on the cell periphery co-localizing with the mannose receptor. In addition, biochemical assays showed that the biotinylation of the cell surface proteins results in the labeling of Neu1 in macrophages but not in the proliferating THP-1 cells and that ~80% of Neu1 in the THP-1-derived macrophages was accessible for the substrate added directly to the cell medium, confirming that it is localized on the cell surface. Similar observations were made for the lysosomal carboxypeptidase, cathepsin A, whose interaction with Neu1 is essential for the activation of Neu1 in the lysosomes.

Both Neu1 and cathepsin A are sorted from the trans-Golgi network to the endosomial pathway. Like the majority of soluble lysosomal luminal proteins, cathepsin A is targeted through the mannose 6-phosphate receptor pathway (56), whereas the intracellular trafficking of Neu1 is mediated by the adaptor proteins through a Tyr-containing motif in its C terminus (9). In the lysosome, the two proteins undergo activation. The activation of cathepsin A involves its processing by a lysosomal endopeptidase, which removes a 2-kDa “linker” polypeptide between the two polypeptide chains of the mature enzyme (57). The exact molecular mechanism that underlies the activation of Neu1 is unknown, but most likely it involves a conformational change of the Neu1 polypeptide after the formation of the complex with cathepsin A. We speculate that in the differentiating cells, both enzymes are first targeted to the endosomal/lysosomal compartment, where they gain enzymatic activity and then are sorted to the vesicles destined to the plasma membrane. The existence of such a lysosome-to-plasma membrane transport pathway has been well documented for other professional antigen-presenting cells, dendritic cells, where it plays a crucial role in targeting the peptide-MHC II complexes.

**FIGURE 9.** Reduced phagocytosis in THP-1-derived macrophages with suppressed Neu1. Control THP-1-derived macrophages (Control), cells transfected with Neu1 siRNA (Neu1 siRNA) and scrambled RNA (Mock), and cells differentiated in the presence of 20 μg/ml anti-Neu1 antibodies (Anti-Neu1 ab) or of the preimmune serum (Preimmune ab) were incubated for 3 h at 36 °C in the presence of fluorescein-labeled E. coli bioparticles (30 particles/cell), fixed, and stained with 4',6-diamidino-2-phenylindole. Slides were examined on a Nikon Eclipse E6000 direct epifluorescence microscope. Magnification was ×200. Panels represent typical images obtained in triplicate experiments; 70–150 cells (10 panels) were studied for each condition in each experiment. Bottom, the ingestion index was calculated for each type of cells as the percentage of macrophages with phagocytized particles multiplied by the average number of particles phagocytized per ingested macrophage. The results show an average ± S.D. of three independent experiments. *, p < 0.001 as compared with nontransfected cells, mock-transfected cells, or cells treated with preimmune serum.

**FIGURE 10.** Effect of anti-Neu1 antibodies on the ionomycin-induced production of IL-10, IL-12(p40), IL-1β, IL-6, and TNF-α in THP-1-derived macrophages. THP-1-derived macrophages were stimulated for 12 h with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the absence (Control) or in the presence of preimmune rabbit serum (100 μg/ml) of anti-Neu1 serum (20 and 100 μg/ml) followed by the analysis of IL and TNF-α production by an enzyme-linked immunosorbent assay. The data show an average of three independent experiments ± S.E.; *, p < 0.05 using unpaired Student’s t test.
(pMHCs) to the cell surface (58). Upon receipt of a maturation stimulus (such as lipopolysaccharide), the internalized antigens are processed in the endosomes, and pMHCs are formed. Several hours later, they appear in the LAMP-negative compartments termed class II-containing vesicles, which are the intermediates in the transfer of pMHCs to the plasma membranes (58). The concentration of pMHCs in class II-containing vesicles reaches a maximum 12 h after the lipopolysaccharide treatment, and after an additional 12 h, the majority of complexes is already transferred to the cell surface. The described targeting of pMHCs in dendritic cells resembles the targeting of Neu1 and cathepsin A in differentiating monocytes and macrophages. We found that 12–24 h after receiving the differentiation stimulus, the lysosomal-endosomal apparatus of monocytes undergoes a dramatic rearrangement. The MHC II-positive and Neu1-positive compartments are segregated from the LAMP-2-positive lysosomes and late endosomes, relocated closer to the cell surface, and fused with the plasma membranes. The same vesicles are stained positively for the internalized antigen HRP or fluorescent dextran, which in the proliferating monocytic cells are normally stored for at least several days in a LAMP-2-positive endosomal/lysosomal compartment. In the differentiating cells, the dextran molecules and HRP peptides (probably in the form of pMHCs) were gradually accumulated on the cell plasma membrane colocalizing at least in part with Neu1 and cathepsin A.

Most importantly, the suppression of Neu1 expression in the THP-1-derived macrophages by siRNA or their treatment with anti-Neu1 antibodies significantly reduced the functional capacity of the cells, such as the ability to engulf bacteria or to produce cytokines, suggesting that the up-regulation of the Neu1 expression is important for activation of macrophages and establishing the link between Neu1 and cellular immune response. Further experiments should clarify if the differentiation-induced translocation of the lysosomal sialidase, Neu1, and the lysosomal carboxypeptidase, cathepsin A, to the plasma membrane is also important for other specific functions of the macrophages and, in particular, for the antigen presentation. Optimal sialylation of the cell surface glycoproteins may also be important for the interaction of macrophages with other cells (e.g. with T, B, and NK cells via sialic acid-binding immunoglobulin-like lectins) (reviewed in Ref. 59). Fully differentiated macrophages are present in all body tissues (microglial cells in the central nervous system, Kupffer cells in liver, etc.). Therefore, it is quite conceivable that if up-regulation of the NEU1 gene is functionally important for macrophage differentiation, then the primary or secondary deficiency of the Neu1 activity may affect functions of all monocyte-derived cells and cause defects in multiple tissues and organs.

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