Expression of cell surface transmembrane carbonic anhydrase genes CA9 and CA12 in the human eye: overexpression of CA12 (CAXII) in glaucoma

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Original Article

Purpose: Carbonic anhydrase enzymes (CAs) are universally involved in many fundamental physiological processes, including acid base regulation and fluid formation and movement. In glaucoma patients, CA inhibitors are very effective in lowering intraocular pressure by reducing the rate of aqueous humour secretion mediated by the CAs in the ciliary epithelium. In this work, we investigated the expression and tissue distribution of two recently discovered CA genes (CA9 (CAIX) and CA12 (CAXII)) in fetal, neonatal, and adult human eyes with and without glaucoma.

Methods: CAIX and CAXII expression in 16 normal and 10 glaucomatous eyes, and in cultured non-pigmented ciliary epithelial cells (NPE) from normal and glaucoma eye donors was assessed by immunostaining. In addition, northern blot hybridisation was performed to assess expression of CA4, CA9, and CA12 mRNA in cultured NPE cells from normal and glaucoma donors.

Results: CAIX was localised primarily to the NPE with its expression prominent during embryonic eye development but which decreased significantly in adults. CAIX expression in the NPE was very low. The epithelium of cornea and lens occasionally expressed both enzymes at low levels during development and in adult eye, and no expression was detected in the retina. The NPE from glaucoma eyes expressed higher levels of CAXII, but not CAIX, in comparison with normal eyes. This expression pattern was retained in cultured NPE cell lines. NPE cells from a glaucoma patient showed a five-fold increase in the CA12 mRNA level with no detectable expression of CA9 mRNA. Also, no expression of the CA4 gene encoding a GPI anchored plasma membrane protein was detected on these northern blots.

Conclusions: Transmembrane CAIX and CAXII enzymes are expressed in the ciliary cells and, thus, may be involved in aqueous humour production. CA12 may be a targeted gene in glaucoma.

Material and Methods

Tissue specimens and cultured cells

A total of 26 eyes were collected from the pathology department at UCI Medical Center (Irvine, CA), St Joseph Hospital (Orange, CA), and San Diego Eye Bank (San Diego, CA). Among 26 eyes studied, 16 were normal eyes with no clinical or histological evidence of glaucoma and 10 were glaucomatous. Thirteen of the 16 normal eyes were from donors, and the remainder were enucleated because of extrabulbar tumour (n=2) and trauma (n=1). The 10 glaucomatous eyes were enucleated because of...
immunochemical studies

The mouse monoclonal antibody (MN75) used to detect the MN/CAIX protein and the rabbit polyclonal antibody to CAXII protein have been described previously.14 Immunohistochemical staining of tissue sections and acetone/methanol fixed cultured cells with anti-CAIX and anti-CAXII antibodies was done using a peroxidase technique as described previously.14 Microwave pretreatment was applied to all tissue sections. Known positive and negative tissue specimens were included in each run. For immunostaining, NPE cell subcultures were grown in chamber slides and then fixed in a solution with one part of acetone and one part of methanol.

RNA analysis

For mRNA isolation, NPE cells were grown in DMEM+10% fetal calf serum to confluence and used before passage 13. mRNA isolation from cultured cells, RNA electrophoresis, and Northern blots were done as described previously.28S rRNA levels were used to ensure equivalent loading of mRNA in all lanes.

Table 1 CAIX and CAXII expression in developing, adult, and glaucoma eyes

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<th>Fetal eyes</th>
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<th>Adult eyes</th>
<th>Glaucomatous eyes</th>
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<td>CAIX</td>
<td>CAXII</td>
<td>CAIX</td>
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<td>Non-pigmented epithelium of ciliary body retina</td>
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<td>+/+</td>
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<td>Inner limiting membrane -</td>
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<td>Outer limiting membrane -</td>
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<td>Anterior epithelium</td>
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<td>Posterior endothelium</td>
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<td>Lens epithelium</td>
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± Extremely weak focal staining.

† The positivity is limited to the ora serrata where the retina merges with the non-pigmented ciliary epithelium.

Intraorbital tumour/inflammation (n=3) and clinically diagnosed glaucoma with uncontrolled eye pain and/or blindness (n=7). The donor’s eyes were obtained either immediately after brain death or within 48 hours after death. The age distribution of the glaucoma cases ranged from 57 to 85 years, with one female of 39 years. All of the cases were diagnosed with ACG (angle closure glaucoma). As far as we could ascertain there were no hereditary cases. The donors included fetal eyes (n=5) with a gestational age of 15 to 20 weeks, neonatal/infant eyes (n=5) with an age of 1 day to 18 months, and adult eyes (n=3). All enucleated eyes were fixed in 10% neutral buffer formalin, paraffin embedded, sectioned, and stained with haematoxylin and eosin (H&E) for light microscopic examination. The study was performed with the approval of the ethics committees of each institution involved in this project and, as far as it applies, followed the tenets of the Declaration of Helsinki. Establishment and culturing of human non-pigmented ciliary epithelial cell lines (NPE) from normal and glaucoma eye donors have been described in detail previously.15 For immunostaining, NPE cell subcultures were grown in chamber slides and then fixed in a solution with one part of acetone and one part of methanol.

RESULTS AND DISCUSSION

To identify the CAs expressed in the ciliary epithelium, we first analysed the expression of the CAIX and CAXII enzymes by immunostaining tissue sections of 26 normal and glaucomatous eyes. Microscopically, all eyes from donors had normal histology. Three non-glaucomatous eyes enucleated for extraorbital tumour/trauma also contained well preserved ciliary bodies with open angles and relatively unremarkable cornea, lens, choroid plexus, retina, sclera, and optic nerve. All glaucomatous eyes (n=10) had closed angles. Among these, two were associated with an orbital tumour and one was the result of inflammation. The rest were from patients with a clinical diagnosis of angle closure glaucoma (ACG) with no known associated disease. Histological sections of the glaucomatous eyes showed the formation of peripheral anterior synchiae, fibrosis, and degeneration of meshwork. Optic atrophy and variable degrees of degeneration of cornea and retina were observed in all glaucomatous eyes.

In normal developing eyes (gestational age ranging from 15 weeks to 20 weeks), the non-pigmented ciliary epithelium, the corneal epithelium and endothelium, and the lens epithelium expressed both CAIX and CAXII (table 1). While expression of CAXII was prominent, the expression of CAIX in the ciliary epithelium was weak and the positive staining was limited to a few epithelial cells (fig 1A, B). In contrast, very low levels of CAIX, but no CAIX immunoreactivity, were observed along the inner membrane (the terminations of the processes of Muller’s cells) of the retina near the ora serrata. After birth the inner membrane of the retina no longer expressed CAIX and the intensity of CAXII immunoreactivity in the epithelium of the cornea, lens, and ciliary body was decreased. In adult eyes there was a persistent expression of CAIX in the non-pigmented epithelium of the ciliary body but the levels of expression were significantly decreased when the intensity of staining was compared with the developing eyes. In addition, the positive immunostaining was focal. In contrast, there was no CAIX immunoreactivity detected in the ciliary epithelial cells in the adult globes (fig 1C, D). The epithelium of the cornea and lens occasionally expressed CAIX and CAXII but the intensity of positive immunostaining was extremely weak. There was no CAIX/CAXII immunostaining in the retina.

In glaucomatous eyes, variable degrees of CAIX/CAXII expression were observed in the epithelium of cornea and lens but the positive immunoreactivity was weak and focal. The most striking finding was high levels of CAXII, but no CAIX expression in the non-pigmented ciliary epithelium (fig 1E, F). The positive CAXII immunostaining was diffuse and the intensity of staining was moderate to strong. Clearly, CAXII expression was preferentially seen in the NPE cells. However, the high pigmentation observed in the pigmented layer
precluded precise quantification of CAXII expression in the NPE cells of the ciliary bilayer. Another interesting observation was the expression of CAIX but not CAXII in the proliferative neuroglial cells of the retina in which there was severe loss of inner and outer nuclear layers. The positive staining was either focal or diffuse. A summary of the distribution of the expression of CAIX/CAXII in developing eyes before and after birth, adult donor/non-glaucomatous, and glaucomatous eyes is given in table 1.

We next determined whether these patterns of expression would be preserved in cultured NPE cells. The cell cultures were grown in the chamber slides and immunostained. In the normal cultured NPE cells, limited numbers of cells showed weak CAXII immunoreactivity. Even fewer cells expressed CAIX, although the level of immunostaining was somewhat stronger than CAXII (fig 1G, H). In contrast, the ciliary NPE cells from the glaucomatous eye showed high levels of CAXII expression consistent with the data obtained with the immunostained eye sections. There was a significant increase in the numbers of cell stained and in the intensity of immunostaining. However, CAIX immunoreactivity was no longer detected in the NPE cells derived from the glaucomatous eye (fig 1I, J).

We then examined the expression of these genes by northern blot analysis of mRNA isolated from these, normal (2 year old), and glaucomatous ciliary NPE cells grown to confluence (fig 2). They showed relatively strong CA9 and CA12 signals on northern blots from a normal subject while, in contrast, NPE cells from a glaucoma patient showed high (five times) overexpression levels of the CA12 mRNA with no detectable expression of CA9, consistent with the immunostaining data (table 1). In this experiment we also confirmed the absence of expression of the CA4 gene as was shown previously by immunostaining of eye sections with specific CAIV antibodies. Thus, the assumed involvement of this enzyme in the ciliary epithelium was not corroborated by our experiments.

Figure 1 Examples of immunostaining of CAIX and CAXII proteins in non-pigmented epithelium of the ciliary body of developing (19 weeks’ gestational age), adult, and glaucomatous eyes (A-F) and of ciliary non-pigmented epithelial cultured cells of normal and glaucomatous eyes (G-J). CAXII is illustrated in A, C, E, G, and I. Immunostaining for CAIX is illustrated in B, D, F, H, and J. Diffuse immunoreactivity for CAXII and focal, very weak positivity for CAIX is seen in the developing eyes (A, B, arrows). In the fully developed eyes (adult) CAXII positive staining is weak and limited to a few cells; no CAIX immunoreactivity is seen (C, arrows, D). In contrast, high levels of CAXII expression is seen particularly in the non-pigmented ciliary epithelial cells of the glaucomatous eyes with no expression of CAIX (E, F). CAIX/CAXII expression is also seen in the cultured normal NPE cells (G, arrows, H). In the cultured glaucoma ciliary cells the intensity of CAXII immunostaining is much stronger with staining seen both in the cytoplasm and the plasma membrane. Conversely, no expression of CAIX was seen (I, J).
In the present work, we have established the nature of the CA9 and CA12 and CA4 expression in cultures of human ciliary non-pigmented epithelial cell lines from a glaucoma patient (GCE-1, passage 12), a normal subject (ODM-C4, passage 9), and, as a positive control, from the renal carcinoma cell line 786-O. 28S RNA levels indicate equivalent loading of mRNA in all lanes. As expected, strong positive hybridization with the CA4 probe (1.35 kb band) was obtained with many human tissues on MTN 7760-1 (Clonetech, Palo Alto, CA, USA) northern blots (data not shown).

Figure 2  Northern blot analysis of CA9, CA12, and CA4 expression in cultures of human ciliary non-pigmented epithelial cell lines from a glaucoma patient (GCE-1, passage 12), a normal subject (ODM-C4, passage 9), and, as a positive control, from the renal carcinoma cell line 786-O. 28S RNA levels indicate equivalent loading of mRNA in all lanes. As expected, strong positive hybridization with the CA4 probe (1.35 kb band) was obtained with many human tissues on MTN 7760-1 (Clonetech, Palo Alto, CA, USA) northern blots (data not shown).

it should also impact on the quest for more selective topical inhibitors of CAXII for the treatment of glaucoma. We have recently identified novel sulphonamide inhibitors selectively inhibiting CAXII or CAIX using purified recombinant CAXII and CAIX enzymes (F Jurnak et al, in preparation).

ACKNOWLEDGEMENTS
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REFERENCES
Similar hereditary motor neuropathies are not allelic disorders

A study in two families has suggested that different forms of peroneal muscular atrophy with vocal chord paralysis are caused by a separate gene or genes and not by an allele of the gene predisposing to one form—distal hereditary motor neuropathy type VII (dHMN-VII).

Both families had a phenotypically similar condition to dHMN-VII. The occurrence of disease among affected and unaffected family members, however, did not fit with the pattern of inheritance of the \textit{DHMNVP} gene, which is responsible for dHMN-VII and maps to chromosome 2q14.

In one family with hereditary motor and sensory neuropathy type II (HMSN-IIC) one affected twin and one unaffected twin had the same haplotype of chromosome 2q14 from their affected mother. Two point LOD scores between the disease and markers of the \textit{DHMNVP} gene were all negative. In the other family, with vocal cord paralysis and sensorineural deafness and distal muscle atrophy, two affected siblings had inherited opposite haplotypes of chromosome 2q14 from their affected mother. Again, LOD scores were all negative.

The two selected families had neurological and electrophysiological examinations, and their DNA was tested for linkage to the \textit{DHMNVP} gene with 10 microsatellite markers spanning the gene. The researchers had already shown that the \textit{DHMNVP} gene on chromosome 2q14 predisposed to dHMN-VII and sought to test the suggestion by others that this condition and HMSN-IIC might be allelic disorders.

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