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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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.

Carbonic anhydrase enzymes (CAs) are universally involved in many fundamental physiological processes, including acid base regulation and fluid formation and movement. The involvement of carbonic anhydrases (CA) in the aqueous humour secretion by the ocular ciliary epithelium was suggested by several types of experiments and directly supported by clinical observations of the efficacy in reducing raised intraocular pressure (IOP) in glaucoma by inhibitors of carbonic anhydrase (CAinh). The clinical efficacy of CAinh plus some experimental data with isolated ciliary epithelial cells in vitro suggested both cytosolic and membrane CAs mediate these effects to reduce aqueous inflow in the eye. Previously, cytosolic (CAII) and membrane GPI anchored (CAIV) enzymes were discovered in different anatomical and cellular sites of the eye. It was suggested they play a fundamental role in shifting protons and bicarbonate and consequently other solutes across membranes, thereby regulating acid base homeostasis and fluid movements. However, the CAIV enzyme was not found in the ciliary epithelium, suggesting that other membrane associated CAs might be involved in aqueous humour secretion and movements.

Recently, we and others discovered a novel class of cell surface transmembrane carbonic anhydrase genes, CA9(10) and CA12(11,12) and showed their expression in a variety of specialised adult normal human tissues and ubiquitous overexpression/induction in human cancers. In a follow up study, we examined the expression and distribution of these proteins by immunostaining tissue sections from human eye donors. In addition, we performed a northern blot analysis of cultured ciliary NPE cells(13) in order to measure CA4, CA9, and CA12 mRNA levels in these cell populations.

Here we show, by immunostaining with specific antibodies, the expression of CAIX and CAXII cell surface transmembrane proteins in different anatomical structures of the human eye, namely, in embryonic, neonatal/infant, and adult eyes, under normal and pathological conditions including angle closure glaucoma. In addition, by northern blot analysis we show the expression of these genes in cultured human ciliary non-pigmented epithelial (NPE) cells from normal and glaucomatous eyes.

**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 extraorbital tumour (n=2) and trauma (n=1). The 10 glaucomatous eyes were enucleated because of...
The mouse monoclonal antibody (MN75) used to detect the CAIX and CAXII expression in developing, adult, and glaucoma eyes.

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

± Extremely weak focal staining.

CAIX and CAXII expression in developing, adult, and glaucoma 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. However, in adult eyes and glaucomatous eyes, variable degrees of CAIX/CAXII expression were significantly decreased when the intensity of positive immunostaining 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.
In the present work, we have established the nature of the CAs expressed and most likely involved in the development and function of the ciliary epithelium and shed new light on the molecular pathogenesis of glaucoma. Glaucoma is a complex, relatively common (affects over 67 million people worldwide) eye disorder, characterised by clinical and genetic heterogeneity. The mode of transmission is complex and involves multiple causative and modifying susceptibility genes. To date, at least 10 loci that potentially confer susceptibility to glaucoma have been mapped and so far only two causative candidate genes have been identified. Previously, it was assumed by many working on the aqueous humour production that CAIV is the crucial membrane enzyme involved in the ciliary process. Contrary to this belief, we establish here that the novel cell surface bitopic transmembrane enzymes CAIX and CAXII are expressed in the ciliary cells and most likely play an important role in aqueous humour production. Classical theories of glaucoma have focused primarily on the assumption that faulty facilities governing the outflow system may underlie the physical and genetic causes of this multifaceted disease. Here, we show for the first time the overexpression of the CAXII enzyme in the ciliary NPE cells of glaucoma patients, suggesting that some glaucomas might result from overproduction of the aqueous humour solely or in combination with damage to the outflow facility. The silencing of the CA9 gene in adult eyes (probably by hypermethylation) could lead in some people to overexpression of the CA12 gene in the ciliary NPE cells, which may in turn cause overproduction of the aqueous humour and subsequently high intraocular pressure and hence lead to glaucoma. Furthermore, it is also possible that the overexpression of CAXII in glaucoma patients may be caused by mutated allele(s) of this gene. Clearly, the overexpression of CA12 could be a diagnostic marker for certain types of glaucomatous ciliary NPE cells and may provide a framework for a better understanding of the fluid equilibrium in the eye. In clinical terms 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 Jurtner et al, in preparation).

ACKNOWLEDGEMENTS

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REFERENCES

Overexpression of CA12 in glaucoma

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 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 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 DHMNVP gene with 10 microsatellite markers spanning the gene. The researchers had already shown that the 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.