High β-Galactosidase and Ganglioside $G_{M1}$ Levels in the Human Parotid Gland

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Background: Ganglioside $G_{M1}$ is a membrane glycolipid typical of nerve cell membranes, where it partakes in neurotransmitter release and is catabolized by the lysosomal β-galactosidase (GM1ase) (EC 3.2.1.23). After demonstrating a novel degenerative disease of the parotid gland in mice deficient in GM1ase, mimicking the human storage disease GM1 gangliosidosis, we studied GM1ase and ganglioside $G_{M1}$ content in the human parotid glands.

Study Design: Levels of GM1ase and ganglioside $G_{M1}$ were determined in samples of parotid tissues and neighboring muscle (as a negative control) for 3 subjects. Tissues were also processed for histochemical demonstration of GM1ase.

Results: The mean specific activity of GM1ase was more than 6-fold higher in the healthy human parotid tissues (1.4±0.5 nmol of 4-methylumbelliferone per minute per milligram of protein) relative to the neighboring muscle tissue (0.23±0.07 nmol of 4-methylumbelliferone per minute per milligram of protein). Activity of GM1ase was histochemically localized mainly to striated duct and acinar cells of the parotid gland. Ganglioside $G_{M1}$ content in the parotid gland was on average 30-fold higher relative to muscle.

Conclusions: Our results are consistent with previous findings reported in the mouse and the rabbit, and probably reflect a general property of the mammalian parotid glands. The novel mechanism we previously proposed for the mouse parotid saliva secretion, mimicking neurotransmitter release in ganglioside GM1–containing nerve cells, is probably applicable also to the human parotid gland. Similarly, the human parotid gland is probably also severely affected in GM1 gangliosidosis.


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RESULTS

Ganglioside $G_{M1}$ is a membrane glycolipid typical of nerve cells, catabolized in the lysosomes of such cells by GM1ase. Previous studies demonstrated a surprisingly high level of GM1ase and ganglioside $G_{M1}$ in the parotid glands of mice, and a novel degenerative disease of the parotid gland in mice deficient in GM1ase, mimicking the human storage disease GM1 gangliosidosis.3,4,6 Our results determined GM1ase-specific activity in healthy human parotid tissues of 3 subjects. Muscle, a tissue previously reported as being low in GM1ase activity, as are almost all mammalian and human tissues, was used as our negative control. Our data demonstrate that mean GM1ase-specific activity in the healthy human parotid samples was significantly and substantially (>6-fold) higher than that in healthy muscle samples (P<.001) (Figure 1).

We used the x-gal substrate under acidic conditions to histochemically demonstrate GM1ase activity as blue staining in the parotid glands (Figure 2A-B), whereas such activity was undetectable in muscle (Figure 2C-D). High levels of x-gal staining, indicative of GM1ase activity, were localized to parenchymal cells of the parotid gland. Highest-intensity staining was found in the apical cytoplasm of striated duct cells and lower-intensity...
MATERIALS AND METHODS

TISSUE SAMPLES

Surgical discard tissues were assayed from 3 subjects (1 woman and 2 men, aged 28 to 50 years) undergoing excision of pleomorphic adenomas of the parotid gland. During surgery, neighboring margins of healthy parotid and muscle tissues were also excised. The samples we used included these healthy parotid and muscle tissues, as identified by the surgeons and verified by results of histological examination.

PROTEIN ASSAYS

Tissue samples were rinsed in ice-cold phosphate-buffered saline solution and homogenized to a concentration of 5 mg/mL of wet tissue weight in a buffer containing 0.01M Tris (pH, 6.7), 0.1% sodium dodecyl sulfate, and serine protease inhibitor (Papabloc, St Louis, Mo) at a concentration of 0.1 mg/mL. Protein assays were performed on homogenates using a commercially available assay (Bio-Rad Laboratories Inc, Hercules, Calif) and bovine serum albumin as a standard.

GM1ase ENZYME ASSAYS

Three different serial (2-fold) dilutions of each homogenate were tested for β-galactosidase activity under acidic conditions by means of a fluorometric assay using 4-methylumbelliferyl-β-D-galactopyranoside (+MU-β-gal) (Sigma-Aldrich Corp, St Louis, Mo) in a solution containing 2mM substrate and 0.1M sodium acetate buffer (pH, 4.4), 0.2M sodium chloride, and 0.25% bovine serum albumin. Poststain fixation was performed on homogenates using a commercially available assay (Bio-Rad Laboratories Inc, Hercules, Calif) and bovine serum albumin as a standard.

HISTOLOGICAL AND HISTOCHEMICAL ANALYSIS

For microscopic histochemical study, a modification of the method described by Sanes et al was performed under acidic conditions. The tissues were prefixed for 2 hours in 10% formaldehyde, made permeable at 37°C for 2 hours in 2M magnesium chloride, 0.01% sodium deoxycholate, 0.02% NP-40, 8.25-mg/mL potassium ferrocyanide, and 10.3-mg/mL potassium ferricyanide, and stained overnight at 37°C in 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal) (Research Organics Inc, Cleveland, Ohio) in 0.1M sodium acetate (pH, 4.4), 0.2M sodium chloride, and 0.25% bovine serum albumin. Poststain fixation was performed in 10% neutralized buffered formalin for 1 hour, with consequent dehydration in a graded series of ethanol to 100%. Tissues were cleared in xylene, infiltrated, and embedded in paraffin, and 5-µm sections were prepared using a Reichardt-Jung microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany).

GANGLIOSIDE ANALYSIS

Our method for quantitative analysis of ganglioside GM1 is based on a modification of the method described by Svennerholm. Parotid and control tissue samples (wet tissue weight, 250 mg) were homogenized, and their total gangliosides were extracted based on differential solubility in a mixture of chloroform, methanol, and water. The amphoteric nature of gangliosides makes such an extraction method possible. The order of addition of the solvents and the exact volume ratios are essential for effective extraction of the gangliosides and effective elimination of the various hydrophilic and hydrophobic contaminants.

The tissue was first homogenized in ice-cold distilled water (3 × volume of tissue weight), then methanol (10.6 × volume of tissue weight) and finally chloroform (5.3 × volume of tissue weight) were added. Solids were pelleted by means of centrifugation, and the pellet was reextracted using the chloroform-methanol-water mixture to ensure maximal extraction of gangliosides from the tissue. Distilled water (0.173 × volume of tissue weight) was added to the combined supernatants containing the gangliosides and other contaminants. The addition of the water caused the separation of the original extraction solution into 2 phases, and the gangliosides partition into the aqueous phase. The 2 phases were separated and each reextracted, and the aqueous phase was further purified using reverse-phase chromatography (Sep-Pak Plus C; Waters Corp, Milford, Mass). Final elution of the column was performed in methanol, and the purified ganglioside samples were then lyophilized, dissolved in a small volume (7 µL) of chloroform and methanol (1:2), and applied to thin-layer chromatography (TLC) silica gel 60 plates (EM Science, Gibbstown, NJ). The gangliosides were resolved by means of TLC in a mixture of chloroform, methanol, and 10mM potassium chloride (55:45:10, vol/vol). The addition of potassium chloride altered the mobility of gangliosides and improved their resolution. The ganglioside bands were visualized using a combination of resorcinol, hydrochloric acid, and copper ion as a staining agent, which requires activation by heating at 120°C for 20 minutes. The ganglioside bands appeared violet-blue while neutral, and sulfuric glycosphinolipids appeared yellow-brown. Pure ganglioside GM1 (2 µg per lane) (Sigma-Aldrich Corp) was used as a migration marker and a quantitative standard. The bands of interest were quantitated by means of densitometric measurements. After correcting for the background for each plate, the net optical density measurements for each tissue were divided to the standard measurement on the same plate.

staining was found in acinar cells of parotid sections, whereas findings in the muscle appeared negative. In the acinar cells, reaction products appear primarily in the basal cytoplasm. Endothelial cells also seem to show weak reaction products.

We further assayed ganglioside GM1 content of the human parotid glands using the TLC technique (Figure 3). When using this method, ganglioside GM1 in the human parotid gland appears as a dark stain consistent with that of an authentic sample of ganglioside GM1. In contrast, no ganglioside GM1 stain is observed in the muscle sample. Scanning of TLC plates indicates that ganglioside GM1 levels in the human parotid gland are at least 30-fold higher than that in human muscle (P<.001). Our results demonstrating high levels of ganglioside GM1 and GM1ase in the human parotid gland are consistent with previous results in rabbit and mouse. Therefore, these findings may be a general feature of the mammalian parotid gland.
Previous studies have examined knockout mice deficient in GM1ase to study the role of this enzyme in the salivary glands. The results demonstrated ganglioside GM1 accumulation in the parotid glands of deficient mice as large (diameter, >10 µm) storage vacuoles in the parotid gland (but not the submandibular or sublingual glands). The histochemical staining of these storage vacuoles with combined fluorescein isothiocyanate and cholera toxin, which binds with high affinity and specificity to ganglioside GM1, confirmed the storage of ganglioside GM1 in these vacuoles.

These results suggest that ganglioside GM1 is the physiologic substrate for GM1ase in the parotid glands of mice as previously reported in the nerve cells. Therefore, our working hypothesis is that ganglioside GM1 and GM1ase are important in the secretory function of the parotid gland, similar to their function in neurotransmitter release in nerve cells. Activity of GM1ase in acinar cells of the parotid gland is most likely related to lysosomal metabolism of ganglioside GM1 and membrane turnover in conjunction with the secretory exocytosis/endocytosis cycle. Thus, our proposed model for the role of ganglioside GM1 and GM1ase in mouse parotid secretion and membrane turnover mechanisms may apply to human parotid glands as well.

It is not clear what the functions of ganglioside GM1 and GM1ase are in duct cells. These cells also exhibit active membrane recycling. According to Hand et al., the endocytosis/exocytosis cycle in parotid duct cells is primarily related to secretion and reabsorption of certain electrolytes, principally by the striated and excretory ducts, and to secretion of proteins and glycoproteins, mainly by the intercalated and striated ducts, which are important functions of these gland components. Thus, ganglioside GM1...
and GM1ase probably participate in essential subcellular membrane-recycling processes in acinar and duct cells of the parotid gland. However, their specific roles in acinar and duct cells of the human parotid glands in health and disease require further investigation.

Our model for mouse parotid secretion (Figure 4) is based on the model presented by Sandhoff and Van Echtern\cite{15} for ganglioside $G_{M1}$ subcellular turnover and metabolism in nerve cells. We hypothesize that this model\cite{14} is applicable to the mammalian parotid gland, in conjunction with the principle β-adrenergic secretory pathway described by Castle et al.\cite{16,17}. Our model differs from that of Sandhoff and Van Echtern\cite{15} by emphasizing the obligatory endocytosis coupled with exocytosis.\cite{18} In both models, the final synthesis of secretory glycoproteins and ganglioside $G_{M1}$ is completed in the network of trans–Golgi acinar cells. Ganglioside $G_{M1}$ is then incorporated into cell membranes and transported to the plasma membrane. In recent years, ganglioside $G_{M1}$ has been established as a major component of caveolae and caveolae-like domains in cell membranes, which form uncoated membrane pits.\cite{19} These domains are essential for vesicular membrane trafficking mechanisms (endocytosis/exocytosis) in specific nerve cell types.\cite{20} Therefore, our model, based on the results of the GM1ase knockout mice studies,\cite{21,22} proposes an important role for ganglioside $G_{M1}$ in membrane trafficking and secretory mechanisms in the parotid gland.

Our results predict that human parotid gland is severely affected in GM1 gangliosidosis, as is the mouse parotid gland in knockout mice deficient in GM1ase. However, the potential abnormalities of the human parotid gland in GM1 gangliosidosis have not been reported so far, possibly as an oversight. Anecdotal evidence suggests high levels of dental caries in patients with GM1 gangliosidosis, which may be attributed to deficient salivary secretion. Studies of oral health and parotid gland function in patients with GM1 gangliosidosis should address the issue.

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