The Expression Profile of De-N-acetyl Polysialic Acid (NeuPSA) in Normal and Diseased Human Tissue*

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Background: Polysialic acid (PSA) containing de-N-acetylated (NeuPSA) was identified in human tumors, but expression in normal tissues was unknown. Results: NeuPSA was found to be present in specific cell types in many normal tissues. Conclusion: PSA/NeuPSA may have a broader role in cell biology than previously recognized. Significance: The results raise questions about the possible role of NeuPSA in human health and disease.

Although sialic acids have a key role in many aspects of human biology, the expression of polysialic acid (PSA) in human tissues is thought to be relatively rare. We identified a derivative of PSA called neuraminic acid-containing PSA or NeuPSA that was highly expressed in primary human melanoma tumors and in several cancer cell lines. Moreover, anti-NeuPSA antibodies could induce apoptosis of cancer cells. However, little was known about NeuPSA expression in normal or diseased tissues. In this study we investigated the complete expression profile of NeuPSA in human tissues and a few primary tumors using the anti-NeuPSA monoclonal antibody, SEAM 3. Almost every human tissue tested spanning a representative sample of all organ types was positive for SEAM 3 binding. Specificity of SEAM 3 binding was established by inhibition with NeuPSA but not closely related meningococcal C polysaccharide and loss of SEAM 3 binding when specimens were treated with periodate at high pH, which specifically destroys NeuPSA. Only subsets of cells in each specimen stained positive, and the relative staining between tissues was variable. The distribution and amount of NeuPSA antigen in tissues was correlated with known levels of polysialyltransferase PST or STX expression. The majority of anti-NeuPSA binding occurred intracellularly in the cytoplasm of cells. Tumors generally exhibited considerably increased staining compared with corresponding normal tissues. Identifying the diverse tissue distribution and intracellular location of NeuPSA provides a foundation for investigating the functional role of NeuPSA in human health and disease.

The expression of poly α2,8 N-acetylnuraminic acid homopolymers (i.e. polysialic acid (PSA))4 in human tissues is developmentally regulated (1). Polysialylated neural cell adhesion molecule (PSA-NCAM), by far the most abundant polysialylated protein, is highly expressed in cells derived from all germ layers during fetal development but is limited in adult tissues (2). For example, PSA-NCAM is expressed in regenerating areas of the adult brain such as the olfactory bulb and hippocampus (2, 3) and also in leukocytes (4), but most other tissues are thought to contain little PSA. In addition to NCAM, synaptic cell adhesion molecule 1 (5), the α subunit of the voltage-sensitive sodium channel (6), the integrin α5 subunit (7), the scavenger receptor CD36 (8), neuropilin-2 (9), and the polysialyltransferases that produce PSA, ST8SialI, and ST8SialIV, also known as STX and PST, respectively (10), are also modified with PSA. Apart from modulating NCAM adhesive interactions (3), the specific functional role(s) of polysialylation, the relative distribution of polysialylated versus unmodified protein, and the reasons for differences in polysialylation of proteins (other than NCAM) are unknown. In addition to the small number of mammalian proteins, PSA is expressed on fish egg glycoproteins (11) and is the capsular polysaccharide of neuroinvasive Neisseria meningitidis group B (NmB) (12) and Escherichia coli K1 bacteria (13).

During the development of a vaccine to prevent disease caused by NmB, we discovered that some antibodies elicited by an N-propionyl derivative of NmB capsular polysaccharide (N-Pr MBPS) (14) were reactive with PSA derivatives that contained de-N-acetyl sialic acid residues (i.e. neuraminic acid) (15). Neuraminic acid-containing polysialic acid (NeuPSA), which was an unintended side product of the preparation of N-Pr MBPS, was shown to be immunogenic in mice and to elicit antibodies protective against NmB and NmC strains (16). The specificity and functional activity of two murine monoclonal antibodies (mAbs), SEAM 2 and SEAM 3, that were produced using the N-Pr MBPS-based vaccine, have been studied extensively (15–17, 30). SEAM 2 recognizes a relatively long chain
PSA epitope containing more than 10 residues where ~50% of the residues are Neu (16). SEAM 3 binds to PSA oligosaccharides as short as three residues where the non-reducing end residue is likely Neu. Additionally, it binds to long chain PSA that is less than 50% de-N-acetylated (15–17, 30). Both mAbs bind to Nm group B bacteria and mediate bactericidal activity (14, 19). Using SEAM 2 and anti-PSA mAbs, we showed that PSA and NeuPSA are expressed as intracellular antigens in Leishmania major parasites but moved to the cell surface during rosette formation (20). Recently, we investigated NeuPSA expression in human melanoma tumors and in melanoma, leukemia, and neuroblastoma cell lines using SEAM 3. SEAM 3-reactive NeuPSA antigens were found to be located mainly intracellularly with only a fraction of cells expressing NeuPSA on the cell surface to varying degrees among the cell lines tested (30). Knocking down expression of both PST and STX with siRNA nearly eliminates SEAM 3 binding (30). SEAM 3 mediated antibody-dependent cytotoxicity against the tumor cells. However, SEAM 3 was also reactive with antigens in normal human skin cells. Because expression of PSA and NeuPSA was unknown in normal human skin, the results raised questions of whether PSA/NeuPSA expression could be prevalent in normal human tissues. NeuPSA is not reactive with anti-PSA antibodies or sialic acid binding lectins and is resistant to degradation by low pH and exonereaminidases. Therefore, NeuPSA may not be detected by methods commonly used to detect PSA.

In the present study we utilized the novel specificity of SEAM 3 against NeuPSA to carry out immunohistochemical studies on normal and malignant adult human tissues. Our primary goal was to produce a complete expression profile for NeuPSA in the human body that would help us better identify any biologically meaningful expression patterns that may exist at a tissue, cellular, and/or intracellular level. This information will for the first time help us understand the potential functional roles NeuPSA may play in the pathophysiology of human cells. Secondary goals of this study include a look at how NeuPSA expression changes at various stages of human development and how expression changes in the face of disease, which in our study is represented by malignancy. The changing pattern of NeuPSA expression noted throughout human development and the changes noted at times of disease ought to help define the functional role of NeuPSA in the human body.

**EXPERIMENTAL PROCEDURES**

*mAbs*—Murine mAbs, SEAM 2 and SEAM 3, were produced by immunizing a CD1 mouse with an N-Pr MBPS tetanus toxoid conjugate vaccine (14). The polysaccharide vaccine antigen contained ~16% Neu (21). The mAbs were obtained from cell culture by precipitation with 50% (w/v) ammonium sulfate. The precipitated antibody was washed with ice-cold 30% ammonium sulfate solution, dialyzed against PBS buffer, and purified by affinity chromatography as described previously (19). Irrelevant isotype control mAbs were obtained from Southern Biotech (Birmingham, AL).

**Tissue Acquisition and Processing**—Unless stated otherwise, all human tissues were purchased from the National Disease Research Interchange, and procedures for their use were performed under a protocol approved by the Children’s Hospital and Research Institute Oakland (CHRCO) Institutional Review Board. Tissues were provided as frozen tissue, optimal cutting temperature compound-embedded blocks, and formalin-fixed paraffin-embedded (FFPE) blocks. Placental tissue was provided by the laboratory of Dr. Vladimir Serkov and fetal tissue by Dr. Alexander Lucas at the Children’s Hospital Oakland Research Institute (CHORI), both of which were obtained under CHRCO IRB-approved protocols.Mouse normal skin and basal cell carcinoma specimens were provided by the laboratory of Dr. Ervin Epstein, also at CHORI.

**Immunohistochemistry**—Cryosections of frozen tissue (6 μm thick) and microsections of FFPE tissue (4 μm thick) were prepared at the Alta Bates Medical Center’s Department of Pathology Services (Berkeley, CA). Immunohistochemistry was carried out manually with a Nexium 3600 stainer (BioCare Medical, Pleasanton, CA) at CHORI or using a Ventana BenchMark XT automated stainer located at the CHRCO Department of Pathology. Microtome-sliced paraffin sections were mounted onto Superfrost Plus slides and stored at room temperature until use. Before manual staining, slides were incubated in a gravity convection oven at 60 °C for 60 min. Slides next underwent deparaffinization with 3 treatments of xylene (5 min each), then rehydrated by serially dipping the slide in 100, 95, and 70% v/v ethanol for 5 min each with a final PBS wash. Antigen retrieval was performed using a Decloaking Chamber (BioCare Medical) device in combination with Reveal Decloaker antigen retrieval solution per manufacture protocol (BioCare Medical). After washing the slides with deionized water, they were treated with 3% peroxide for 10 min to block endogenous peroxidases followed by blocking with 2.5% normal horse serum (Vector Laboratories) for 30 min at room temperature to prevent nonspecific binding. Sections were incubated with 100 μl of primary antibody (~0.5 μg/ml SEAM 3, purified SEAM 2, or irrelevant mouse IgG2b control) in a humidified chamber for 1 h at room temperature or 4 °C overnight. Bound primary antibody was detected using ImmPRESS universal Ig-peroxidase conjugate reagent from Vector Laboratories (Burlingame, CA) incubated for 30 min at room temperature. The slides were developed using the AEC substrate kit (Vector Laboratories) per the manufacture protocol. Slides were counterstained with hematoxylin QS (Vector Laboratories) and mounted in aqueous mounting medium Vectamount AQ (Vector Laboratories).

Automated staining was performed using a BenchMark XT Ventana stainer at the CHRCO Department of Pathology. A program based on the manual staining protocol described above was used to control the automated staining procedures. Before staining, the automated stainer performs deparaffinization with the manufacturer’s dewaxing solution and cell conditioner. Slides were treated with inhibitors to block endogenous peroxidases, and then the program was stopped for manual application of primary antibody (0.5 μg/ml of SEAM 3, purified SEAM 2, or murine IgG control). A universal secondary rabbit anti-mouse Ig reagent was then applied, and chromogenic detection was carried out with 3,3-diaminobenzidine substrate.
Slides were counterstained with hematoxylin and mounted with a coverslip.

For periodate treatment, FFPE tissues were deparaffinized in xylene and then rehydrated in a series of 95, 90, 75, and 50% alcohol incubations. Antigen retrieval was conducted using a Decloaking Chamber (BioCare Medical) and Reveal Decloaker (BioCare). After antigen retrieval, tissues were treated with 0.1 M carbonate buffer, pH 10.3, or 100 mM periodate in 0.1 M carbonate buffer, pH 10.3, for 30 min. Tissues were briefly rinsed with water then treated with 10% ethylene glycol for 10 min. Tissues were rinsed with water, then stained using a Nemesis 3600 (BioCare) automated stainer and IntellivPATH FLX Universal HRP Detection kit (BioCare). Samples were blocked for 5 min with peroxidase block and incubated for 1 h with 0.5 μg/ml SEAM 3, 20 min with secondary antibody, 20 min with tertiary antibody, 5 min with 3,3-diaminobenzidine, and counterstained with hematoxylin.

Cryosections were thaw-mounted onto Superfrost Plus slides and stored at −80 °C until use. Before staining, slides were kept at room temperature for 60 min. No fixation step was used on cryosections. Slides were treated with 3% peroxide to block endogenous peroxidases and then treated with avidin/biotin blocking reagent. All sections were then incubated with primary antibody (0.5 μg/ml of purified SEAM 3, purified SEAM 2, or irrelevant murine IgG control). Primary mAbs were incubated for 1 h at ambient temperature or overnight at 4 °C. Protocols for incubations with a biotinylated secondary antibody and appropriate streptavidin conjugates and substrate used were those recommended by the manufacturer (Vector Laboratories).

**Inhibition Studies Using N-Pr MBPS—**N-Pr MBPS was diluted in Da Vinci Green (Biocare Medical) to a final concentration of 50 μg/ml. A solution containing primary antibody plus N-Pr MBPS inhibitor was applied to specimens during the manual application step of automated immunohistochemistry approximately 1 h after preparation.

Separate inhibition studies were carried out using Nmb and Nmc capsular polysaccharide (MBPS and NmC capsular polysaccharide (MCPS), respectively). MBPS or MCPS was added to a solution containing primary antibody and was applied to specimens during the manual titration step of automated immunohistochemistry as described above for N-Pr MBPS.

**Histological Analysis—**Stained slides were reviewed by Dr. Hua Guo of the Department of Pathology at CHRCO. Slides were graded based on staining intensity using a scale from zero (no staining) to four plus (+ + + + +, most intense staining). The characteristics of staining specificity were further documented noting the tissue and cell types stained (for example, epithelial and endothelial versus interstitial cell involvement) and noting the staining distribution intracellularly (cytoplasmic versus nuclear).

**Western Blot Analysis of CHO Cells—**CHO cells were graciously donated by the laboratory of Donald Reason (CHORI). Cells were grown in liquid media (RPMI complete) and collected for quantification with the Guava EasyCyte capillary flow cytometer using the Guava ViaCount reagent (Millipore). The cells were washed in PBS twice, and 300 μl of NuPAGE buffer containing reducing agent (Invitrogen) was added. DNA was sheared by repeated pipetting using a 20-gauge needle. The samples were heated to 70 °C for 15 min followed by centrifugation for 5 min at 15,000 × g. Wells of a prepared 10% NuPAGE (Novex) gel (Invitrogen) were loaded with 10 μl of sample, and the proteins were resolved by electrophoresis. The proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. After transfer, the membrane was cut into strips according to lanes loaded and the strips were soaked in blocking buffer (LI-COR Biosciences, Lincoln, NE). Primary antibody SEAM 3 was diluted in blocking buffer and incubated with the PVDF strips for 1 h. The strips were then washed 4 times for 5 min and incubated with IR dye-labeled secondary antibody in blocking buffer (both from LI-COR Biosciences, Lincoln, NE). Strips were washed 4 times, and the staining was quantified on a LI-COR Odyssey scanner.

**Light Microscopy—**All slides were analyzed using a Zeiss Axioplan 2 Upright Light/Fluorescence Microscope with digital video capture using Q Capture software (Technical Instruments, San Jose, CA).

**Qualitative Analysis of Angata et al. (22) PST/STX mRNA Expression Data—**An electronic image of the Northern blot published in Angata et al. (22) was downloaded from the Journal of Biological Chemistry web site, and the relative intensities of the longest mRNA transcript from each tissue studied was measured using Image J. The integrated pixel density of a band portion on each blot was subtracted from the pixel density of PST/STX transcript band and divided by the background pixel density to produce a measure of the relative increase over background for each transcript. Qualitative mRNA expression values represented by +, ++, ++++, ++++++, and ++++++ for a 2, 3, 4, 5, and 6-fold increase over background are indicated in Table 2.

**RESULTS**

**Expression of SEAM 3 Reactive Antigens in Human Placental Tissue—**To develop an efficient and reproducible immunohistochemistry (IHC) protocol for SEAM 3 that minimized nonspecific binding, we chose to work first with human placental tissue, which is known to express polysialylated antigens. The placenta is a simple organ made up of vasculature and a villous structure composed of only a few cell types. FFPE slides of normal human placental tissue were prepared as stated under “Experimental Procedures.” Staining procedures were initially developed manually but were finally automated for consistency using a Ventana stain. Controls included secondary antibody-only and an irrelevant murine isotype-matched IgG mAb. Both controls (IgG2b shown in Fig. 1, left panel; the IgG3 control for anti-CD34 is not shown) were negative for any binding activity (graded zero out of a possible four plus for stain intensity). An anti-CD34 mAb was used to identify hematopoietic stem cells in the placenta. As shown in Fig. 1, anti-CD34 was reactive with placental cells (right panel indicated with arrows). Anti-CD34 is known to mark areas of the vasculature (endothelial cells of arteries and veins) of the placenta that are richly populated with stem cells (23, 24) but does not mark trophoblast cells at the external surfaces of the tissue. In contrast to antigens marked by the anti-CD34 mAb, light microscopy images showed that SEAM 3 binding was specific for the outer
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**FIGURE 1. Immunohistochemical characterization of NeuPSA expression in human placenta.** Cells marked by the anti-NeuPSA mAb SEAM 3 in placenta (center panel) were limited to trophoblasts present on the outer surfaces of chorionic villi (indicated by arrows). Staining was localized to areas around the nuclei and the cytoplasm. In contrast, the external surfaces of hematopoietic stem cells marked by anti-CD34 (indicated by arrows in the right side panel) were not reactive with SEAM 3. Bars = 20 μm.

Trophoblast cells of the placental villi (Fig. 1, middle panel indicated by arrows). Staining occurred primarily in the intracellular space including the cytoplasm and around the nuclei of trophoblast cells. The surrounding intervillous space and all vasculature components of the placental tissue remained unstained. Therefore, hematopoietic stem cells did not express NeuPSA antigens, which were highly expressed in trophoblasts. The lack of reactivity with hematopoietic stem cells is of particular interest because, as described below, SEAM 3 was strongly reactive with thymic lymphocytes.

**NeuPSA Expression Profile in Normal Human Tissue Marked by SEAM 3**—Using SEAM 3 as a marker for NeuPSA expression, representative specimens from all major normal human organs were collected and screened for binding activity using the automated staining program described above (Table 1). The choice of tissues to include in this study was based on the Food and Drug Administration requirements created for the development of standard normal tissue arrays used for testing mAbs for human use in the United States (25). As shown in the examples in Fig. 2 and summarized in Table 1, nearly all normal human tissues tested show some degree of reactivity with SEAM 3. However, only specific cell types within a particular organ were positive for staining, and there was a wide range of reactivity with SEAM 3. All samples were graded for staining intensity both by the authors and separately by Dr. Hua Guo (CHRCO) who was kept blind from information regarding the antibody used during staining and any changes in staining protocol. Each organ system demonstrated its own unique staining pattern. The following are a few representative tissue types in the human body.

The colon is a classic representative organ of the gastrointestinal tract that demonstrates unique tissue layers, including the mucosa, submucosa, and muscularis. Staining with SEAM 3 revealed a pattern that was specific for the epithelial mucosa and for the crypts of glands present in the mucosal layer (Fig. 2). Also of significance, the lamina propria remained mostly negative except for infiltrative lymphocytes that stained positive with SEAM 3. The area around nuclei of these lymphocytes, which make up a majority of the cell structure, appear to be where binding and, therefore, staining occurred. Some weak staining was noted in the submucosal tissue of the colon but was reported as nonspecific binding during pathologic analysis. The underlying muscularis remained negative.

The thyroid is a classic representative of an endocrine organ with secretory function in the human body. Treatment of the tissue sample with SEAM 3 resulted in specific staining of only the thyroid epithelial cells, whose function is the production and secretion of thyroid hormone (Fig. 2). Histologically, the thyroid epithelial cells are found in a circumferential pattern around larger colloid filled follicles. Similar to the staining described for cells of the colon, the specific intracellular structures that appeared positive include the area around the nuclei and cytoplasm of the thyroid epithelial cells. The surrounding connective tissue that makes up the interfollicular space remained unstained.

To evaluate NeuPSA expression in the central nervous system, white and gray matter of the cerebral cortex were used in this study as they demonstrate the two major tissue types that are found throughout the central nervous system. The cells that make up these tissues include the small glial cells and the larger cytoplasmic–rich neurons. Our automated IHC assay using SEAM 3 demonstrated staining of both glial cells and to neurons (Fig. 2). Only the area around the nuclei of the glial cells, which are smaller, rounded cells with a centrally located nucleus, were stained. The neurons, on the other hand, showed significant positive staining in the cytoplasm as well as around the nuclei. With higher magnification, the pattern of intracellular cytoplasmic staining appeared very granular in nature (Fig. 2). Most of the axonal meshwork that makes up the majority of the cerebral cortex specimen was also marked with faint staining. However, under careful pathologic evaluation, the weak staining was considered nonspecific.

Recently, it was shown by Drake et al. (4) that peripheral blood leukocytes express PSA antigens and that polysialylation has a role in leukocyte trafficking. In addition, relatively high levels of polysialyltransferase PST and STX mRNAs are expressed in the thymus (22). As shown in Fig. 2, SEAM 3 reacted strongly with thymocytes even though, as described above, SEAM 3 was not reactive with CD34-positive hematopoietic stems cells in the placenta.

With almost every human organ showing some degree of positive staining (Table 1), it is important to note those tissues that showed little or no staining. For example, the hepatocytes of the liver showed almost no staining with SEAM 3 and appeared similar in staining to the negative control IgG2b mAb...
Five tissues (colon, skin, thymus, brain, and liver) were tested with a second anti-NeuPSA mAb that recognizes a longer more highly de-N-acetylated epitope, SEAM 2 (14, 16, 17). The results for the tissues tested were similar to those obtained with SEAM 3 (data not shown). Because SEAM 2 recognizes a different NeuPSA epitope, the results provide further support for the expression of NeuPSA antigens identified with SEAM 3.

**Effect of Periodate Treatment of Tissue Specimens on SEAM 3 Binding**—Tests for the specificity of anti-PSA binding include selective hydrolysis of PSA with mild acid or exo- or endosialidase treatment. However, NeuPSA is resistant to acid hydrolysis and is not degraded by commonly available sialidases (16, 26). Alternatively, mass spectrometry may be used to determine the structure of PSA released from glycans by peptide N-glycosidase. Unfortunately, mass analysis of NeuPSA is complicated by the zwitterionic character of NeuPSA and the propensity of the polysaccharide to form high mass aggregates that can only be dissociated with high concentrations of salt (15). Therefore, we sought to develop a chemical method that would selectively degrade NeuPSA, resulting in loss of anti-NeuPSA binding.

The non-reducing end residue of poly α2,8 sialic acid can be cleaved by the reaction of periodate with vicinal hydroxyl groups on C7, C8, and C9. The remaining residues in the polymers are not affected by periodate treatment. However, de-N-acetylation produces residues with vicinal amino and hydroxyl groups on C5 and C4, respectively, that are susceptible to perio-

## TABLE 1

| ORGAN                     | Tissue type          | Staining                  | Positive cell type          | Staining pattern                      |
|---------------------------|----------------------|---------------------------|-----------------------------|---------------------------------------|
| Brain                     | Cerebrum white matter| ++                        | Glial cells                 | Perinuclear staining                  |
|                           | Cerebrum grey matter | ++++                      | Glial and neuronal cells    | Perinuclear staining of gial cells, granular cytoplasm and perinuclear staining of neurons |
|                           |                      |                           |                             |                                        |
|                           | Pons                 | ++                        | Neuronal cells              | Perinuclear area and granular cytoplasm stained |
| Thyroid                   | Thyroid              | ++++                      | Thyroid epithelial cells    | Granular cytoplasm-stained            |
|                           |                      |                           |                             |                                        |
| Heart                     | Cardiac muscle       | ++++                      | Cardiac muscle cells        | Perinuclear staining                  |
| Gastrointestinal tract    | Colon                | ++++                      | Epithelial cells of villi   | Perinuclear staining                  |
|                           | Small intestine      | ++++                      | Epithelial cells of villi   | Perinuclear staining                  |
| Blood                     | Lymphocytes          | ++++                      | B and T cell lymphocytes    | Granular cytoplasm-stained            |
|                           | Liver                | +                        | Hepatocytes                 | Granular cytoplasm-stained            |
| Pancreas                  | Acinar cells         | +                         | Acinar cells                | Granular cytoplasm-stained            |
|                           | Islet cells          | ++                        | Islet cells                 | Granular cytoplasm-stained            |
| Adrenal gland             | Adrenal cortex       | ++++                      | Large cytoplasm rich cells  | Granular cytoplasm-stained, weak      |
|                           |                      |                           | of the cortex               | perinuclear staining                  |
|                           |                      |                           |                             |                                        |
| Thymus                    | Thymus               | ++++                      | B and T cell lymphocytes    | Granular cytoplasm and perinuclear staining |
|                           |                      |                           |                             |                                        |
| Spleen                    | Spleen               | ++++                      | B and T cell lymphocytes    | Perinuclear staining                  |
| Kidney                    | Cortex               | +                        | Epithelial cells of tubules | Granular cytoplasm stained            |
|                           | Glomeruli            | -                         |                             |                                        |
|                           |                      |                           |                             |                                        |
|                           | Ovary                | +                         | Cortical stroma             | Granular cytoplasm-stained            |
|                           | Fallopian tube       | +                         | Mucosal columnar epithelium| Granular cytoplasm-stained            |
|                           | Cervix               | +                         | Epithelial cells            | Granular cytoplasm-stained            |
| Bladder                   | Bladder              | +                         | Epithelial cells            | Granular cytoplasm-stained            |
| Prostate                  | Lobule               | ++                        | Lobule epithelial cells     | Granular cytoplasm-stained            |
|                           | Fibromuscular stroma | –                         |                             | Granular cytoplasm-stained            |
|                           |                      |                           |                             |                                        |
| Skin                      | Skin                 | ++++                      | Squamous cells              | Granular cytoplasm-stained            |
|                           | Retina               | +                         | Retinal cells               | Granular cytoplasm-stained            |
|                           | Ciliary body         | –                         |                             | Granular cytoplasm-stained            |
| Placenta                  | Chorionic villi      | ++++                      | Trophoblasts                | Granular cytoplasm-stained            |
|                           | Blood vessels        | –                         | Endothelial cells           | Granular cytoplasm-stained            |

*(Fig. 2). The kidney showed mild staining of the epithelial cells that line the tubules, but no staining was noted in the structures of the glomeruli (data not shown).*
date oxidation but only at pH values where a significant fraction of the amino groups are in the free amine form (28). We have measured the average pK_a of the amino group in fully de-N-acetylated PSA by titration as ~11. Therefore, NeuPSA antigens should be stable when treated with periodate at low pH but would be expected to be degraded when treated with periodate at high pH. SEAM 3 binding to several specimens tested were not affected when treated with periodate at pH 5.5, but SEAM 3 reactivity was lost after periodate treatment at pH 10.3 compared with the same specimens treated with buffer alone. A representative example of thymic tissue, which is strongly reactive with SEAM 3 (Table 1, Figs. 2 and 3), treated with buffer alone and with periodate at high pH, is shown in Fig. 3.
NeuPSA Expression in Fetal Tissues—PSA-NCAM is known to be highly expressed during fetal development, as are the polysialyltransferase PST and STX mRNAs (22). Because Neu is unstable as a free molecule (29), NeuPSA must be derived from CMP-NANA or PSA by de-N-acetylation of sialic residues already linked by glycosidic bonds. Therefore, it would be expected that NeuPSA expression would be increased in tissues in which PSA is highly expressed. To evaluate the expression of NeuPSA in fetal tissue, IHC was performed using SEAM 3 and an irrelevant mAb as a negative control on a section of tissue containing the developing fetal spinal cord. Fig. 2 shows that antigens expressed by cells within the fetal spinal cord were highly reactive with SEAM 3. As with all of the other tissues tested, SEAM 3 binding was almost completely inhibited with N-Pr MBPS (Fig. 2).

Comparison of NeuPSA Expression in Normal Human Ovarian Tissue and a Primary Ovarian Carcinoma—Previously, we had shown that NeuPSA antigens recognized by SEAM 3 were highly overexpressed in 13 primary human melanoma tumors compared with normal melanocytes (30). To compare differences in NeuPSA expression between normal and cancerous tissue, we evaluated binding of SEAM 3 to human primary melanoma, Wilm’s, and ovarian tumors. An example of SEAM 3 reactivity with normal human ovarian tissue and with an ovarian carcinoma is shown in Fig. 4. Presenting commonly as a painful abdominal mass, mucinous adenocarcinomas of the ovary tend to have a poor clinical prognosis. Like most other forms of ovarian cancer, by the time a tumor is symptomatic and diagnosed, the tumor is already quite large and likely has metastasized. Research to advance early detection of mucinous adenocarcinomas of the ovary will play a key role in improving clinical outcomes of this horrible disease. Staining of normal ovarian tissue with SEAM 3 results in weakly positive staining around the nucleus (×1000). However, staining the mucinous ovarian adenocarcinoma tissue showed intense staining (×1000) with the same dilution of SEAM 3 (Fig. 4). The histology of the tumor as compared with normal ovarian tissue demonstrated a gross change in architecture involving large cavernous, mucinous spaces lined by larger epithelial cells that appeared almost villous in structure. The larger epithelial cells tended to pick up the most intense staining, but the underlying ovarian stromal cells also demonstrated increased staining intensity.

Comparing SEAM 3 Binding to Murine Normal Skin and Basal Cell Carcinoma (BCC) Tumors—Humans are unique among mammals in expressing sialic acid antigens devoid of N-glycol Neu (Neu5Gc) as a result of an ancestral inactivation of the gene encoding Neu5Ac oxidase (18). Therefore, it was of interest to determine whether SEAM 3 was reactive with nor-

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**FIGURE 3.** Specificity of SEAM 3 binding demonstrated in representative thymic tissue by inhibition with NeuPSA derivatives and the effect of high pH periodate treatment. Shown is SEAM 3 binding to thymus tissue specimens in the absence of inhibitor compared with the mAb preincubated with 50 μg/ml of N-Pr MBPS (NeuPSA-containing PSA derivative) or MCPS (poly-2,9-N-acetylenuraminic acid) as indicated. At high pH the vicinal free amino group at C5 and hydroxyl group at C4 result in NeuPSA being susceptible to oxidative cleavage by periodate. Treatment of thymic tissue with 0.1 M carbonate buffer, pH 10.3, does not affect SEAM 3 binding (indicated by brown staining in the figure), but carbonate buffer containing 0.1 M meta-periodate results in complete loss of SEAM 3 reactivity. Bars = 45 μm.
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normal and cancerous murine tissues. Fig. 5 shows the results of IHC with normal mouse skin, an early stage "micro" BCC and a BCC tumor with SEAM 3. Compared with normal human skin (30), SEAM 3 showed almost no reactivity with murine skin (Fig. 5) but was reactive with both the micro-BCC and BCC tumor. Interestingly, hair clusters of follicular cells near the micro-BCC tumor were stained, whereas similar clusters of cells in the normal, tumor free skin were not stained. We have observed previously that there were marked differences in the expression of NeuPSA antigens when Nmb bacteria were grown in the presence of bovine blood-derived culture media compared with human serum, which we hypothesized were the result of Neu5Gc present in the former but not the latter (19). The result suggests that SEAM 3 may be less reactive with PSA containing Neu5Gc and that PSA expressed by the murine BCC is composed primarily of Neu5Ac.

Comparison of PST/STX mRNA Expression Levels with NeuPSA Staining Patterns by IHC (Table 2)—As the data summarized in Table 1 show, the presence of NeuPSA in the human body appears to be widespread. This result was unexpected given the apparently limited expression of PSA in adult human tissues (2). However, the α2,8-polysialyltransferases ST8SialII and ST8SialIV, which are also known as STX and PST, respectively, appear from the work of Angata et al. (22) to be widely expressed in most human tissues. The relative abundance of NeuPSA in human tissues determined by IHC is compared in Table 2 with STX and PST mRNA expression determined from Northern blots published by Angata et al. (22). Although the comparison is qualitative, there was a close correlation between the two observations/results. In particular, we observed the most staining of NeuPSA in brain, heart, spleen, thymus, small and large intestine, and peripheral blood leukocytes, which also had the highest levels of PST/STX mRNA expression. Correspondingly, tissues in which there was little or no reactivity with SEAM 3 such as the liver, the mRNA levels of PST/TX are nearly undetectable (22). The results are consistent with studies in cancer cell lines where NeuPSA expression coincided with PST and STX expression (30).

DISCUSSION

PSA expression in human adults appears to be limited to a few tissues and a relatively small number of proteins, of which PSA-NCAM is most abundant (2). Using the murine anti-NeuPSA mAb SEAM 3 to mark NeuPSA expression by IHC, we found NeuPSA in all normal tissues tested. Although such widespread expression was unexpected given the limited expression profile of PSA-NCAM, the presence of NeuPSA antigens was correlated with known mRNA expression of the polysialyltransferases PST and STX. A distinctive feature of the staining pattern was predominance of antigens marked by SEAM 3 in the cytoplasm and around nuclei of cells. We have described a similar cellular distribution of NeuPSA antigens in cancer cell lines (30). Although it is difficult to make broad statements about the pattern of NeuPSA expression, each organ system studied individually demonstrates specific features that together reveal a previously unrecognized pattern of PSA expression in the human body.

IHC studies with human placental tissue provided evidence that NeuPSA expression was cell-type-specific within particular tissues. Only the outer trophoblast cells stained positive in the placental samples treated with SEAM 3. Early on, during embryo development, the trophoblast cells play the vital role as invasive, eroding cells that penetrate the maternal endometrium, allowing for implantation. After the placenta has fully developed, the trophoblast cells are the basic epithelial cells that make up the placenta simple villi structure. Using anti-CD34 binding to identify hematopoietic stem cells located in the endothelial structures of the placenta, showed that the stem cells and developing blood vessels were not reactive with SEAM 3 and, thus, do not express NeuPSA. Because cells derived from hematopoietic stem cells, such as thymocytes, reacted strongly with SEAM 3, NeuPSA expression appears to be governed by a developmental program.

The apparent lack of specificity of anti-de-N-acetyl GD3 mAbs has made it unclear what cells and tissues express de-N-acetylated sialic acid antigens (27). For this reason, we tested whether SEAM 3 binding to NeuPSA antigens could be inhibited by the nominal polysaccharide N-Pr MBPS used to produce the mAb (14). In instances of even the strongest staining, the majority of SEAM 3 binding was inhibited by N-Pr MBPS but less so by PSA (MBPS) and not at all by a structurally similar polysialic acid, MCPS. Staining particularly in the cytoplasm and around the nucleus was almost completely inhibited by N-Pr MBPS. Importantly, SEAM 3 binding was lost when tissues were treated with periodate at high pH where NeuPSA, in particular, is susceptible to oxidation. Unlike anti-de-N-acetyl GD3 mAbs (27), we found no reactivity with whole CHO cell...
extracts on Western blots. Taken together with the correlation of SEAM 3 reactivity and polysialyltransferase mRNA expression (Table 2), the results provide strong evidence for widespread NeuPSA expression in human tissues.

Working with FFPE tissues allowed us to obtain a large number of samples that maintain the integrity of the original tissue structure. However, antigen retrieval steps that are required in all IHC assays with formalin fixed-tissues expose the sample to elevated temperatures and various reagents. It is possible that such treatments and the conditions for antigen retrieval could have inadvertently produced NeuPSA in tissues expressing PSA. To validate the use of FFPE tissues, we carried out the same IHC assays using unfixed frozen tissues. At no point was the tissue exposed to fixatives or elevated temperatures. The automated Ventana program was modified to skip all deparaffinization and antigen retrieval steps. Working with frozen unfixed tissue is known to be very difficult, and only some of the frozen tissues we studied were able to maintain structures characteristic of the particular tissue after processing and staining. Of these tissues, many showed a similar staining pattern as seen with the FFPE specimens, albeit with reduced staining intensity. Examples included samples of the small intestine and adre-
nal gland. Therefore, we conclude that the board expression of NeuPSA observed in human tissues did not result from antigen retrieval procedures.

Considering the full expression profile of NeuPSA in normal human tissue (Table 1) raises the question of whether there is a trend or pattern of expression in specific cell types that showed increased expression. Most human organs had a basic epithelial cell type that demonstrated positive NeuPSA expression. Examples include the thyroid epithelial cell, the epithelial lining of the small and large intestine, and the tubule epithelial lining in the kidney. An additional interesting trend in cells with high NeuPSA expression was seen in cells of endocrine origin that have significant secretory function. Examples include the acinar and islet cells of the pancreas, cortical cells of the adrenal gland, and thyroid epithelial cells. The staining pattern observed in the secretory cells was granular in nature and located in the cytoplasm and around nuclei. We hypothesize that the granular staining pattern represents marking of intracellular vesicular structures en route to the cell membrane for secretion of various cell type-specific products. In cell lines we have showed that SEAM 3 reactivity co-localizes with Golgi apparatus and endoplasmic reticulum markers, especially Tuba, which is a protein found associated with the Golgi, cell junctions, and cytoskeleton (30). It is of interest in this regard that the polysialyltransferases PST and STX, which are also polysialylated, can be found intracellularly associated with the endoplasmic reticulum and Golgi on the cell surface and secreted outside the cell (10). Given that there are very few proteins known to be polysialylated and none that have a cellular distribution similar to the polysialyltransferases, it is possible that one or both transferases are modified with NeuPSA and may be involved in vesicular trafficking in secretory cells.

The purpose of PSA de-N-acetylation is unclear at the present time, but the consistency of high expression in invasive cells ranging from trophoblasts and cancer cells (30) to pathogenic bacteria (14–16, 19) and parasites (20), implies an important role in cellular interactions. Modification of proteins such as NCAM with PSA appears to serve mainly to block binding interactions (3). In contrast, PSA oligomers containing even a relatively small fraction of Neu form aggregates of high molecular mass, which suggests that NeuPSA modification of proteins could promote cellular interactions. Also, NeuPSA is stable under acidic conditions and is not degraded by exoneuraminidases (16, 27). The simple process of enzymatic de-N-acetylation, therefore, may provide a relatively simple mechanism for shifting the properties of PSA from anti- to binding and chemically labile to stable.

Recognizing widespread expression of NeuPSA in human tissues raises fundamental questions about the role of PSA in human health and disease while at the same time offering many new possibilities for intervention. NeuPSA was discovered during the development of vaccines to prevent disease caused by NmB bacteria (14–16). Anti-NeuPSA antibodies are protective against invasive NmB stains (14, 16), and some anti-NeuPSA mAbs have cytotoxic functional activity against cancer cells (30). However, the widespread expression of NeuPSA in normal human tissues, particularly fetal tissues, would appear to preclude the use of NeuPSA-based vaccines for preventing meningococcal disease and, possibly, treating cancer. Further studies will be required to fully evaluate the risks and benefits of such approaches. Alternatively, enzymes constituting the NeuPSA pathway including the α2,8-polysialyltransferases and putative PSA de-N-acetylas have the potential to be unique targets for preventing and treating diseases where NeuPSA plays an important role. The potential applications for agents that can be used to manipulate the NeuPSA pathway await further studies currently in progress to identify the components of the pathway and the purpose of NeuPSA expression.

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