Polysialylated Neuropilin-2 Is Expressed on the Surface of Human Dendritic Cells and Modulates Dendritic Cell-T Lymphocyte Interactions*

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Polysialic acid (PSA) is a unique linear homopolymer of α2,8-linked sialic acid that has been identified as a posttranslational modification on only five mammalian proteins. Studied predominantly on neural cell adhesion molecule (NCAM) during development of the vertebrate nervous system, PSA modulates cell interactions mediated by NCAM and other adhesion molecules. An isoform of NCAM (CD56) on natural killer (NK) cells is the only protein known to be polysialylated in cells of the immune system, yet the function of PSA in NK cells remains unclear. We show here that neuropilin-2 (NRP-2), a receptor for the semaphorin and vascular endothelial growth factor families in neurons and endothelial cells, respectively, is expressed on the surface of human dendritic cells and is polysialylated. Expression of NRP-2 is up-regulated during dendritic cell maturation, coincident with increased expression of ST8Sia IV, one of the key enzymes of PSA biosynthesis, and with the appearance of PSA on the cell surface. PSA on NRP-2 is resistant to digestion with peptide N-glycosidase F but is sensitive to release under alkaline conditions, suggesting that PSA chains are added to O-linked glycans of NRP-2. Removal of polysialic acid from the surface of dendritic cells or binding of NRP-2 with specific IgG promoted dendritic cell-induced activation and proliferation of T lymphocytes. Thus, this newly recognized polysialylated protein on the surface of dendritic cells influences dendritic cell-T lymphocyte interactions through one or more of its distinct extracellular domains.

Sialyltransferases comprise a large family of enzymes that add sialic acid by several different glycosidic linkages to cell surface glycoconjugates (1). In vertebrates, single molecules of sialic acid can be added by α2-3 or α2-6 linkages to the penultimate sugar (usually galactose) of glycoconieties. Glycoproteins can also be modified by linear homopolymers of α2-8-linked sialic acid with a degree of polymerization greater than seven that are referred to as polysialic acid (PSA) (2). Expression of PSA is great during ontogeny and is drastically reduced in the adult, where mostly neuroectoderm-derived tissue contains PSA (2–5). Polysialic acid has been identified in only five mammalian proteins: NCAM, CD36, α-subunit of the voltage-gated sodium channel, and two sialyltransferases, ST8Sia II and ST8Sia IV (2, 6–9). Polysialic acid modification of NCAM during neuronal development has been studied extensively and has been shown to play a significant role in cell migration, axonal guidance, synapse formation, and functional plasticity by preventing the formation of stable cell contacts mediated by NCAM and other cell surface molecules (10, 11). Neural cell adhesion molecule is heavily polysialylated in undifferentiated neurons but loses its PSA content in mature neurons that have formed permanent intercellular synapses (10, 11). The function of PSA modification on the other four proteins remains to be determined. An isoform of NCAM, namely CD56, that is present in natural killer (NK) cells of the immune system is known to be polysialylated, yet the role of polysialylated CD56 in NK cell function remains unknown.

Circulating peripheral blood monocytes can differentiate into either macrophages or dendritic cells after exposure to specific stimuli. Dendritic cells present antigen to naïve T lymphocytes at specific intercellular junctions called the immunological synapse (12). Dendritic cell-T lymphocyte contact at these sites is mediated by numerous proteins that include among others major histocompatibility complex class I and II molecules, T cell receptor, adhesion molecules such as LFA1, LFA3, ICAM1, ICAM3, the B7 family members (CD80 and CD86), CD28, and DC-SIGN. Immunological and neuronal synapses are similar in the capacity to establish cell-cell adhesion, to activate signaling pathways, and to secrete and respond to extracellular soluble mediators. Dendritic cells and neurons share an additional feature of expressing members of the neuropilin transmembrane receptor family (13–15).

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§ The abbreviations used are: PSA, polysialic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; endothNF, endothelial-cell adhesion molecule; NK, natural killer; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; FCS, fetal calf serum; RT, reverse transcription; nt, nucleotides; mAb, monoclonal antibody; NRP-2, neuropilin-2; PNGase F, peptide N-glycosidase F; IFN, interferon; DC, dendritic cells; ImDC, immature DC; mDC, mature DC.
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The two members of the neuropilin family, NRP-1 and NRP-2, are best known for their role in facilitating axonal guidance during the development of the neuronal system (13, 14, 16, 17). Neuropilin-1 and neuropilin-2 are also expressed in vascular endothelial cells where they affect proliferation, migration, and angiogenesis (18, 19). In contrast, only NRP-2 has been found to be expressed on lymphatic endothelium (20, 21). Neuropilin-2 has also been described in neuroendocrine cells located along the human digestive tract, pancreatic islet cells, and different malignant cells (22–25). The neuropilins share 45% homology and have overall similar structure with five main extracellular domains; two complement binding domains, two coagulation factor V/VIII homology domains, and a MAM (meprin, tyrosine phosphatase domain) region (13, 26, 27). The neuropilins have a single transmembrane segment and a short cytoplasmic domain that is devoid of signaling motifs. NRP-1 and NRP-2 can bind ligands such as members of the semaphorin and VEGF families and can activate cellular signaling pathways by interacting with other cell surface receptors such as VEGF receptors and plexins (18, 28–33). In the immune system, NRP-1 is expressed in dendritic cells and a subset of lymphocytes and is important in the regulation of dendritic cell-T lymphocyte interactions (15, 34). In contrast, NRP-2 has not yet been identified in cells of the immune system.

In this report, we identify NRP-2 as a polysialylated cell surface protein expressed in human monocyte-derived dendritic cells. We show that NRP-2 expression is up-regulated during dendritic cell maturation, coincident with a marked increase in expression of ST8Sia IV, one of the key enzymes of PSA biosynthesis, and with the appearance of PSA on the cell surface. We also show that either binding NRP-2 with specific IgG or removing PSA from the cell surface enhances the ability of dendritic cells to activate T lymphocytes in a mixed lymphocyte reaction system (Gambro Healthcare, Lund, Sweden). The purity of monocyte-derived dendritic cells were harvested at the indicated times, and total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) following the protocol suggested by the manufacturer. The RNA preparation was treated with DNase I (Invitrogen) at 37 °C for 30 min to remove contaminating DNA. DNase was then removed by binding to DNase inactivation reagent (Ambion, Austin, TX). Semiquantitative real-time RT-PCR was performed using a Quantitect SYBR Green RT-PCR kit (Qiagen) with an ABI sequence detection system (ABI PRISM 5700) as described previously (35). Ten nanograms (10 ng) of total RNA was used to detect gene expression of ST8Sia II (GenBank™ accession number U33551), ST8Sia IV (GenBank™ accession number L41680), NRP-1 (GenBank™ accession number AF016050), NRP-2 (GenBank™ accession number AF016098), and NCAM (GenBank™ accession number NM_000615). Gene expression of 18S rRNA (GenBank™ accession number X03205) was also measured as an internal control. The following primers were selected using DNAsis Max software (Hitachi, Japan) and were synthesized by Qiagen (Germantown, MD): ST8Sia II (forward, nt 402–421, 5'-GATGGCATTCCACATGTTG-3', and reverse, nt 746–727, 5'-ACCAATGAGAATTCGCGT-3') yielding a 138-bp product; ST8Sia IV (forward, nt 608–625, 5'-ACCATGAGAATTCGCGT-3', and reverse, nt 746–727, 5'-AGCAGAACTCCACACAGG-3') yielding 149-bp product; ST8Sia III (forward, nt 551–532, 5'-CACCAGTACGACGCTG-3') yielding a 149-bp product; ST8Sia IV (forward, nt 608–625, 5'-ACCATGAGAATTCGCGT-3', and reverse, nt 746–727, 5'-AGCAGAACTCCACACAGG-3') yielding a 138-bp product; NRP1 (forward, nt 143–164, 5'-ATCACCCAGTTGAAAATCGCA-3', and reverse, nt 236–216, 5'-TCCCTCAAATCAGAGTGGG-3') yielding a 93-bp product; NRP2 (forward, nt 684–667, 5'-GGATGCCATTCACATTTTTG-3', and reverse, nt 800–780, 5'-ACCAGGTAGTACGCAGAGG-3') yielding a 152-bp product; NCAM (forward, nt 1313–1333, 5'-GTACCTCTTACTCTGTAGG-3', and 5'-GTACCTCTTACTCTGTAGG-3').
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reverse, nt 1483–1464, 5′-TCCGGCATCAGTGTACTGGA-3′, yielding a 170-bp product; 18 S rRNA (forward, nt 1279–1298, 5′-CGGACAGGTGACAGATTG-3′, and reverse, nt 1397–1378, 5′-ATGCCAGAGTCTCGGTCTGT-3′), yielding a 119-bp product. To synthesize cDNA, reverse transcription was performed at 50 °C for 30 min. After a 15-min hot start at 95 °C, DNA amplification was allowed to proceed for 35 cycles (15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C). All reactions were run in triplicate. Semiquantitative analysis was based on the cycle number (C_T) at which the SYBR Green fluorescent signal crossed a threshold in the log-linear range of RT-PCR. The accuracy of each reaction was confirmed by analysis of melting curves and product size on gel electrophoresis.

Analysis of Cellular Proteins by SDS-PAGE and Immunoblot—Monocytes and monocyte-derived immature and mature dendritic cells were collected, and proteins from 2 × 10^6 cells were solubilized in 0.1 ml of radioimmune precipitation assay solution containing 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, a protease inhibitor mixture (1:100 dilution of protease inhibitor mixture from Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium vanadate. Cells were incubated at 4 °C for 20 min, and the lystate was centrifuged at 10,000 × g for 10 min. The amount of protein in the supernatant was measured by the Bradford method using a Bio-Rad protein assay kit. Proteins (1–10 μg) from each cell lystate were resolved by electrophoresis on a 10% SDS-polyacrylamide gel using Tris-glycine-SDS running buffer (gel and running buffer were from Invitrogen), electrotransferred by a semi-wet gel using Tris-glycine-SDS running buffer (gel and running buffer were from Invitrogen), and probed with 0.5 μg/ml mouse anti-PSA mAb 735 or mouse mAb against NRP-2 and goat anti-NRP-1 IgG (both from Santa Cruz Biotechnology, Santa Cruz, CA), developed using an ECL reagent (Amersham, Arlington Heights, IL), and exposed to X-ray film.

To immunoprecipitate specific proteins for analysis by SDS-PAGE, a lystate from 1 × 10^7 mature dendritic cells prepared in 0.4 ml of radioimmune precipitation assay solution as described above was first mixed with 2 μg of preimmune mouse IgG (Sigma-Aldrich) and 30 μl of protein G plus/protein A-agarose (Calbiochem) and incubated for 2 h at 4 °C with end-over-end rotation. Agarose beads and nonspecifically bound protein were removed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant was then mixed with protein G plus/protein A-agarose (30 μl) that had been preincubated with 2 μg of mouse anti-PSA mAb 735 or mouse mAb against NRP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for 16 h at 4 °C with end-over-end rotation. The beads and bound protein were collected by centrifugation at 10,000 rpm for 10 min at 4 °C, washed 3 times with radioimmune precipitation assay solution, and resuspended in SDS-PAGE sample buffer. 1/20 of the total immunoprecipitated product was analyzed by SDS-PAGE immunoblot as described above, whereas 1/2 of the total immunoprecipitated product was resolved by SDS-PAGE and stained with Simply Blue™ SafeStain (Invitrogen) for subsequent protein identification.

Immunofluorescent Staining of Cell Surface Proteins and Analysis by Flow Cytometry—Purified monocytes and immature/mature dendritic cells were collected as described above, and 1 × 10^6 cells were resuspended in 0.5 ml of PBS, pH 7.4, containing 2% human serum AB (Gemini Bioproducts, Calabasas, CA). Cells were then stained at 4 °C for 30 min with 10 μg/ml mouse anti-PSA mAb 735, mouse mAb against NRP-1 (Miltenyi Biotec, Auburn, CA), or mouse anti-NRP-2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with biotinylated rabbit anti-mouse IgG and then phycoerythrin-conjugated streptavidin (both from Dako, Carpinteria, CA). Cells were also stained with phycoerythrin-, allophycocyanin-, or fluorescein isothiocyanate-conjugated monoclonal antibodies to CD1a, CD3, CD14, CD19, CD40, CD83, CD86 CD206, HLA-DR, and isotypic control IgG (all mAbs from BD Pharmingen) following the procedure recommended by the manufacturer. After incubation with antibodies, the cells were washed with 2 ml of phosphate-buffered saline and fixed with 1.0% paraformaldehyde. Fluorescence was analyzed by flow cytometry using a BD Biosciences FACSCalibur, and data were analyzed using FlowJo data analysis software.

Digestion with Endosialidase NF and Peptide N-Glycosidase F (PNGase F)—Monocytes and monocyte-derived dendritic cells were treated with endosialidase NF (endoNF) (37, 38) at 1.5 μg/ml for 2 h at 37 °C in a CO_2 incubator. Monocytes were treated with endoNF after being resuspended at 2 × 10^6 cells/ml in RPMI 1640 containing 10% FCS. Mature dendritic cells were exposed to endoNF after 5 days in culture by the addition of the enzyme directly to the medium of wells in the tissue culture plates. Cells were collected after the treatment and were washed 2 times in RPMI 1640 containing 10% FCS and were either directly analyzed or placed back in culture in a mixed lymphocyte reaction. NK cells were similarly treated with endoNF after cells were resuspended at 2 × 10^6 cells/ml in RPMI 1640 containing 10% FCS. To heat-inactivate endoNF, a solution of enzyme was placed in a boiling water bath for 10 min.

To release N-linked glycans from NRP-2 and CD56, both proteins were immunopurified with the respective Abs indicated above, and an equal amount of each in 0.01 ml was denatured in a solution containing 0.5% SDS and 0.04 μM dithiothreitol at 100 °C for 10 min and either mock-treated or incubated with 1000 units of PNGase F (New England Biolabs, Ipswich, MA) for 1 h at 37 °C. Mock- and PNGase F-treated proteins were analyzed by SDS-PAGE and immunoblot using anti-PSA mAb 735 and anti-CD56 IgG. The relative intensity of bands on exposed films was quantitated using a PharusFX™ Plus Molecular Imager and Molecular Analyst software (Bio-Rad).

β-Elimination of O-Linked Glycans—To release O-linked glycans from NRP-2 and CD56, both proteins were immunopurified, and an equal amount of each was separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes as described above. Duplicate lanes were placed in either HOH or a solution of 0.1 N NaOH at 37 °C for 1 h as described previously (39). The membranes were then probed on immunoblot
with anti-PSA mAb 735, and the relative intensity of bands on the exposed films was determined by densitometry as noted above.

**Mixed Lymphocyte Reaction**—Monocytes were differentiated into mature dendritic cells in vitro and were treated with endoNF or heat-inactivated endoNF or were mock-treated for 2 h as indicated above after 5 days in culture. After the 2-h treatment, cells were harvested from the wells by gentle pipetting and washed twice with RPMI medium 1640 and 10% FCS, and 2 × 10^4 mature dendritic cells were mixed with 1 × 10^5 allogenic resting T lymphocytes in 0.20 ml of RPMI 1640, 10% FCS without IL-4 and GM-CSF in 96-well U-bottom plates (Nunc, Denmark). Alternatively, dendritic cells were preincubated with preimmune goat IgG (Sigma-Aldrich) or goat polyclonal anti-NRP-2 (R&D Systems, Minneapolis, MN) at 40 μg/ml for 1 h at 37 °C before mixing with resting lymphocytes. The final concentration of each antibody in the mixed lymphocyte reaction was 20 μg/ml. Where indicated, cells were first treated with endoNF and then incubated with anti-NRP-2 IgG. After 48 h in culture, an aliquot of the medium was sampled to determine the amount of IFN-γ by enzyme-linked immunosorbent assay (R&D Systems), and 1 μCi of [3H]thymidine (5.0 Ci/mmol; Amersham Biosciences) was added to each well. Cells were maintained in culture for an additional 18 h and harvested onto glass fiber filters (Wallac Oy, Turku, Finland) with a filtermate 196 harvester (Wallac Oy), and the amount of [3H]thymidine incorporation was measured using a TopCount microplate liquid scintillation counter (1450 Microbeta Trilux Wallac Oy).

**Mass Spectrometry**—Proteins from specific regions of the SDS-polyacrylamide gel were analyzed by mass spectrometry at the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia Health Sciences Center, Charlottesville, VA as previously described (40). Briefly, protein from gel slices was reduced, alkylated, and trypsinized and analyzed on a Finnigan LTQ-FT mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8-cm × 75-mm inner diameter Phenomenex Jupiter 10-mm C18 reversed-phase capillary column. The nanospray ion source was operated at 2.8 kV. The resulting mass spectroscopy data were analyzed by data base searching using the Sequest search algorithm against human International Protein Index. The probability of a protein being present based on the mass spectrometry was determined by the PeptideProphet™ algorithm (41).

**RESULTS**

**Differentiation of Monocytes into Dendritic Cells Results in Increased Expression of Polysialic Acid on the Cell Surface**—Change in the PSA content of NCAM during the differentiation of neurons helps guide the ultimate formation of correct neurologic synapses. Maturing dendritic cells, like neurons, must also interact selectively with other cells and components in the extracellular milieu as they migrate from the periphery to lymphatic tissue to interact with T lymphocytes. To determine whether PSA was present on the surface of monocytes and/or monocyte-derived dendritic cells, monocytes were purified from the peripheral blood of human donors and maintained in culture conditions that promoted differentiation into dendritic cells. When grown in the presence of GM-CSF and IL-4, monocytes differentiated into immature dendritic cells that were characterized phenotypically by the loss of cell surface
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CD14 and increased expression of CD206 (mannose receptor), CD86, and CD1a (data not shown). After exposure to E. coli lipopolysaccharide, these immature dendritic cells differentiated into mature dendritic cells that expressed cell surface CD83 and demonstrated further increases in amount of surface CD86 and CD1a (data not shown).

Monoclonal Ab 735 specifically recognizes chains of PSA containing at least eight α2,8-linked sialic acids (36). When analyzed as a positive control by flow cytometry, natural killer NK-92 cells that express polysialylated NCAM stained strongly with mAb 735 (Fig. 1D, bold line) and lost reactivity with mAb 735 after exposure to endoNF, an endoglycosidase that specifically cleaves chains of at least five α2,8-linked sialic acids (Fig. 1D, shaded region). When freshly isolated monocytes were stained with mAb 735, there was a heterogeneous pattern of faintly stained cells detected by flow cytometry (Fig. 1A, bold line). After 4–6 days in culture, the monocyte-derived immature dendritic cells stained heterogeneously with mAb 735, but the staining was more intense (Fig. 1B, bold line). Further maturation of dendritic cells after exposure to LPS resulted in a relatively uniform population of cells that stained even more intensely with mAb 735 (Fig. 1C). The specificity of staining polysialylated proteins in monocytes and mature dendritic cells with mAb 735 was confirmed by loss of staining after intact cells had been exposed to endoNF (Fig. 1, A and C; shaded regions). The loss of staining was complete for mature DCs (Fig. 1C), but some weak staining of monocytes remained after exposure to endoNF (Fig. 1A). This pattern of staining for monocytes and monocyte-derived dendritic cells by flow cytometry was seen in cells differentiated in the presence of FCS, IL-4, and GM-CSF or in serum-free medium with IL-4 and GM-CSF (data not shown), suggesting that the expression of PSA on the cell surface was related to differentiation induced by factors independent of serum components. These results demonstrate that the content of α2,8-linked PSA increases on monocytes as they differentiate into immature dendritic cells and increases further during maturation.

Characterization of Polysialylated Protein(s) in Monocytes and Monocyte-derived Dendritic Cells—To analyze the polysialylated protein(s) in monocytes and monocyte-derived dendritic cells, cellular proteins were separated by SDS-PAGE and immunoblotted using PSA-specific mAb 735. Polysialylated NCAM from a lysate of NK cells was used as a positive control by flow cytometry (Fig. 1, A–C) and suggest that a polysialylated protein or proteins distinct from polysialylated NCAM is expressed on monocytes and on dendritic cells at different stages of maturation.

Identification of Neuropilin-2 as a Polysialylated Protein in Monocyte-derived Dendritic Cells—To identify the polysialylated protein(s) in monocyte-derived cells, proteins from a lysate of mature dendritic cells were immunoprecipitated with mAb 735 and analyzed by immunoblot, mass spectrometry, and amino acid sequencing. Analysis of the immunoprecipitate by SDS-PAGE and an immunoblot using anti-PSA mAb 735 as described under “Experimental Procedures.” Ten micrograms of cellular protein from monocytes (lane 1) and monocyte-derived dendritic cells (lane 2) were analyzed. Five micrograms of protein from a lysate of NK-92 cells was similarly evaluated (lane 5). A portion of intact mDC (lane 4) and NK cells (lane 6) was treated with endoNF for 2 h at 37 °C before cell lysis and analysis by immunoblot. These results from monocytes and monocyte-derived cells from one donor are representative of data from seven donors.

Flow cytometry (Fig. 1, A–C) and suggest that a polysialylated protein or proteins distinct from polysialylated NCAM is expressed on monocytes and on dendritic cells at different stages of maturation.

Identification of Neuropilin-2 as a Polysialylated Protein in Monocyte-derived Dendritic Cells—To identify the polysialylated protein(s) in monocyte-derived cells, proteins from a lysate of mature dendritic cells were immunoprecipitated with mAb 735 and analyzed by immunoblot, mass spectrometry, and amino acid sequencing. Analysis of the immunoprecipitate by SDS-PAGE and an immunoblot using anti-PSA mAb 735 revealed a diffuse band ranging from 100 to 260 kDa, with the most intense staining detected in the molecular mass range of 130–200 kDa (Fig. 3, lane 1). This band of polysialylated protein is broader than that seen in Fig. 2, lane 3, where protein was analyzed directly from a lysate of mature dendritic cells. The relative breadth of this band may be partly due to degradation of PSA and/or NRP-2 that may occur during immunoprecipitation and immunoblotting. Proteins in four slices of a companion SDS-PAGE gel with molecular masses of 100, 120, 150, and 250 kDa were analyzed by mass spectrometry and amino acid sequencing, and peptides matching the detected sequences with >95% probability were identified (Table 1). Although these peptides are present in >19 distinct proteins, only one identified protein, isoform A22 of neuropilin-2 precursor, that resulted from analysis of the 150-kDa band, is a cell surface protein whose molecular mass in the absence of PSA (104.8 kDa) is consistent with the results in Fig. 2, lane 3.
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To determine whether NRP-2 is a polysialylated protein in mature dendritic cells, protein that was immunoprecipitated using anti-NRP-2 IgG from a lysate prepared from mature dendritic cells was analyzed by immunoblot with anti-PSA mAb 735. A diffuse protein band spanning 130–200 kDa was detected when the gel was immunostained with mAb 735 (Fig. 3, lane 2). Protein in this molecular mass range was also detected when the gel was probed with anti-NRP-2 IgG (Fig. 3, lane 3). The size of this band was reduced to 110 kDa when mature dendritic cells were treated with endoNF before immunoprecipitating with anti-NRP-2 IgG and were immunostained with anti-NRP-2 IgG (Fig. 3, lane 4), confirming that NRP-2 is indeed polysialylated. No band was evident when endoNF-treated mature dendritic cells were immunoprecipitated with mAb 735 and analyzed on immunoblot using mAb 735 (data not shown). These results demonstrate that NRP-2 is a polysialylated protein that is expressed in mature dendritic cells and that its molecular mass likely varies up to 200 kDa depending on the extent of polysialylation.

Neuropilin-2 Is Up-regulated at the RNA and Protein Levels during Differentiation of Monocytes to Dendritic Cells—

Although NRP-2 and NRP-1 have been identified in several different cell types and expression of NRP-1 is induced during monocyte differentiation (15), NRP-2 expression in cells of the immune system has not been described previously. To determine the expression pattern in monocytes and monocyte-derived dendritic cells, the amount of RNA encoding NRP-2 was quantitated in monocytes and in immature and mature dendritic cells. Change in the amount of NRP-2 RNA was normalized to expression of 18S rRNA that changed minimally during differentiation. Although no RNA encoding NRP-2 was detected in freshly isolated monocytes, expression was induced de novo in immature dendritic cells and was up-regulated 12.1 ± 3.4-fold in mature dendritic cells (Fig. 4A). Consistent with the RNA data shown in Fig. 4A, immunoblot analysis of cell lysates using anti-NRP-2 IgG revealed no band in monocytes and 2 faint bands between 120 and 130 kDa in immature dendritic cells (Fig. 4B, lanes 1 and 2). In addition, a more diffuse and intense band in the range of 130–180 kDa and a weaker band at 120 kDa were detected in mature dendritic cells (Fig. 4B, lane 3). When mature dendritic cells were treated with endoNF before cell lysis and analysis by SDS-PAGE and immunoblot, the size of the 130–180-kDa protein was reduced to 130–150 kDa, and the intensity increased, whereas the 120-kDa band did not change in size (Fig. 4B, lane 4). This pattern of change of total cellular NRP-2 was also reflected in flow cytometry by the staining of intact cells with anti-NRP-2 IgG. Minimal to no staining of monocytes was seen, whereas a diffuse bimodal staining pattern was detected in immature dendritic cells (Fig. 4C). Further differentiation of immature dendritic cells resulted in generation of a homogeneous population of very...
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**FIGURE 4. Expression of NRP-2 increases during differentiation of dendritic cells.** Change in expression of NRP-2 specific RNA and NRP-2 protein during differentiation of monocytes into immature (imDC) and mature (mDC) dendritic cells was determined. A, total RNA was isolated from monocytes and monocyte-derived imDC and mDC and was analyzed using primers that were specific for NRP-2 by SYBR Green semiquantitative real-time RT-PCR as described under “Experimental Procedures.” The -fold change in NRP-2 RNA in mDC compared with imDC is shown relative to the change in the expression of 18 S rRNA that was measured as an internal control. The expression of NRP-2 RNA in imDC was set to 1, as noted by the dotted, horizontal line. B, the amount of NRP-2 protein in monocytes, imDC, and mDC was determined by separation of 5 μg of total cellular protein from each sample by SDS-PAGE and evaluation by immunoblot using anti-NRP-2 IgG as described under “Experimental Procedures.” C, the amount of NRP-2 on the surface of intact monocytes, imDC, and mDC was determined by flow cytometry after cells were stained with anti-NRP-2 IgG (bold line) or isotype control IgG (thin, dotted line). These results shown in A–C from one donor are representative of data from five different donors.

**FIGURE 5. Expression of ST8Sia II and ST8Sia IV during monocyte differentiation.** Change in expression of ST8Sia II (A)- and ST8Sia IV (B)-specific RNAs during differentiation of monocytes into immature (imDC) and mature (mDC) dendritic cells was determined. Total RNA was isolated from monocytes and monocyte-derived imDC and mDC and was analyzed by SYBR Green semiquantitative real-time RT-PCR as described under “Experimental Procedures.” The -fold change in each RNA in imDC and mDC compared with monocytes is shown relative to the change in the expression of 18 S rRNA. The expression of ST8Sia II and ST8Sia IV RNA in monocytes was set to 1, as noted by the dotted, horizontal line. Data represent the mean ± S.E. of samples from one donor run in triplicate and are representative of data from five experiments using cells from different donors.

brightly stained cells (Fig. 4C). The increased expression of NRP-2 on the surface of differentiating monocytes (Fig. 4C) corresponds closely to the pattern of increase in cell surface PSA that is seen in Fig. 1. These data further support that NRP-2 is a polysialylated protein whose presence on the cell surface is induced de novo in monocyte-derived immature dendritic cells and further increases during dendritic cell maturation.

**ST8Sia II and ST8Sia IV Expression during Monocyte Differentiation—** Two sialyltransferases, ST8Sia II and ST8Sia IV, are responsible for catalyzing PSA synthesis of NCAM in neurons. To determine whether either may be involved in the polysialylation of NRP-2, the amount of RNA encoding each was determined in monocytes and monocyte-derived cells. RNA encoding both enzymes was detected in monocytes; crossover threshold CT values during PCR for 18 S rRNA and ST8Sia II and ST8Sia IV RNAs were 17.2 ± 0.4, 29.7 ± 0.8, and 32.0 ± 0.5, respectively. The amount of RNA encoding ST8Sia II increased 2.5 ± 0.8-fold in relation to 18 S rRNA during differentiation into immature dendritic cells and stayed constant during further maturation (Fig. 5A). In contrast, whereas the amount of RNA encoding ST8Sia IV increased 5.9 ± 1.4-fold during differentiation into immature dendritic cells, it increased 52.1 ± 6.2-fold after cells were exposed to LPS (Fig. 5B). This marked increase in RNA encoding ST8Sia IV paralleled the increase in expression of polysialylated NRP-2 and the appearance of PSA on the surface of dendritic cells.

**PSA Chains on NRP-2 Are Sensitive to β-Elimination by Alkaline Treatment but Not to Digestion with PNGase F—** To determine whether PSA is present on N-linked glycans, as occurs with NCAM in neurons (2), or on O-linked glycans of NRP-2, polysialylated NRP-2 was analyzed on immunoblotting using anti-PSA mAb 735 after treatment with either PNGase F, an enzyme that releases O-linked oligosaccharides or 0.1 N NaOH, a condition that releases O-linked glycans (8, 39). When immunopurified NRP-2 was separated by SDS-PAGE, transferred to membranes, and either mock-treated or treated with 0.1 N NaOH for 1 h, there was an 86% reduction in staining with mAb 735 of the alkali-treated NRP-2 (Fig. 6A, lanes 1 and 2). As a control for nonspecific loss of anti-PSA staining during this procedure, purified CD56 was similarly analyzed and was found to incur a 67% loss in staining with mAb 735. To support the finding of potential O-linkage of PSA to NRP-2, purified NRP-2 was also treated with PNGase F, separated on SDS-PAGE, and evaluated by immunoblotting using mAb 735. As expected, there was no loss in staining with mAb 735 or change in size of NRP-2 (Fig. 6B, lanes 1 and 2). When analyzed as a positive control for digestion by PNGase F, purified CD56 lost reactivity with mAb 735 on immunoblot after exposure to PNGase F (Fig. 6B, lanes 3 and 4). In addition, although there was a significant decrease in the
molecular mass of CD56 after PNGase F treatment, there was no shift in the molecular mass of NRP-2 (data not shown). These data suggest that most PSA chains are bound to NRP-2 by an O-linked glycosidic linkage.

**Polysialic Acid and Neuropilin-2 on the Surface of Dendritic Cells Modulate the Ability of Dendritic Cells to Activate T Lymphocytes**—Neuropilin-2 and the PSA moiety of NCAM on the surface of differentiating neurons both influence axonal pathfinding and plasticity. To determine whether NRP-2 and PSA on the surface of maturing dendritic cells similarly affect dendritic cell interactions with other cells, the ability of dendritic cells to activate T lymphocytes in a mixed lymphocyte reaction was determined after removing PSA from the cell surface using endoNF. Immediately after endoNF treatment, there was no detectable PSA on the surface of dendritic cells as measured by staining with mAb 735 and analysis by flow cytometry; when measured up to 48 h after cells were placed back in culture, the amount of cell surface PSA returned to no more than 6% that of the pretreatment levels. In addition, after treatment with endoNF, there was no visible change in the morphology of individual dendritic cells or in cell–cell interactions as observed by light microscopy. Removal of PSA from the surface of dendritic cells led to a 1.7-fold increase in proliferation of T lymphocytes compared with mock-treated control cells (Fig. 7A). Along with the increase in proliferation, the production of IFN-γ in these cultures increased 7.2-fold (Fig. 7B). Treatment of dendritic cells with heat-inactivated endoNF led to a minimal 1.1- and 1.6-fold increase in incorporation of [3H]thymidine and synthesis of IFN-γ, respectively, compared with mock-treated cells, demonstrating the specificity of endoNF action.

To determine whether binding of anti-NRP-2 IgG, which blocks binding of VEGF to NRP-2, also affects the ability of dendritic cells to activate T lymphocytes, dendritic cells were preincubated with preimmune or anti-NRP-2 IgG and were added along with the respective IgG to lymphocytes in a mixed lymphocyte reaction as described above. As was seen after removal of PSA from the surface of dendritic cells, lymphocyte proliferation increased 1.5-fold in reactions containing dendritic cells with bound anti-NRP-2 IgG in comparison to dendritic cells that were pretreated with preimmune IgG (Fig. 7A). The production of IFN-γ in cultures containing anti-NRP-2 IgG also increased 3.1-fold compared with that of control cells (Fig. 7B). To determine whether the enhancement in T lymphocyte proliferation seen when dendritic cells were treated with endoNF occurred independently from the increase detected with bound anti-NRP-2 IgG, a mixed lymphocyte reaction was performed using dendritic cells that were treated with endoNF and incubated with anti-NRP-2 IgG.

The increases in [3H]thymidine incorporation and production of IFN-γ under this condition were greater than the increase with either condition alone (Fig. 7, A and B). Thus, it appears that both PSA and NRP-2 on the surface of dendritic cells have an inhibitory effect on T lymphocyte activation and proliferation, possibly by interfering with ligand or coreceptor interactions.

**DISCUSSION**

We describe in this report that NRP-2, a receptor for the semaphorin and vascular endothelial growth factor families in neurons and endothelial cells, respectively, is present on the surface of human dendritic cells throughout their maturation. Neuropilin-2 is not detected in freshly isolated monocytes at either the RNA or protein
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level but is induced in monocyte-derived immature dendritic cells that are differentiated in vitro in the presence of IL-4, GM-CSF, and FCS. Neuropilin-2 is also expressed in cells that are grown in medium containing human serum or in serum-free medium (data not shown). Further maturation of cells after exposure to LPS leads to an increase in the amount of RNA encoding NRP-2 and increased expression of NRP-2 on the cell surface. The other member of the neuropilin family NRP-1 is also expressed on the surface of immature dendritic cells but is not detected in monocytes (15). We confirmed these results but found a difference in the expression of NRP-1 and NRP-2 during maturation from immature to mature dendritic cells. We did not detect transcripts encoding NRP-1 in mature dendritic cells and found a reduced amount of NRP-1 on the surface of mature compared with immature dendritic cells (data not shown), in contrast to the increased expression of NRP-2 that we detected in mature dendritic cells.

In this report we show not only that NRP-2 is expressed in dendritic cells but that it is also modified by polysialylation. This is convincingly shown by analysis of proteins immunoprecipitated with anti-NRP-2 IgG from lysates of mature dendritic cells. When analyzed by SDS-PAGE and immunoblot using anti-PSA mAb 735 or anti-NRP-2 IgG, a diffuse band ranging from 130 to 180 kDa was detected by both antibodies. When dendritic cells were treated with endoNF before analysis, the band was sharper, and the size of the protein stained with anti-NRP-2 IgG was reduced to 110 kDa, close to the molecular mass of unmodified NRP-2. There was marked variability in the intensity of staining of immature dendritic cells using mAb 735 as determined by flow cytometry. There was an equally heterogeneous pattern of staining of these cells with anti-NRP-2 IgG. Because RNAs encoding two sialyltransferases, ST8Sia II and ST8Sia IV, that mediate synthesis of sialic acid polymers were present in differentiating monocytes, it is likely that the limiting factor for cell surface polysialylation in dendritic cells is the rate of synthesis of NRP-2. Further maturation of dendritic cells after exposure to LPS resulted in up-regulation of transcripts encoding ST8Sia IV and of expression of NRP-2. This resulted in a homogeneous population of mature dendritic cells that stained brightly with mAb 735 on analysis by flow cytometry, suggesting that NRP-2 was maximally expressed and polysialylated in all mature dendritic cells.

We detected a 250-kDa protein in monocytes that was polysialylated and have determined by RNA analysis and immunoblot that it was neither NRP-2 nor any of the 3 isoforms of NCAM (data not shown for NCAM), and its identity remains to be determined. Removal of PSA from the surface of monocytes with endoNF was incomplete, suggesting that PSA on this polysialylated protein was protected from complete digestion by the endoglycosidase. Our data clearly demonstrate that NRP-2 is polysialylated in dendritic cells. Our results presented in Table 1 and Fig. 3 also raise the possibility that proteins in addition to NRP-2 in dendritic cells may be polysialylated. There are other proteins identified by mass spectrometry and listed in Table 1 that may be carriers of PSA on dendritic cells, and we are currently evaluating some of the nonstructural proteins for PSA. Given the relatively homogenous band centered at 150 kDa, but ranging from 130 to 200 kDa, that is detected on an immuno-

blot of protein in a cell lysate probed with mAb 735, though, we propose that NRP-2 is the predominant carrier of PSA in dendritic cells. The broadness of a band of polysialylated protein on immunoblot does not necessarily indicate the presence of multiple proteins, as PSA chains vary greatly in size and generate heterogeneity in protein molecular mass (42, 43). It is of note that although NRP-1 and NRP-2 share extensive amino acid sequence homology, we found that NRP-1 is not polysialylated in dendritic cells (data not shown).

There are several possible explanations for why the pattern on immunoblot of mAb 735-immunoprecipitated protein (Fig. 3, lane 1) is so much broader than the band that is detected in the original cell lysate (Fig. 2, lane 3) or in the material immunoprecipitated with anti-NRP-2 IgG (Fig. 3, lanes 2 and 3). It should be noted that maintaining the integrity of a polysialylated protein in a cell lysate is complicated by the inherent instability of PSA (44). In addition, we are using mAb 735 that has the ability to recognize multiple, repeating epitopes on a single extended chain of PSA and monoclonal anti-NRP-2 IgG that recognizes a single epitope on polysialylated NRP-2. Thus, mAb 735 has greater potential than anti-NRP-2 IgG to recognize breakdown products of polysialylated NRP-2 that may be generated during the immunoprecipitation and immunoblotting. It is also possible that not all isoforms of NRP-2 are recognized by the anti-NRP-2 IgG. Because protein in the original lysate is relatively free of manipulation before preparation for SDS-PAGE, we consider the protein pattern from the lysate on the anti-PSA-stained immunoblot (Fig. 2, lane 3) as the most accurate representation that supports the presence of a predominant polysialylated protein, namely NRP-2, in dendritic cells.

Removal of PSA from the cell surface or binding NRP-2 with specific IgG enhances the ability of dendritic cells to stimulate lymphocytes in a mixed lymphocyte reaction. Because NRP-2 is likely the predominant carrier of PSA in dendritic cells, this suggests that at least two features of polysialylated NRP-2, the PSA posttranslational modification and the protein domain(s) that is recognized by anti-NRP-2 IgG, modulate the effect of dendritic cells on lymphocyte activation. Polysialylation of NRP-2 may interfere with the activity of any of the five distinct extracellular domains of the protein (i.e. complement binding distal regions, coagulation factor-like mid domains, and the membrane-proximal MAM region) (13, 26, 27). Each of these five regions can potentially promote protein-protein interactions (e.g. between NRP-2 and semaphorins, VEGFs, and plexin and VEGF coreceptors) that could be impeded by long chains of negatively charged polysialic acid. Removal of PSA from NRP-2 on the surface of dendritic cells may lead to enhanced lymphocyte activation by promoting the interaction of dendritic cells with lymphocytes and/or by directly stimulating intracellular signaling pathways in dendritic cells. Activation of dendritic cells would likely occur through an interaction with other cell surface receptors such as plexins and VEGF receptors, as NRP-2 has a short cytoplasmic tail devoid of signaling motifs. Previous results from our laboratory (45) and from others (46) showed that removing sialic acid from the surface of monocytes enhanced the LPS-responsiveness of cells, activated the MEK (mitogen-activated/extracellular signal-regulated kinase
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kinase)/ERK (extracellular signal-regulated kinase) and p38 MAPK intracellular signaling pathways, and resulted in increased cytokine production (45, 46). Future experiments will determine whether removal of PSA from the surface of dendritic cells similarly leads to enhanced responsiveness to extracellular stimuli, such as LPS.

We also show that binding of anti-NRP-2 IgG to the surface of dendritic cells potentiates dendritic cell activation of lymphocytes. It is possible that bound IgG prevents an interaction with factors that interfere with the ability of dendritic cells to activate T lymphocytes. Despite their homology and capacity to bind to similar ligands, NRP-2 and NRP-1 appear to have opposing roles in the dendritic cell-T lymphocyte interaction. T lymphocyte proliferation was reduced when dendritic cells were incubated with anti-NRP-1 IgG (15), in contrast to what we found with NRP-2.

The region(s) of NRP-2 that is polysialylated remains to be identified. There are numerous possible sites for O-linked chains of PSA throughout the full length of NRP-2 but only four potential N-linked glycosylation sites. In contrast to PSA chains that are N-linked to NCAM, PSA chains on NRP-2 are resistant to digestion with PNGase F. Using 0.1 n NaOH in a technique that was previously reported to show the O-linkage of PSA to CD36 and to a protein(s) from leukemia cells (8, 39), we demonstrate that PSA is markedly released from NRP-2 under alkaline conditions. One limitation of this technique, though, was that PSA was also released from CD56, as also shown by others (8), suggesting that the PSA is nonspecifically lost during alkaline conditions. However, the more dramatic loss in PSA from NRP-2, in contrast with that from CD56, along with the resistance of PSA on NRP-2 to digestion with PNGase F suggests that NRP-2 is polysialylated on O-linked glycans. Previous studies have shown that polysialyltransferases bind to a specific domain in the first fibronectin type III repeat of NCAM and polysialylate N-linked glycans in the adjacent immunoglobulin domain (Ig5) (47). With alteration of this polysialyltransferase fibronectin type III (FNI) binding domain, the enzyme loses its specificity and adds PSA to O-linked glycans in FNI (48). With the presumed absence of these NCAM recognition sites in dendritic cells, it remains to be determined whether a specific recognition site exits on NRP-2 for the relevant polysialyltransferase(s) and whether a preferred site exists in NRP-2 for the addition of PSA. Future structural analyses will help identify the site(s) on NRP-2 that are polysialylated.

We hypothesize that an understanding of the full role of polysialylated NRP-2 and unmodified NRP-2 in the activity of dendritic cells may be inferred from what is known about neuropilin function in other cells and about polysialylation of NCAM in differentiating neurons. Neuropilins 1 and 2 interact with two large families of ligands, the chemorepulsive semaphorins and the proliferation-inducing VEGFs, to mediate axonal guidance of neurons and angiogenesis of endothelial cells (13, 14, 16–19). Polysialic acid modification of NCAM expressed by differentiating neurons also affects cell plasticity by protecting the generating axon from inappropriate interactions with other cells and the extracellular matrix until the final correct synapse is formed (10, 11). Similarly, it is possible that polysialylation of NRP-2 provides a protective shield around differentiating dendritic cells during their migration to lymph nodes and secondary lymph organs, when there are numerous opportunities to interact inappropriately with vascular and lymphatic endothelial cells. Once dendritic cells are correctly localized in lymphatic tissue, polysialylated NRP-2 may play a role in modulating the extent of dendritic cell interactions with T lymphocytes. The expression of NRP-2 in dendritic cells as well as in neurons further highlights similarities between the immune and nervous systems and suggests a critical role for polysialic acid in the immune system.

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