Arylsulfatase B Activity in Cultured Retinal Pigment Epithelium: Regional Studies in Feline Mucopolysaccharidosis VI

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Feline mucopolysaccharidosis VI (MPS VI) is a recessively inherited lysosomal storage disease resulting from a deficiency of arylsulfatase B (ASB). Previous histopathologic findings have indicated that the disease is expressed morphologically in non-pigmented retinal pigment epithelial cells (RPE) in the posterior pole and superior equatorial regions by the accumulation of vacuolated inclusions and eventual cellular hypertrophy, while pigmented regions in the periphery are minimally affected. To determine if the regional and age-dependent variations in disease severity result from differences in residual enzyme activity, primary cultures of feline MPS VI-affected RPE were initiated from defined regions of the eye and maintained in vitro for 14 days. Cultures initiated from nonpigmented areas of affected adult eyes (posterior pole, superior equatorial) were more diseased than those from pigmented (inferior–equatorial, peripheral) areas. In the nonpigmented cultures, the disease was expressed by the accumulation of single membrane-bound inclusions and cellular hypertrophy. These inclusions were indistinguishable in their morphologic appearance and distribution from those found in situ. In contrast, the cultures initiated from pigmented areas remained normal or minimally affected. The same spatial disease distribution was present in young affected eyes, but the expression of the disease was much less severe. It is apparent that temporal, spatial, and pigmentation factors were correlated with disease expression in vitro as well as in situ. Arylsulfatase B activity was measured biochemically, and found to be deficient in all regions of young and adult eyes. It was notable that there was no correlation between the level of residual enzyme activity, and the pigmentation or spatial position from which the cells were obtained. Thus, variations in residual enzyme activities cannot account for the spatial and temporal differences in disease severity. Invest Ophthalmol Vis Sci 27:1050–1057, 1986

Arylsulfatase B (ASB; EC 3.1.6.1.) is a lysosomal hydrolase that degrades the glycosaminoglycan (GAG) dermatan sulfate. In mucopolysaccharidosis VI (MPS VI; Maroteaux-Lamy syndrome), the activity of ASB is deficient and results in the increased urinary excretion of dermatan sulfate and intracellular storage of this GAG.[1] In affected humans and cats, this recessively inherited lysosomal storage disease is characterized clinically by dysostosis multiplex, hepatosplenomegaly, corneal opacities, mitral insufficiency, and vacuolated leukocytes.[2,3,4]

In the retinal pigment epithelium (RPE) of affected cats, storage of non-degraded or partially degraded GAGs within lysosomes results in the accumulation of membrane bound inclusions.[5] The nonpigmented RPE in the posterior pole is affected to a greater extent earlier in the disease, with the accumulation of vacuolated inclusions eventually resulting in cellular hypertrophy. The pigmented RPE in the peripheral regions remains normal. While the reason for this spatial distribution of the disease is not known, it implies that, at least in terms of GAG metabolism, the RPE does not function as a uniform monolayer.

In MPS VI-affected tissues, such as peripheral leukocytes, cultured fibroblasts, and cultured RPE cells, 5–10% of normal ASB activity is present.[6] This low level of activity, termed residual enzyme activity, is insufficient to prevent the morphologic manifestations of the disease. In RPE cultures from heterozygotes, the activity of ASB is reduced to about 30% of normal.

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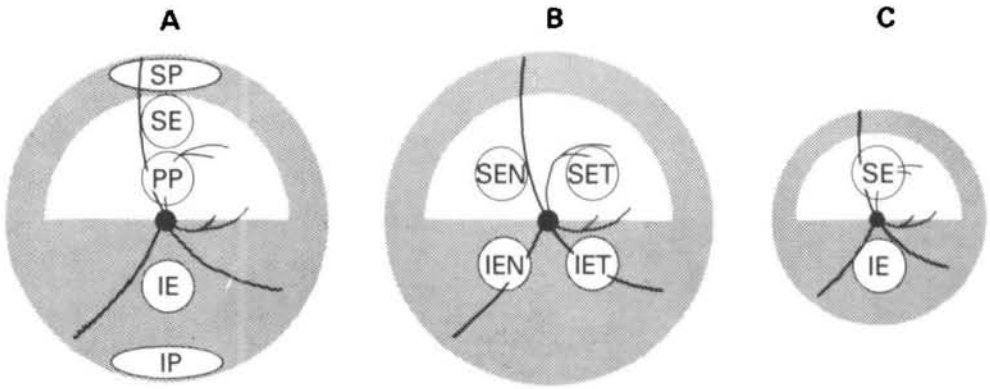


Fig. 1. Schematic diagram of the left eyes of adult (**A, B**) and young (**C**) cats illustrating the general regions of the eye that were sampled. SP = superior periphery, SE = superior equatorial, PP = posterior pole, IE = inferior equatorial, IP = inferior periphery, SEN = S E nasal, SET = S E temporal, IEN = I E nasal, IET = I E temporal.

Since these animals are phenotypically normal and their tissues exhibit no abnormal storage, it is apparent that the level of ASB activity is high enough to prevent expression of the disease. Therefore, ASB activity must be reduced below some critical level in affected animals before the disease is expressed morphologically.

The regional difference in RPE disease expression in affected animals may be the result of regional differences in residual enzyme activity. That is, higher levels of ASB activity may be present in affected pigmented cells in comparison to nonpigmented cells, resulting in minimal disease expression in the former. Our previous studies demonstrating a deficiency of ASB activity in MPS VI-affected RPE utilized mixed cultures derived from the entire eyecup in which both pigmented and nonpigmented cells were present.[6] This precluded the correlation of residual enzyme activity, disease severity, and pigmentation. The purpose of this study is to further characterize the regional in vitro pathology of MPS VI-affected RPE, and, using cultures derived from defined regions of the eye, to determine if regional differences in residual ASB activity account for the spatial and pigment-related variations in disease severity.

Materials and Methods

Animals

Mucopolysaccharidosis VI-affected (n = 8) and homozygous normal (n = 12) feline eyes were used in this study. The eyes were obtained as previously described from ketamine anesthetized cats, which were subsequently euthanatized.[7] Affected animals were identified on the basis of their characteristic clinical phenotype and deficient ASB activity in peripheral blood leukocytes and/or cultured fibroblasts. The animals were raised and maintained in standard animal care facilities with cyclic illumination. The affected animals ranged in age from 1.3–23 months, while the normals were between 4–6 months of age. As this study examined enzyme activities in different regions of a given eye, it was not necessary to age-match the normal and affected animals. All procedures were performed in full compliance with the ARVO Resolution on the Use of Animals in Research.

Cell Cultures

The technique for initiating feline RPE cultures from defined regions of normal and MPS VI-affected animals has been described in detail previously.[6] Briefly, the eyecup was flattened with several radial cuts. The neuroretina was gently removed and hollow glass cylinders or ovals were placed over the areas to be sampled. The junction between the base of the cylinder and the RPE monolayer was sealed with silicone grease, and the cells within the cylinder rinsed with Puck's saline F. Trypsin (0.25% in Ca^{++}-Mg^{++} free Puck's saline F (pH 8.0)) was introduced into the cylinder. After incubation for 2 hr at 37°C, the cells were released from Bruch's membrane with gentle pipetting, pelleted by centrifugation (50 xg for 5 min) and resuspended in Dulbecco's Minimum Essential Media supplemented with 20% fetal bovine serum, 20 mM glucose, 5 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were seeded at a density of 2×10^5 cells/cm^2 in Falcon multiwell plates and incubated at 37°C in a humidified chamber with a 95% air-5% CO_2 atmosphere. Figure 1 illustrates the regions of the eye sampled. All the studies were performed using primary cultures of cells isolated from these regions.

Microscopy

Cultures of normal and MPS VI-affected RPE cells were examined daily with an inverted phase microscope. At 14 days in culture, representative confluent cultures were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, and processed for light and electron microscopy as previously described.[7]

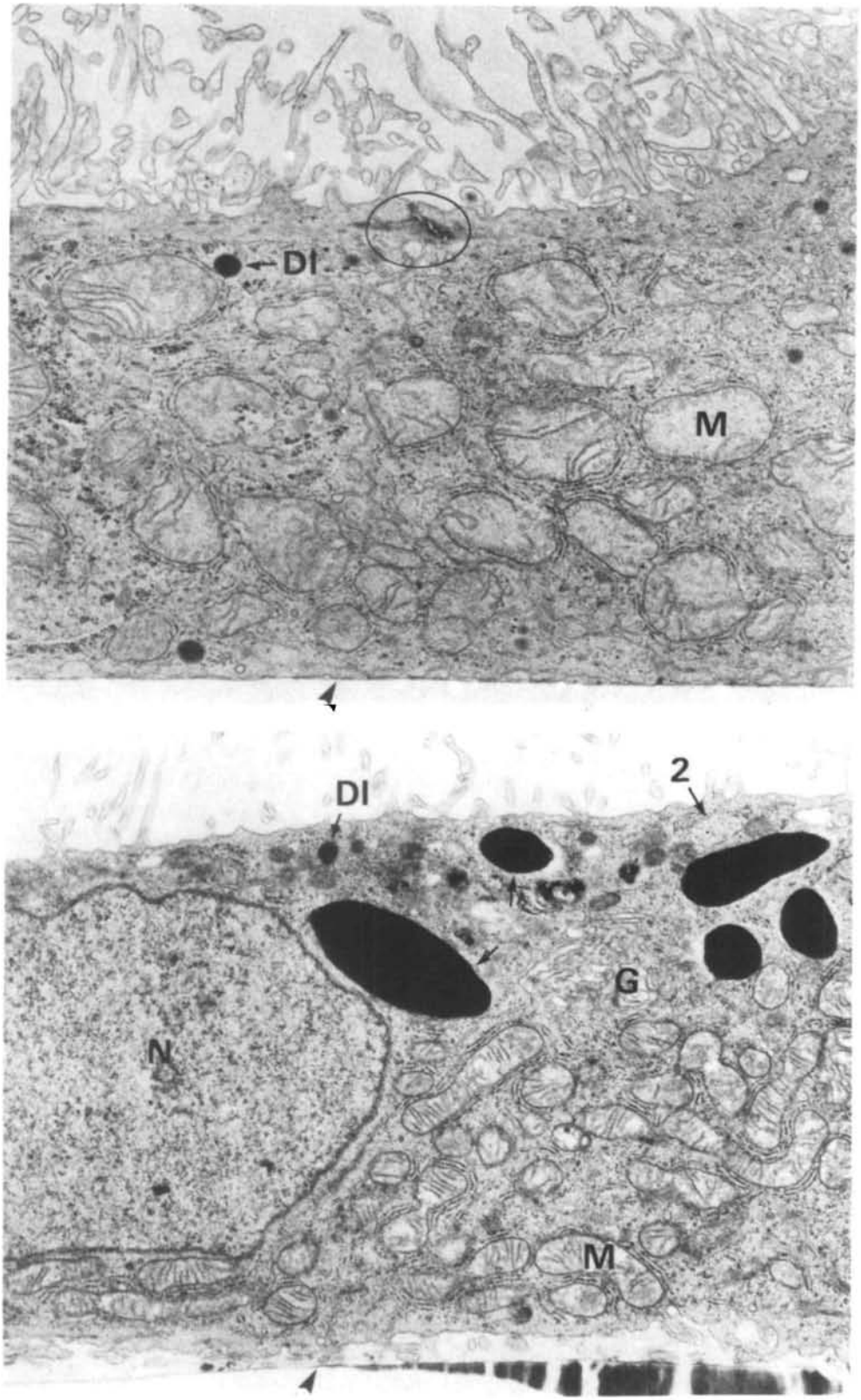


Fig. 2. Normal RPE initiated from nonpigmented superior-equatorial (top) and pigmented inferior-equatorial (bottom) regions of the eye remain well differentiated after 14 days in culture. M = mitochondria, G = Golgi apparatus, small arrows = melanin pigment granule (class 2), DI = electron dense inclusion, 2 = granular inclusion, arrowhead = culture plate surface, circle = apicolateral junction. (Top = ×12,070; Bottom = ×12,208).

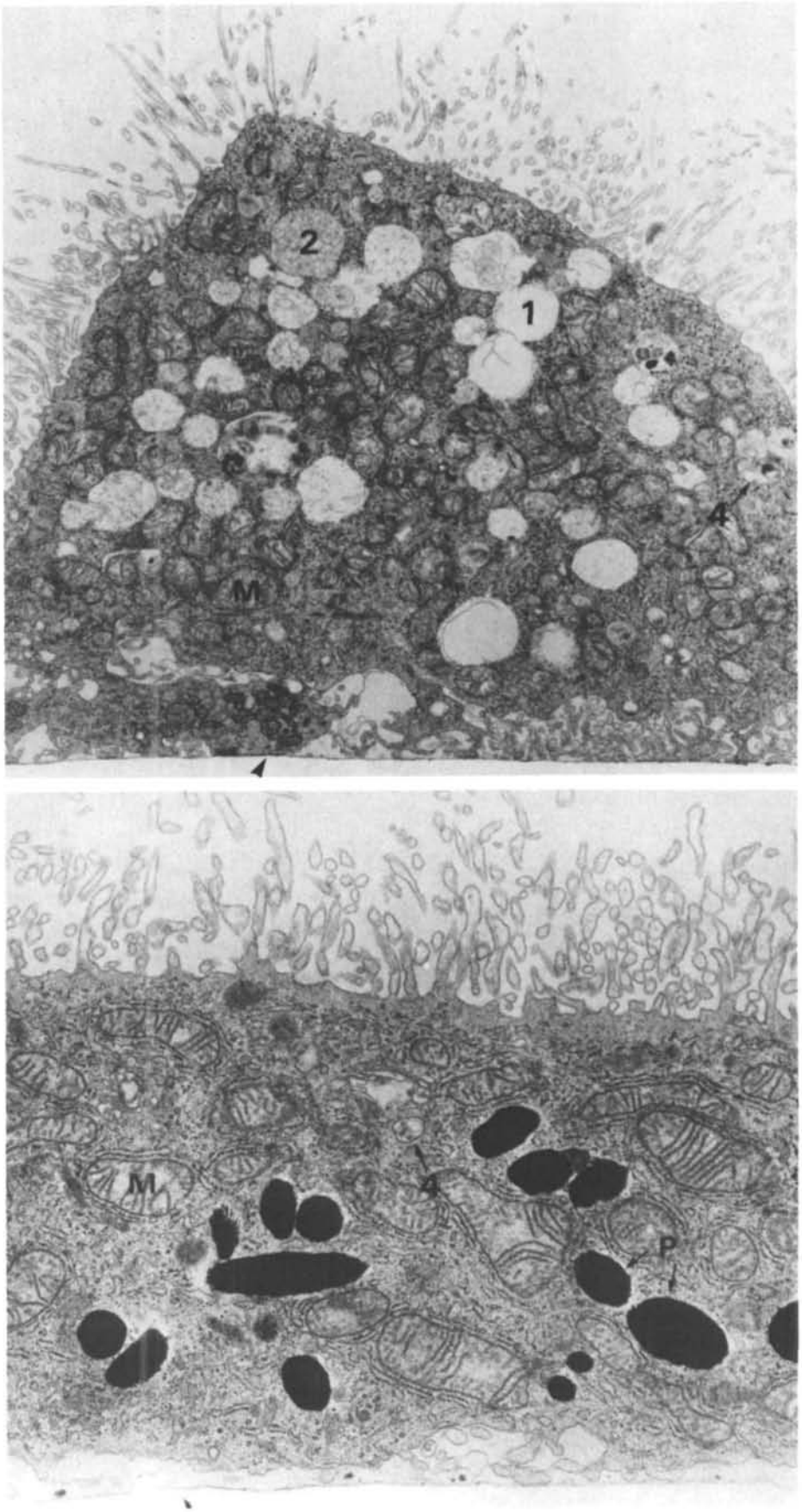


Fig. 3. MPS VI-affected RPE (age = 23 months) initiated from nonpigmented (superior equatorial, top) and pigmented (inferior equatorial, bottom) regions of the same eye after 14 days in culture. Nonpigmented MPS VI-affected RPE cell is hypertrophied and contains numerous single membrane-bound inclusions characteristic of the disease; electron lucent inclusions (1) and inclusions with a granular matrix (2) occupy much of the cytoplasm. Pigmented RPE cell from the same eye is minimally diseased and appears indistinguishable from normal. 4 = inclusion with mixed content, M = mitochondria, P = melanin pigment granule. (Top = ×9,110; Bottom = ×2,010).

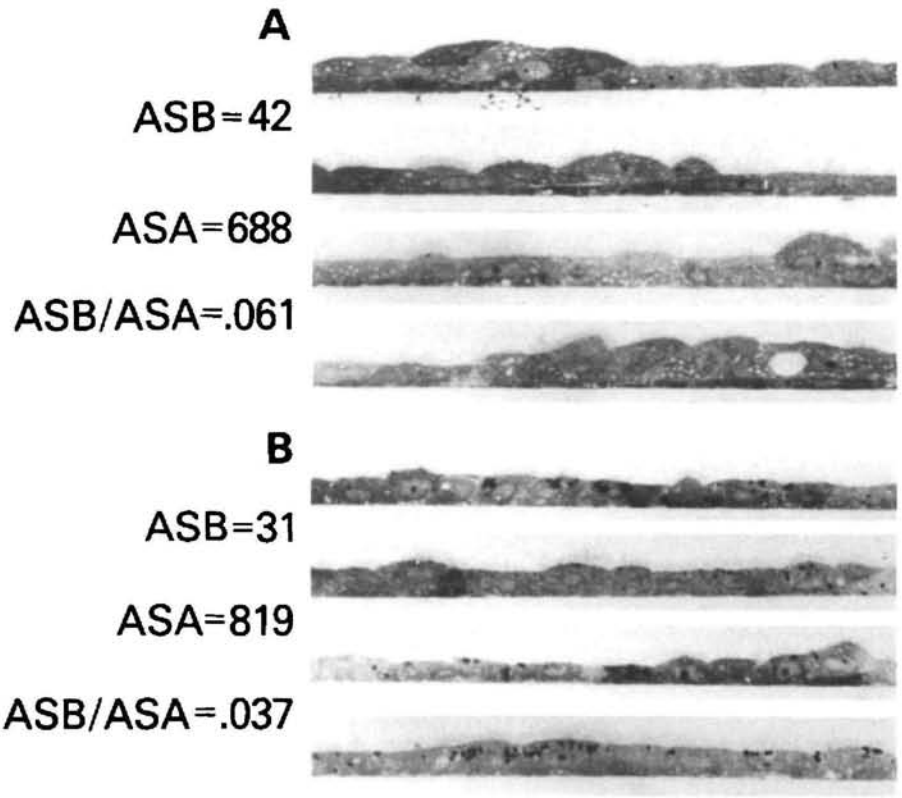
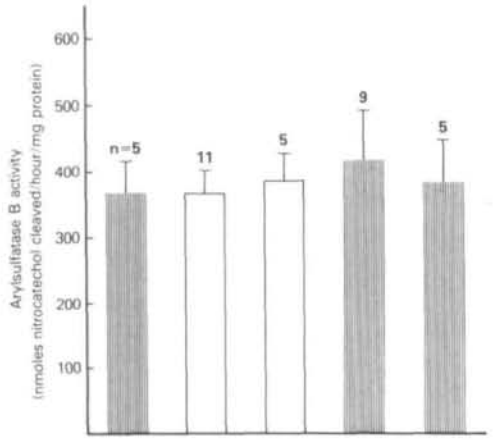


Fig. 4. Fourteen-day old primary culture of MPS VI-affected RPE isolated from defined regions of the same eye of a 1.3-month-old animal. Non-pigmented cells isolated from the superior equatorial region **(A)** contain many small vacuolated inclusions but the cells are not hypertrophied. Pigmented cells from the inferior equatorial region **(B)** are minimally diseased. The residual ASB activity is similar in both regions and to values obtained from adult affected animals. ASB/ASA ratios are uniform and low in all areas sampled. The levels of ASA are within normal levels. (×482).

Enzyme Assays

The ASB activity was measured in confluent cultures of normal and MPS VI-affected RPE after 14 days in culture. As an independent control of lysosomal enzyme activity, the arylsulfatase A (ASA) activity was also measured. The cultures were thoroughly rinsed with 0.85% NaCl and the cells harvested with a rubber policeman. The cells were pelleted by centrifugation (50 xg for 10 min) and were frozen at −70°C until assayed. The cell pellets were lysed in 1.0 ml distilled water with three freeze-thaw cycles. The resulting suspension was centrifuged for 10 min at 10,000 xg, and the supernatant used for analysis.



Regions sampled	Superior peripheral	Superior equatorial	Posterior pole	Inferior equatorial	Inferior peripheral
Cat age	5.0±1.6m	6.0±2.2m	5.1±1.7m	6.2±2.3m	5.1±1.7m
Number of cats	5	6	4	7	4
Number of eyes	5	6	4	7	5

Fig. 5. Arylsulfatase B activity is uniform in normal primary RPE cultures initiated from five defined regions of the eye. Pigmented regions are represented by shaded bars, nonpigmented regions by empty bars. n = number of cultures analyzed, mean ± SEM.

Arylsulfatase A and B activities were determined using a modification of the method described by Baum, et al.[8] The samples were incubated with 10 μM p-nitrocatechol sulfate in 0.5 M sodium acetate buffer (pH 5.6) for 1 hr at 37°C. The reaction was terminated by the addition of 2 N NaOH, and the absorbance of the released p-nitrocatechol was determined spectrophotometrically at 515 nm. Protein content was determined using the Biorad assay.[9]

Results

Morphology

Normal and MPS VI-affected cultures exhibited similar growth characteristics in all regions of the eye sampled. The isolated cells, either singly or in clusters, attached to the culture plate surface by 24 hr and formed confluent monolayers of polygonal cells between 5–7 days. These cultures were stable for several months. The high seeding density employed was effec-

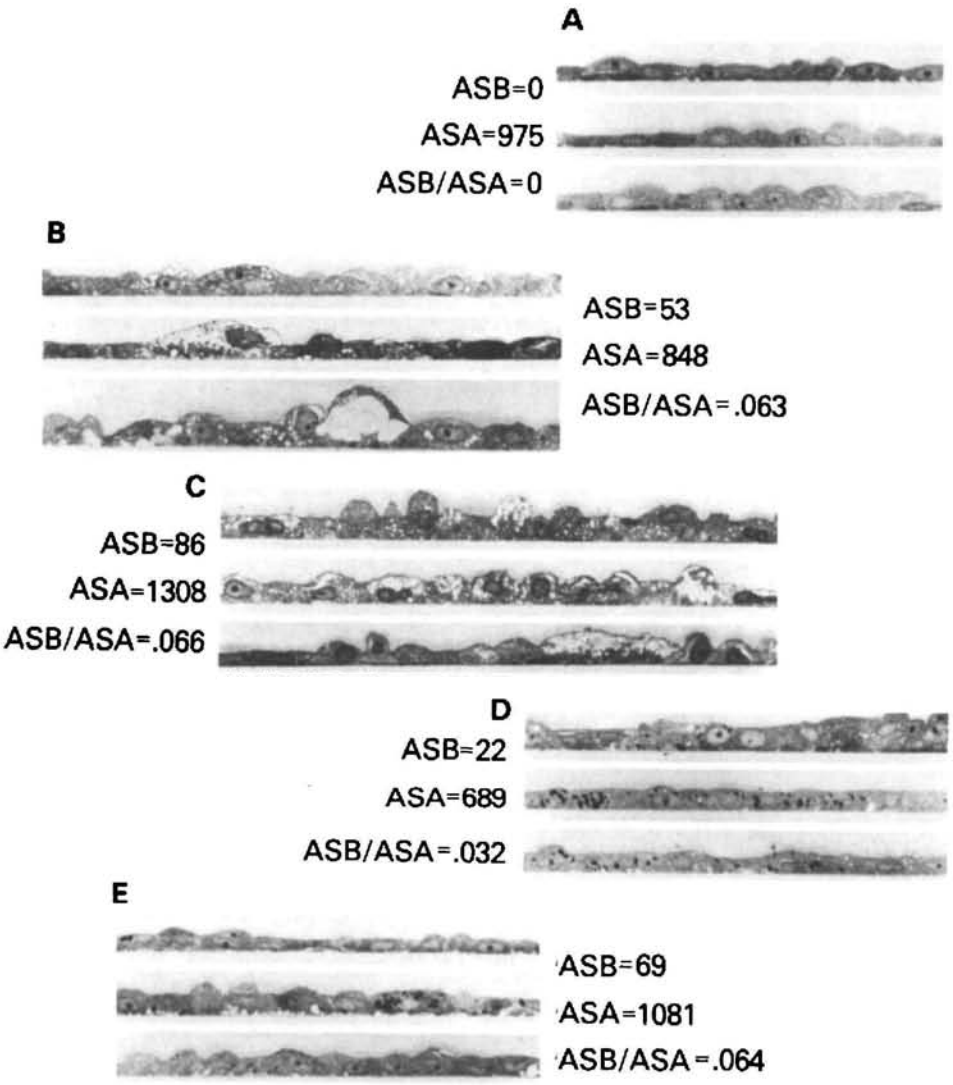


Fig. 6. Fourteen-day-old primary cultures of adult (age 10 months) MPS VI-affected RPE isolated from defined regions of the same eye. Nonpigmented cells are severely diseased (**B** = superior equatorial, **C** = posterior pole), while pigmented cultures are minimally affected (**A** = superior periphery, **D** = inferior equatorial, **E** = inferior periphery). ASB/ASA ratios are uniform and low in all areas sampled. The levels of ASA are within normal levels. The low residual ASB activity is not correlated with disease severity. (×518).

tive in maintaining pigmentation in those cultures initiated from pigmented regions of the eye; cells isolated from nonpigmented regions remained nonpigmented.

Normal RPE cells remained well differentiated in culture (Fig. 2). With the exception of pigment granules, there were no differences between the cells isolated from the various regions of the eye. The cells were polarized with respect to the culture plate surface, with prominent apical microvilli and apicolateral junctions. In addition to abundant numbers of mitochondria, electron dense inclusions and occasional inclusions with a granular matrix were described in these normal cells. The Golgi apparatus was well developed and located in the perinuclear region of the cells.

MPS VI-affected cells isolated from nonpigmented regions (superior equatorial, posterior pole) of adult eyes contained single membrane bound inclusions similar to those described in situ[5] (Fig. 3, top). Most of the inclusions appeared electron lucent. However, inclusions with a homogeneous granular matrix and, to a lesser extent, lamellar and mixed matrices were also present. When numerous, the inclusions resulted in cellular hypertrophy. In contrast, cells isolated from pigmented regions showed little or no accumulation of inclusions (Fig. 3, bottom). Hypertrophy was not observed in these cells. The variation is disease severity in cultures from different regions of the eye was similar to previous histopathologic observations of intact retinal pigment epithelium.[5]

Retinal pigment epithelial cells from a young (1.3 months) affected animal contained fewer vacuolated inclusions than in adult cells, although the same spatial disease distribution was present as observed in older animals. Nonpigmented cells from the superior equatorial region contained more vacuolated inclusions than those from the pigmented inferior equatorial region; however, cellular hypertrophy was not observed in any region (Fig. 4).

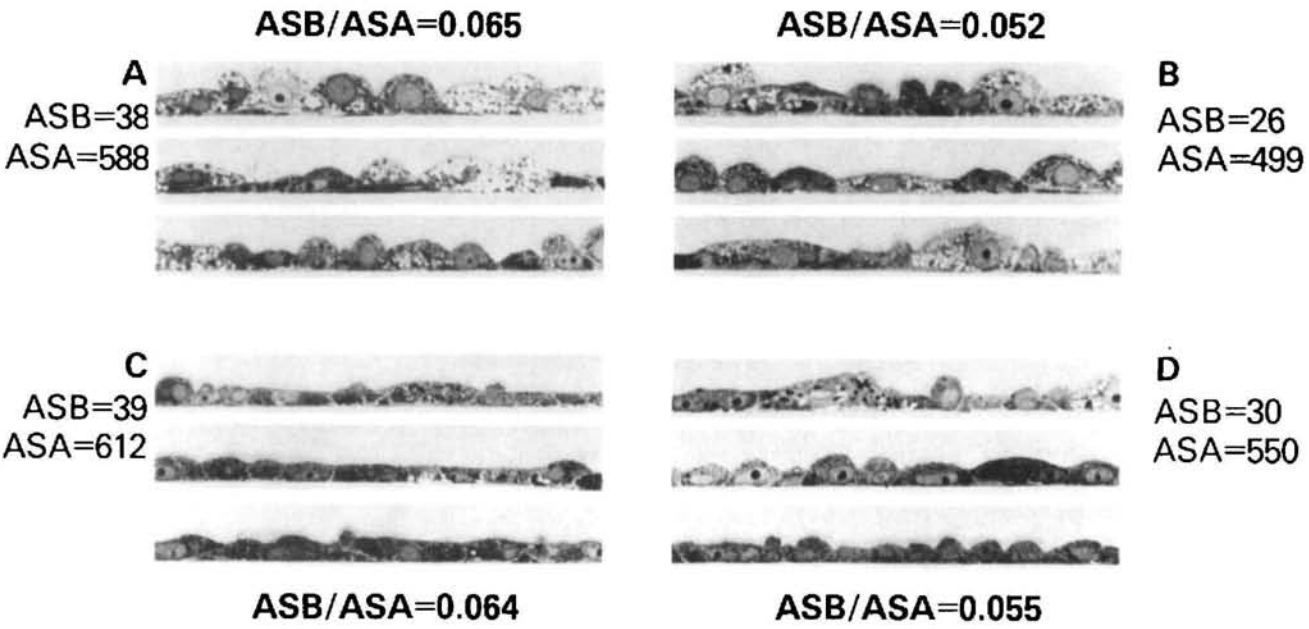


Fig. 7. Fourteen-day-old primary cultures of MPS VI-affected RPE isolated from defined regions of the eye of a 17-month-old animal. Severely diseased nonpigmented cultures (**A** = superior equatorial temporal, **B** = superior equatorial nasal) have similar low residual ASB activity levels as minimally affected pigmented cultures (**C** = inferior equatorial temporal, **D** = inferior equatorial nasal). ASB/ASA ratios are uniform and low in all areas sampled. The levels of ASA are within normal limits. (×585).

Enzyme Activities—Normal Feline RPE

In order to determine if the ASB activity in normal cat RPE was correlated with the spatial position within the eye from which the cells were obtained, or with pigmentation, enzyme activity was measured in 14-day-old cultures initiated from three pigmented regions (superior periphery, inferior equatorial, inferior periphery) and two nonpigmented regions (superior equatorial, posterior pole) of the eye (Figs. 1A, 5). No significant differences in ASB activity existed between any of the regions sampled; normal ASB activity was not correlated with the spatial position from which the cells were obtained or with pigmentation.

Enzyme Activities—MPS VI-Affected Feline RPE

Arylsulfatase B activities were determined in cultures initiated from spatially defined regions of adult (10 and 17 months) MPS VI-affected cats and correlated with the cellular morphology of paired cultures obtained from the same eye (Figs. 6, 7). Arylsulfatase A, another lysosomal enzyme involved in sulfatide metabolism, was measured as a control. The ASB activities were reduced in all regions of the eyes sampled to levels that were approximately 10% of normal activity. Cultures initiated from pigmented regions of the eyes (superior peripheral, inferior equatorial, inferior peripheral) had residual enzyme activities similar to those from nonpigmented regions (superior equatorial, posterior pole). This uniformly low level of ASB activity was present regardless of the disease severity status of the cultures (e.g., compare the severe pathology of the nonpigmented cultures to the minimal or absence of pathology in the pigmented ones). Arylsulfatase A activities were normal in all regions sampled.

The levels of residual enzyme activity present in cultures from young (1.3 months; Fig. 4) MPS VI-affected eyes were similar to those observed in adults. There was no correlation between the levels of residual enzyme activity and the region of the eye sampled, pigmentation, or disease as evidenced by the accumulation of vacuolated inclusions.

Discussion

Feline MPS VI was expressed in cultured RPE cells both morphologically and biochemically. In spite of the enzyme deficiency, the growth characteristics of affected cells remained normal. The expression of the RPE disease in vitro was similar to that observed in situ. In both, the severity of the disease was correlated with the spatial position of the cell within the eye. Nonpigmented cells from the superior equatorial region and the posterior pole contained numerous cytoplasmic inclusions, while pigmented cells from the inferior equatorial and peripheral regions remained normal or minimally affected.

Morphologic expression of the disease in the RPE was also correlated with the age of the animal. Although the same spatial disease distribution and deficient enzyme activity observed in adults was present in the RPE cultures from a young animal, the morphologic

expression of the disease was much less dramatic. Temporal as well as spatial and pigmentation factors, therefore, are also correlated with disease expression in vitro as well as in situ.

The limited amount of RPE material available in situ from spatially defined regions of the eye precluded ASB assays on freshly isolated cells. In vitro techniques overcame this limitation by amplifying the amount of material available for study. Even after this in vitro amplification process, the essential morphologic features of the in situ disease were retained by the primary cultures.

We previously suggested the active role of ASB in RPE metabolism because of the high activities present in these cells when compared to cultured fibroblasts or peripheral blood leukocytes.[7] In the absence of ASB, the massive accumulation of inclusions, probably secondary lysosomes containing undegraded or partially degraded substrate, provided further evidence for the active role of this enzyme in the RPE.

The relationships between ASB activity, pigmentation, and the spatial region from which the cells were obtained were characterized in this study using cultures initiated from either pigmented or nonpigmented regions. In normal RPE cultures, ASB activities were uniform in the five areas of the monolayer sampled. No correlation was found between ASB activities and the presence or absence of pigment. In MPS VI-affected RPE cell cultures, ASB activity was reduced to approximately 10% of normal; this low level is similar to that observed in cultured skin fibroblasts (11%) and peripheral blood leukocytes (6%).[2] Residual enzyme activity in affected RPE cultures was not correlated with the region of the eye from which the cells were obtained, the presence of pigment, the age of the animal, or the severity of the disease in the RPE. In addition, ASB/ASA ratios were uniform and low in all regions sampled. Similarly, low ratios have been found in other MPS VI-affected tissues.[10] Therefore, the regional and temporal variations in disease expression do not result from regional or temporal variations in residual ASB activity. Both severely affected nonpigmented RPE cells and relatively nonaffected pigmented RPE cells from both young and adult animals have similar levels of residual enzyme activity. Spatial differences in GAG production, secretion, and/or uptake may be responsible for the non-uniform disease expression; these possibilities are currently under investigation.

Key words: arylsulfatase B, cat, mucopolysaccharidosis VI, regional studies, retinal pigment epithelium, tissue culture

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