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Expression of polysialylated neural cell adhesion molecules on adult stem cells after neuronal differentiation of inner ear spiral ganglion neurons

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ABSTRACT

During brain development, polysialylated (polySia) neural cell adhesion molecules (polySia–NCAMs) modulate cell–cell adhesive interactions involved in synaptogenesis, neural plasticity, myelination, and neural stem cell (NSC) proliferation and differentiation. Our findings show that polySia–NCAM is expressed on NSC isolated from adult guinea pig spiral ganglion (GPSG), and in neurons and Schwann cells after differentiation of the NSC with epidermal, glia, fibroblast growth factors (GFs) and neurotrophins. These differentiated cells were immunoreactive with mAb's to polySia, NCAM, β-III tubulin, nestin, S-100 and stained with BrdU. NSC could regenerate and be differentiated into neurons and Schwann cells. We conclude: (1) polySia is expressed on NSC isolated from adult GPSG and on neurons and Schwann cells differentiated from these NSC; (2) polySia is expressed on neurons primarily during the early stage of neuronal development and is expressed on Schwann cells at points of cell–cell contact; (3) polySia is a functional biomarker that modulates neuronal differentiation in inner ear stem cells. These new findings suggest that replacement of defective cells in the inner ear of hearing impaired patients using adult spiral ganglion neurons may offer potential hope to improve the quality of life for patients with auditory dysfunction and impaired hearing disorders.
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1. Introduction

Regeneration of sensory neurons can occur in the inner ear of human and adult animals [1,2]. In the vestibular organ and the spiral ganglia of adults, cells showing the identical phenotypic characteristics of neural stem cells (NSCs) have been identified [3]. These adult NSCs form neurospheres that are characterized by self-renewal and can differentiate into neurons and glia cells [1].

In the proliferation and differentiation of neural stem cells, distinct cell surface glycoconjugates including, glycoproteins, glycosphingolipids, and extracellular matrix molecules play a critical role in regulating development and differentiation [4,5]. A well-studied member of this class of cell surface glycoproteins is the polysialic acid (polySia) that posttranslationally modifies neural cell adhesion molecules (NCAMs). The polySia glycan is an extended, linear homopolymer consisting of α2,8-ketosidic linked residues of N-acetylneuraminic acid (Neu5Ac; Sia). Their degree of polymerization (DP), or chain length, can exceed 400 Sia residues when accurately determined in the absence of acid hydrolysis [6,7]. Thus, polySia is a large polyanionic "space filling" molecule that functions as an anti-adhesive glycan on cell–cell and cell–matrix interactions [7–10].

The predominant protein carrier of polySia in brain is NCAM, although this glycotope is also expressed on a restricted set of other glycoproteins including the α-subunit of the sodium channel in adult rat brain [11], neuropilin-2 in human dendritic cells [12] and on CD36, a human milk glycoprotein [13]. Autopolysialylation of the two mammalian polysialyl transferases, ST8Sia II (STX) and ST8Sia IV (PST), has also been described [14,15]. More recently, the synaptic CAM, SynCAM1 (*Cadm1*), which is expressed selectively on NG2 glia cells, has been reported to be polysialylated and to modulate SynCAM 1 function [9,16].

The embryonic (E) or heavily polysialylated form of NCAM is a cell surface glycoprotein that modulates many key functional interactions between cells, including cell–cell and cell–matrix adhesion, neural migration, neurite outgrowth, fasciculation, axon

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path finding, synaptic plasticity, cell signaling/cytokine response and myelination [9,17–19]. More recent studies showed polySia was expressed on human and murine leukocytes and to regulate immune responses [6]. It is also expressed on human dendritic cells where it modulates T-lymphocyte–dendritic cell interactions [12]. While expression of the polysialylated form of NCAM is usually restricted to early stages of embryonic and postnatal development [10,20], it is persistently expressed in selective regions of adult brains that are associated with synaptic plasticity and neurogenesis, including the hippocampus, hypothalamus, dentate gyrus and olfactory bulb. A further important role for the polySia glycan is its support of dynamic changes associated with peripheral nerve regeneration [21].

Due to its polyanionic charge, the polySia chains that modify N-linked glycans on NCAM prevents both the homophilic and heterophilic binding interactions between NCAM expressing cells [9,22]. In this context, polySia functions as an anti-adhesive glycotope preventing cell adhesion and cell migration. As such, re-expression of polySia on a number of adult human cancers allows the detachment of these cells from their original tumor site, thereby aiding their malignant potential by facilitating metastatic spread [10,23–26].

The cell surface expression of polySia is developmentally regulated in both the central nervous system (CNS) and the peripheral nervous system (PNS). Thus, growth and migration of nerve cells, fasciculation, synapse formation, and myelination processes occur as noted above [10,11,27,28]. Polysialylated NCAM is also a known neurological marker in neural stem cells formed in the CNS and is involved in their migration and differentiation [29,30]. While these latter studies focused primarily on differentiation of CNS cells, there is a dearth of information on the role of adult neural stem cells present in the inner ear. Accordingly, the objective of this study was to determine if polySia–NCAM was expressed on adult NSC isolated from guinea pig spiral ganglion, and in neurons and Schwann cells differentiated from these stem cells. Our new findings show that polysialylated NCAM is expressed on these adult stem cells, and in neurons and Schwann cells after differentiation with epidermal, glia, fibroblast growth factors (GFs) and neurotrophins. The results suggest a functional role for polySia in neuronal differentiation in inner ear stem cells, a finding that has not been previously reported.

2. Materials and methods

2.1. Isolation and culturing of guinea pig spiral ganglion neurons

Spiral ganglion neurons were isolated from adult guinea pigs weighing ca. 300 g. The animals were first anesthetized with pentobarbital by infusion via the intra-abdominal route. Spiral ganglia were obtained by dissection and transferred to a tube containing Dulbecco's modified Eagle's media (DMEM; Gibco, Carlsbad, CA, USA, 41966). The rest of procedures follow as described in Rask-Andersen et al. [1].

2.2. Immunofluorescent histochemical staining of neurospheres differentiated from cultured spiral ganglion neurons

To examine the neurospheres obtained from the cultured spiral ganglia for cell proliferation, they were transferred to a 6-well slide, the surface of which was protected with a cover slide. 3.5 μL of 10 mM bromodeoxyuridine (BrdU; ABD Serotec; Raleigh, NC, USA; Cat. No. MCA 2060) were added and the cultures incubated for 4–12 h at 37 °C in 5% CO_2 atmosphere.

After incubation, the differentiated cells were embedded in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and washed with PBS. For the first 5 min, cells were incubated with 0.2% Triton-X100 in PBS (Junsei Chemical, Tokyo, Japan) followed by 2 M HCl for 1 h at room temperature and then for 5 min with 0.1 M $Na_2B_4O_7$ (Sigma, St. Louis, MO, USA) before rinsing with PBS. Blocking was carried out using 10% goat serum (Vector Lab, Inc., Peterborough, England,) for 5 h at room temperature.

For the double antibody staining experiments, antibodies specific for BrdU, Nestin (1:200; Chemicon; Cat. No. MAB 353), polysialylated NCAM (1:100; Miltenyl Biotec, Bergisch Gladbach, Germany, Cat. No. 130–093-274), non-polysialylated NCAM (1:100; Abacam, Cat. No. ab8233), β-III tubulin (1:100; Abcam, Cat. No. ab6046), and S-100 (1:100; Sigma, St. Louis, MO, USA) were used. Cells were added to PBS containing 10% goat serum and incubated with the primary antibodies at 4 °C for 12 h, before rinsing with PBS. The secondary antibodies, including the cyanine-conjugated antibody, goat anti-rat IgG antibody (Alexa Fluor-red, Cambridge, England), sheep anti-mouse Cy2 (Cy2-green color for nestin (Jackson ImmunoResearch, Baltimore, MD USA), NCAM (Abcam,), β-III tubulin, (Abcam), and goat anti-rabbit fluorescein isothiocyanate (FITC, green color for S-100; (Abcam, diluted 1:200) were added and incubated in PBS – 10% goat serum. After rinsing with PBS, mounting was carried out using the mounting medium for fluorescence (Vector Lab, Inc.). For nuclear staining, 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.) was used. For nuclear staining, whole mounting was done using DAPI-conjugated mounting medium (VECTASHIELD, Burlingame, CA, Cat. No. H01200).

2.3. Immunohistochemical staining of cells differentiated from spiral ganglion stem cell neurospheres

Cells that were differentiated from the adult cultured spiral ganglion stem cells were prepared for immunohistochemical staining using antibodies specific for NCAM (1:100; Sigma), polysialic acid (1:100; Miltenyl Biotec (as above), β-III tubulin (1:300; Chemicon, Millipore, Billerica, MA USA), and S-100 (1:100; DAKO, Glostrup, Denmark). After sub-culturing for one week in the cell differentiation medium, the cells were repeatedly washed with PBS by re-suspension before embedding in 4% paraformaldehyde in PBS (10 min).

For the staining of neurons and Schwann cells obtained after differentiation of the neurospheres as described above, the primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA). After addition of the primary antibody, cells were incubated for 1 h at room temperature before rinsing three times for 5 min each with 1% saponin in PBS. The secondary antibodies were added and incubated at room temperature for 30 min, before adding the avidin-horseradish peroxidase (Vectastatin ABC kit, (Vector Laboratories) for visualization in the light microscope.

3. Results

3.1. Growth of adult neural stem cells isolated from guinea pig spiral ganglion neurons

After isolation of the adult neural stem cells (NSC) from guinea pig spiral ganglia (GPSG) and three sub-passages in a growth medium containing epidermal growth factor (EGF) and fibroblast growth factor (FGF), cell colonies with a round-shaped phenotype characteristic of neurospheres were obtained. These neurospheres were capable of self-renewal upon sub-culturing. The population of neurospheres expressed both polysialylated and non-polysialylated NCAMs, markers known to be associated with neural stem cells (Fig. 1) [31,32].

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