Abstract A novel fluorescent cytochemical method for sialidase activity was developed using 5-bromo-4-chloroindol-3-yl-α-D-N-acetylneuraminic acid (X-Neu5Ac) as the substrate. Intact nuclei were isolated from porcine liver and incubated at 37°C for 3 h with 1 mM X-Neu5Ac at pH 4.8. The nuclei were stained with blue color that was derived from the oxidized compound of the reaction product X (5-bromo-4-chloro-3-hydroxyindole). A specific sialidase inhibitor, 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid, suppressed the staining in a dose-dependent manner. Despite the specificity of the cytochemical reaction, the staining was too weak to analyze the staining distribution and pattern of individual nuclei. To attain more sensitive detection of sialidase activity, the nuclei were incubated with X-Neu5Ac in the presence of Fast Red Violet LB. Individual nuclei of porcine liver were clearly stained with fluorescence that was produced by the conjugated compound of product X with Fast Red Violet LB. This fluorescent cytochemical method was also employed successfully for detection of sialidase activity of intact GOTO neuroblastoma cells in culture. The present method should provide a useful tool for investigating the localization and stage-specific expression of sialidase activity in tissues and cells.

Keywords Sialidase · X-Neu5Ac · Cell nucleus · Porcine liver · Neuroblastoma cells

Introduction

Sialidase is an exoglycosidase that cleaves α-linked non-reducing sialic acid residues from sialoglycoconjugates such as sialooligosaccharides, sialoglycoproteins, and gangliosides (Saito and Yu 1995). In mammalian cells, sialidase plays a crucial role in the catabolism of sialoglycoconjugates; the cleavage of terminal sialic acid residues by the enzyme is the first step in the degradation of carbohydrate portions of sialoglycoconjugates (Ledeen 1989). Mammalian sialidase exists as isoenzymes in different subcellular organelles. Two are soluble enzymes in the cytosol (Venerando et al. 1975; Miyagi and Tsuiki 1985) and intralysosomal matrix (Miyagi and Tsuiki 1984), and the other two are membrane-bound enzymes associated with plasma membranes (Schengrund et al. 1972; Yohe and Rosenberg 1977) and lysosomal membrane (Tulsiani and Carubelli 1970; Michalski et al. 1986). The presence of nuclear membrane-bound sialidase has also been suggested (Saito et al. 1996). Some of these sialidase isoenzymes have recently been cloned, and their amino acid sequences have been determined (Miyagi et al. 1993a, 1999; Bonten et al. 1996).

Sialidase is assumed to participate in diverse cellular functions. Expression of sialidase activity is developmentally regulated (Carubelli and Tulsiani 1971; Venerando et al. 1982; Yohe et al. 1986; Saito et al. 1992, 1995; Hasegawa et al. 2001). Sialidase activity may be implicated with processes of cellular growth and differentiation (Nojiri et al. 1982; Landolfi et al. 1985; Pitto et al. 1989; Lambre et al. 1990; Kopitz et al. 1994, 1997) or neoplastic transformation (Schengrund et al. 1973; Miyagi et al. 1994). The possible involvement of sialidase in cell-to-cell interactions has also been suggested (Saito and Yu 1993).

To explore the cellular function of sialidase, it would be useful to establish a specific and sensitive in situ detection method for the enzyme in tissues and cells. Thus, the immunological localization of cytosolic sialidase in rat skeletal muscle was examined using a specific antibody (Akita et al. 1997). Regarding histo- and cytochem-
tional detection of sialidase activity, a very few studies have been conducted. Gossrau et al. (1977) reported a histochemical method for sialidase activity using 5-bromo-3-indolyl-α-D-N-acetylneuraminic acid as the substrate. While this method appeared to be useful, some essential aspects, for example, its specificity to sialidase activity and sensitivity of the method, were not fully characterized. Thus, this method has not widely been used for localization of sialidase activity in tissues and cells. No other histo- or cytochemical method has been reported.

Previously, we synthesized a novel sialic acid derivative, 5-bromo-4-chloroindol-3-yl-α-D-N-acetylneuraminic acid (X-Neu5Ac). This compound was effectively hydrolyzed by bacterial sialidase (Fujii et al. 1993). Using this compound, Wiggins et al. (2000) devised a spot test for analysis of bacterial sialidase activity. In the present study, we developed a novel fluorescent cytochemical method for in situ detection for sialidase activity using X-Neu5Ac and Fast Red Violet LB. The method was successfully applied to the detection of sialidase activity of isolated nuclei from porcine liver and intact neuroblastoma cells in culture.

Materials and methods

Materials

Fresh porcine liver was obtained from a local slaughterhouse. A GOTO neuroblastoma cell line was purchased from Riken Gene Bank (Riken, Tsukuba-shi Ibaragi, Japan). The cells were cultured in a 1:1 mixture of RPMI 1640 and Eagle’s MEM containing 10% fetal bovine serum. The chromogenic substrate for in situ detection for sialidase activity using X-Neu5Ac and Fast Red Violet LB. The method was successfully applied to the detection of sialidase activity of isolated nuclei from porcine liver and intact neuroblastoma cells in culture.

Assay of sialidase activity using 4MU-Neu5Ac

Sialidase activity in subcellular fractions was assayed in a test tube reaction using 4MU-Neu5Ac as the substrate (Kobayashi et al. 2000). The reaction mixture contained 0.1 M sodium acetate buffer (pH 4.8), 0.2 mM 4MU-Neu5Ac, and an enzyme preparation in a final volume of 0.1 ml. After incubation at 37°C for 1 h, the reaction was terminated by the addition of 0.18 M glycine-NaOH buffer (pH 10.8-2.9 ml), followed by spectrophotometric measurement with excitation and emission wavelengths at 365 and 445 nm, respectively. Enzyme activity was calculated based upon the fluorescence intensity of authentic 4MU solutions of known concentrations.

Cytological and fluorescent cytochemical detection of sialidase activity using X-Neu5Ac

Intact nuclei from porcine liver were placed in plastic microtiter plate wells (96 wells) and incubated with 1 mM X-Neu5Ac and 0.1 M sodium acetate buffer (pH 4.8) at 37°C for 3 h (total volume 0.1 ml). The reaction was terminated by placing the plate on ice. To measure the color intensity of the blue-stained nuclei in wells, a color photograph of the microplate was taken and was scanned using a densitometric scanner at 605 nm with a reflection mode (Shimadzu Chromatoscanner CS-910; Shimadzu, Kyoto, Japan).

Alternatively, isolated nuclei were placed in a plastic microtube and incubated with 1 mM X-Neu5Ac and 0.1 M sodium acetate buffer (pH 4.8) in the presence of Fast Red Violet LB (0.1 mg/ml) at 37°C for 3 h. The nuclei were then washed with PBS twice and examined under fluorescence microscopy (IX70 with IX-FLA; Olympus, Tokyo, Japan) with filters for excitation wavelengths ranging from 520 to 550 nm and emission wavelengths of 580 nm and above.

Fluorescent cytochemistry of GOTO cells was carried out after fixation with 4% paraformaldehyde in PBS at room temperature for 10 min. The fixed cells were treated with X-Neu5Ac plus Fast Red Violet LB in a similar manner as described above, except that incubation was done at room temperature for 1 h.

Results and discussion

While the nuclear localization of sialidase activity was originally suggested using rat brain preparations, its specific activity was relatively low (Saito et al. 1996). We recently found that nuclear membranes from porcine liver possess a higher sialidase activity. Accordingly, intact nuclei and nuclear membranes were prepared and used for cytochemical analysis of nuclear sialidase activity. Sialidase activity in subcellular fractions was examined using tube reaction with 4MU-Neu5Ac as the substrate. The enzyme activity in nuclear membranes was significantly higher than that of cytosolic fraction (112 vs 16 pmol/mg protein/min), but lower than those in liver homogenate and microsomal membranes (316 and 225 pmol/mg protein/min, respectively). These results suggest the presence of intrinsic sialidase activity in porcine liver nuclear membranes.

Sialidase activity in isolated nuclei was further examined using a newly developed cytochemical method with X-Neu5Ac as the substrate. This synthetic sialyl compound is known to be hydrolyzed specifically by sialidase, producing Neu5Ac and compound X (i.e., 5-bromo-4-chloro-3-hydroxyindole); the latter is easily oxidized to a blue-colored, water-insoluble compound (Fig. 1; Fujii et al. 1993). Thus, sugar derivatives of

Preparation of intact nuclei, nuclear membranes, and other subcellular fractions from porcine liver

Intact nuclei were isolated from porcine liver based upon the method reported previously (Blobel and Potter 1966). All procedures were carried out at 4°C. Porcine liver was perfused with phosphate-buffered saline (PBS) and homogenized in 1.3 M sucrose containing 5 mM MgCl₂ and 10 mM Tris-HCl (pH 7.4) (called 1.3 M sucrose buffer). The homogenate was centrifuged at 1,000 × g for 20 min. The pellet was suspended in 2.2 M sucrose buffer and centrifuged at 100,000 × g for 60 min. The resultant pellet was suspended in 2.2 M sucrose buffer and centrifuged in the same condition. Purified nuclei were recovered as the pellet. Phase contrast microscopic examination demonstrated that the nuclear preparation was essentially free of contamination by other subcellular organelles or membrane-like structures (data not shown). The isolated nuclei were then disrupted by sonication for 2 min. Nuclear membranes were obtained as the pellet after centrifugation at 100,000 × g for 1 h.

Other subcellular fractions were prepared as follows. A porcine liver homogenate in 0.25 M sucrose buffer was centrifuged at 10,000 × g for 10 min. The supernatant was further centrifuged at 100,000 × g for 1 h. The resultant pellet and supernatant were used as microsomal membrane and cytosolic fractions, respectively.