INTRODUCTION

Cytochrome P450s (CYP450s) are a multigene family of enzymes involved in oxidative metabolic activation and detoxification of many compounds. CYP450s act on endogenous substrates introducing oxidative, peroxidative and reductive changes into small molecules of various chemical structures. CYP450s also metabolize exogenous drugs, environmental chemicals and pollutants, resulting in either detoxification or generation of toxic metabolites which contribute to cancer risks and/or other toxic effects. The best example of such a role lies in Cyp1b1, where deleterious mutations result in abnormal development of the trabecular meshwork (TM) in both mouse and human eyes. Human Cyp1b1 consists of three exons (371, 1044 and 3707 bp in length) and two introns (390 and 3032 bp) spanning 8.5 kb of genomic DNA (GenBank accession no. U56438). Its coding region starts at the 5'-end of the second exon and ends with the last exon. Conservatively, mouse Cyp1b1 gene exhibits a structure with three exons and two introns as well. Its exon1 is composed of 371 bp. The open reading frame (1.63 kb) is encoded by exon 2 (starting at the second nucleotide; functional group (ROH) is consequently created, while the other oxygen atom is reduced to water.

Conserved expression of most CYP orthologs strongly supports similar evolutionary conserved functions in mice and humans during development. The best example of such a role lies in Cyp1b1, where deleterious mutations result in abnormal development of the trabecular meshwork (TM) in both mouse and human eyes. Human Cyp1b1 consists of three exons (371, 1044 and 3707 bp in length) and two introns (390 and 3032 bp) spanning 8.5 kb of genomic DNA (GenBank accession no. U56438). Its coding region starts at the 5'-end of the second exon and ends with the last exon. Conservatively, mouse Cyp1b1 gene exhibits a structure with three exons and two introns as well. Its exon1 is composed of 371 bp. The open reading frame (1.63 kb) is encoded by exon 2 (starting at the second nucleotide;
In humans, Cyp1b1 is expressed in many adult and fetal extrahepatic tissues, including most of the parenchymal and stromal tissues from brain, kidney, prostate, breast, cervix, uterus, ovary, lymph nodes, heart, placenta, lung and skeletal muscle. Cyp1b1 is also detected in ocular tissues. However, it is barely detected in the liver. In mice, Cyp1b1 also exhibits a tissue specific pattern of expression. During early stages of murine development Cyp1b1 mRNA was detected in the developing eye, hindbrain, branchial arches and forelimb bud. Constitutive Cyp1b1 expression is seen in endocrine-regulated tissues such as mammary gland, uterus and prostate. Cyp1b1 is not constitutively expressed in rodent lung, liver or kidney.

Human Cyp1b1 and mouse Cyp1b1 is believed to be important in ocular development and function due to its conserved expression in both murine and human eyes. Its expression level is also higher in fetal than adult eyes, and it is differentially expressed throughout the eye. Also, the relative transcript level of human Cyp1b1 and mouse Cyp1b1 in the eye is fivefold higher than other P450s further supporting the importance of Cyp1b1 in normal eye development.

**Cyp1b1 AND DEVELOPMENT OF THE ANTERIOR CHAMBER**

The stages of fetal and postnatal development of the human anterior chamber have been determined. By the 15th fetal week, the main anlage of the presumptive chamber angle tissues are formed and separated into corneoscleral and ciliary-iris regions. Around the 22nd to 24th fetal weeks, ciliary muscle and ciliary processes develop. As the prospective Schlemm’s canal grows, endothelial “vacuoles” appear within its lining endothelium. The corneoscleral trabeculae undergo a longitudinal orientation with deposition of collagen in the trabecular cores and formation of trabecular sheets and the iris is elongated. From the 24th to the 36th fetal weeks, more specialized tissue development occurs. The ciliary muscle becomes a distinct unit and the ciliary processes are enlarged. The corneal trabeculae enlarge with trabecular sheets under differentiation, and the uveal trabeculara form cords and sheets. Endothelial “vacuoles” become present in both the inner and outer walls of Schlemm’s canal. At birth, the anterior chamber tissues have developed almost completely. Trabecular sheets are enlarged in the corneal, but the uveal meshwork undergoes collagen formation and intracellular differentiation. Schlemm’s canal is longitudinal and shift anteriorly with endothelial “vacuoles” confined to the inner wall. During postnatal years 1-4, the chamber angle reaches the configuration of an adult eye. Further formation of collagen fibers and intracellular differentiation are seen in the ciliary body, TM and Schlemm’s canal. The juxtacanalicular meshwork becomes distinguished with its narrow inter-trabecular spaces and small trabecular sheets.

In general, human Cyp1b1 expression is of much higher level in fetuses than in adults. In detail, Cyp1b1 has been detected in the fetal primitive ciliary epithelium as early as 26 days post-conception in human embryos, much earlier than anterior chamber development. Cyp1b1 staining in the corneal epithelium, keratocytes and iris stromal cells is positive in fetal but not in adult eyes. Intense intracytoplasmic Cyp1b1 staining is observed in the non-pigmented ciliary epithelium, iris pigmented epithelium and iris dilator muscle both in fetuses and adults. However, Cyp1b1 could be detected in either fetal or adult TM.

Development of the murine eye occurs relatively late in gestation. On embryonic day 15 (E15), the anterior space is formed. Some perioptic mesenchymal tissues extend into the anterior chamber to form the pupillary membrane, and the rest of the mesenchyme resides at the margin of the optic vesicle to form the future TM, the stroma of the iris and the ciliary body. The ciliary body and iris have not yet formed by E17.5. After then, the rudimentary ciliary body begins to grow out of the marginal neural retina and pigment epithelium. Most of the anterior chamber structure develops postnatally. In the mouse eye, on postnatal day 1 (P1), the ciliary process slightly projects anteriorly to the lens. From P1 to P2, a faster proliferation of the outer ciliary epithelium initiates the formation of the ciliary processes, resulting in a discrete ciliary body by P7 consisting of inner (non-pigmented) and outer (pigmented) ciliary epithelium. By P7 the iridocorneal angle is formed, although many developmental changes continue to occur. Trabecular beams are immature with minimal inter-trabecular spaces on P10-12. By P14, TM beams are mature with abundant extracellular matrix proteins. Intertrabecular spaces are anteriorly open but are minimally open posteriorly. The angle recess becomes deep, and Schlemm’s canal becomes mature with giant vacuoles. By P18-21 major morphogenesis is finished and TM development is completed by P35 to P42.

Different from the age-related decrease of human Cyp1b1 in human eyes, mouse Cyp1b1 staining during fetal development is much lower than adult eyes. On E12.5 Cyp1b1 protein in ocular tissues is not consistently detectable. It is detected diffusely in the lens epithelium influencing the development of the adjacent ciliary body on E15.5. It is much more strongly expressed later during E17.5 to P7. After P7, it declines to low levels. Before E17.5, Cyp1b1 is barely detectable in the inner ciliary epithelium, however, postnatally, it increases rapidly, reaching adult levels by P28. In contrast, Cyp1b1 mRNA is expressed in the outer/pigmented ciliary epithelium.
at P4 and remains positive during eye development.\[22\] On P14, the levels of Cyp1b1 protein appear to parallel development of the ciliary body following the appearance of ciliary processes.\[18\]

The above-mentioned observations suggest that Cyp1b1 protein expression in the eye appears earlier than anterior chamber development in both humans (E15)\[19\] and mice (E15-E16.5).\[22\] In fact, Cyp1b1 is the first gene in the CYP450 family in which mutations are demonstrated to be involved in a primary developmental defect in humans.\[8\] Consistently, it has been reported that loss of Cyp1b1 activity results in similar developmental defects of the TM in mice.\[7\] Moreover, CYP1B1/Cyp1b1 orthologs from human and mice are of high sequence identity.\[6\] Cyp1b1 1 amino acid sequences are 81% similar among humans, rats and mice,\[4\] suggesting the importance of this gene in ontogeny. All these facts suggest that Cyp1b1 expression plays a key role in the normal development of TM tissue.\[20\]

Human Cyp1b1 mRNA has been detected in an adult human TM cDNA library\[20\] and a human TM cell line.\[19\] A recent study in our group also revealed positive CYP1B1/Cyp1b1 protein staining in human and mouse TM cells.\[28\] However, other studies found human Cyp1b1 and murine Cyp1b1 protein as well as mouse Cyp1b1 mRNA to be absent in normal fetal and adult human\[20\] or mouse TM tissue.\[18,22\] These low levels of Cyp1b1 expression in TM tissue in vivo may make its detection difficult.\[18,20,22\] In contrast to the small quantities of Cyp1b1 within the TM, abundant CYP1B1/Cyp1b1 1 protein is shown in the ciliary body, which is responsible for aqueous humor (AH) formation.\[22\] Cyp1b1 may generate biologically active metabolites in situ in the ciliary body, which are then secreted into the AH and diffuse to the TM tissue.\[20\] This notion is supported by studies that suggest proper embryonic TM development may be disrupted due to lack of crucial metabolites from ciliary body in individuals carrying Cyp1b1 mutations.\[6,20\]

**Cyp1b1 MUTATIONS AND GLAUCOMA**

Glaucoma, the second leading cause of blindness worldwide, is a type of optic neuropathy leading to progressive damage of the optic nerve together with visual field defects which can further result in loss of central vision if left untreated.\[27\] It is categorized according to the etiology (primary and secondary), anatomy of the anterior chamber (open versus closed angle), and the time of onset (congenital, infantile, juvenile and adult).\[28\] Under the category of primary developmental glaucoma, primary congenital glaucoma (PCG) is reserved for cases in whom the major observable anatomical defect is trabeculodysgenesis,\[29,30\] that is, a malformation of the TM. The TM is an ocular tissue around the base of the cornea and near the ciliary body which is responsible for draining AH, a transparent fluid from the anterior chamber and maintaining homeostasis of intraocular pressure (IOP). Failure of proper development and differentiation of TM leads to decreased AH outflow and increased IOP in PCG patients.

PCG is the most common form of glaucoma in infants\[17,19,31\] with prevalence varying geographically from a rate of 1:10,000 to 1:20,000 in Western countries\[32\] and 1:2500 in the Middle East\[8\] to 1:1250 in the Gypsy subpopulation of Slovakia.\[33\] It is familial in 10-40% of cases, with an autosomal recessive trait with high (73-100%) penetrance.\[30\] Molecular screening of the PCG families linked to the 2p21 locus has determined that mutations in Cyp1b1 are responsible.\[34-36\]

All CYP450s share a set of conserved structural elements including 4 helix bundles (D-, I-, L-and the antiparallel E-helices), J-helix, K-helix, beta-sheets 1 and 2, the “meander” region and the heme-binding region.\[12\] As shown in Figure 1a, modified from Sarfarazi and Stoilov,\[30\] Cyp1b1 has a 53 residue long membrane-spanning domain located at the N-terminal of the molecule, a 10 residue proline-rich “hinge” region permitting flexibility between the membrane-spanning and cytoplasmic domain of the molecule, and a 480 residue long cytosolic globular domain which includes a substrate binding region and highly conserved core structures (CCSs) composed of J-helix, K-helix and heme-binding regions [Figure 1b].\[30-37\]

Cyp1b1 mutations occur in 87% of familial and 27% of sporadic cases of PCG worldwide.\[39\] Five major groups of mutations in Cyp1b1 have been found in PCG: Missense mutations, frame shift or truncating mutations,
mutations triggering the nonsense-mediated mRNA decay of Cyp1b1,[30] and mutations in the promoter or control regions of the gene.[38]

Critical missense mutations are located either at the N-terminus hinge region or C-terminus CCSs [Figure 1c]. They are expected to interfere with fundamental properties of Cyp1b1 including proper folding, heme binding, substrate accommodation with the reductase and/or the ability to transfer electrons, thus impairing enzyme functions.[30] Molecular dynamics simulations further confirm the potential of Cyp1b1 mutations in structural alterations of missense mutations found with high frequency in patients with PCG.[37]

W57C affects the position that precedes the last proline residue in the proline-rich hinge. It can impair heme incorporation and reduce enzyme activity by interfering with proper protein folding of Cyp1b1.[12] The G61 is one of the most highly conserved positions in the hinge region,[12] with G61E changing the three-dimensional folding basis of the Cyp1b1 molecule. The different chemical properties of the substituted amino acids also impair heme binding.[36] G61E is one of the three major Cyp1b1 mutations in Saudi Arabian PCG patients.[39]

A115P is within the B/B’ loop at the C-terminal end of B-helix and is close to the heme-binding region. This mutation restricts conformational freedom of the site with a larger substituent from P, and precludes hydrogen-binding interactions due to the absence of amino hydrogen.[37] M132R right in the heme-binding region harms the local H-bond interactions and congests the packing of the heme-binding loop.[37] Q144P located at the crucial junction of the I-, C- and B’/C-helix breaks the continuity of the C-helix, and thus impairs the structure of the heme-binding region.[37]

P193L in the N-cap position of E-helix affects the stability of the E-helix and consequent packing in this region.[37] E229K in the vicinity of substrate binding region affects the local charge distribution and disturbs essential salt bridges.[37] It also disrupts the three-dimensional structures of E- and F-helices leading to severe conformational changes in the mutant protein.[36] S239R located in the F/G loop region is close to the substrate access channel and disrupts the dynamics of substrate access.[37]

G365W affects the orientation of J-helix[12] of the CCS responsible for active heme binding. R368H located in between the J- and K-helices weakens the bonds among other close amino acids.[37] It impairs the three-dimensional folding basis of the K-helix of the CCS.[36] As E387 and R390 are landmark residues which are absolutely invariant among all members of CYP450 family,[12] E387K, R390H and R390C disrupt the orientation of K-helix[12] and critically interfere with the formation of stable hemo-protein complex. D374N and P379L are in close proximity to these crucial residues and exert their negative effect in a similar fashion. Actually, D374N is one of the three major Cyp1b1 mutations in Saudi Arabian PCG patients,[39] and all patients with R390C homozygous mutation show very severe phenotype with poor prognosis.[38]

P437L and R444Q impair the structural integrity of the conserved meander region which precedes the heme-binding region.[12,40] G466 and C470 are conserved in all CYP450 proteins to accommodate the limited volume between the heme-binding loop and B-helix. G466D interferes with the binding of the reductase protein.[57] The large and rigid side of W in R469W precedes the invariant C470 and impairs the conformation of the conserved cysteine pocket.[12] It is also one of the three major Cyp1b1 mutations in the Saudi Arabians PCG patients.[39]

Other critical mutations involve frame shift mutations in the Cyp1b1 open reading frame that introduces premature stop codons upstream of the coding sequences for conserved regions and eliminates multiple essential domains of Cyp1b1. In a cohort study on 146 PCG patients among whom 45 subjects were detected with Cyp1b1 mutation, the deleterious effect of frame shift mutations on the protein function resulted in 100% blindness.[39]

In conclusion, Cyp1b1 mutations identified in patients with PCG most probably interfere with the structures of the heme-binding region, the hinge or CCS region of the Cyp1b1 (12). Different combinations of Cyp1b1 mutations may produce various degrees of histologically observed structural alterations in the TM and Schlemm’s Canal[36] that closely correlate with the severity of disease and the difficulty in achieving IOP control.[56,38] At least part of pharmacological treatments for PCG should be focused on supplementing nutritional molecules, for instance, anti-oxidants, which could have been potentially lost due to lack of Cyp1b1 metabolic activity.

OXIDATIVE STRESS AND GLAUCOMA

Oxidative stress early in development may cause trabecular dysgenesis and result in PCG.[31] AH contains H2O2 and superoxide anion[41] which impose oxidative stress on the TM and contribute to the onset of pathogenic alterations in the TM. Ascorbic acid is the main defense mechanism involved in ocular protection to remove H2O2. The concentration of ascorbic acid is about 1 mM in the AH of diurnal mammals, a relatively high concentration that is 20-50 times greater than that in the plasma.[42] Other antioxidants such as glutathione (GSH) and superoxide dismutase (SOD) isoenzymes (Cu-Zn SOD, Mn SOD, and extracellular superoxide dismutase (EC-SOD)) are also present in AH to scavenge free radicals.[43] In fact, the AH of human glaucoma patients has a significant reduction in total reactive antioxidant potential (GSH, ascorbate and tyrosine) due to oxidative stress in the glaucomatous...
eye. These results are also consistent with decreased reducing power in the AH of congenital glaucomatous rabbits, which exhibited a 90% reduction in ascorbic acid and a 69% reduction in uric acid, concomitantly with increased oxidative stress.

Besides the antioxidant defense in AH, the TM itself also has a sophisticated self-protection mechanism against reactive oxygen species (ROS) including SOD, catalase and glutathione. The developing TM in PCG patients has been found to be deficient in antioxidant enzymes and more susceptible to H$_2$O$_2$ permeating the tissue cells, ultimately resulting in TM damage and consequent AH outflow resistance. Consistently, $Cyp1b1^{-/-}$ TM cells are less viable under H$_2$O$_2$ treatment, indicating an insufficient cellular antioxidant capacity to detoxify ROS. Furthermore, mitochondrial DNA sequence variations associated with oxidative stress, including G10398A in NADH dehydrogenase subunit 2, A12308G in tRNA leu gene and G13708A in NADH dehydrogenase subunit 3 have been reported to be significantly more frequent in PCG patients than in controls. These variations can consequently lower ATP levels of TM cells and disable cellular elimination of ROS, impairing the growth, development and differentiation of TM tissue.

Recent studies conducted in our laboratory have demonstrated that the CYP1B1/Cyp1b1 activity in the TM directly determines their oxidative status. The TM tissues from $Cyp1b1^{-/-}$ mice exhibited increased oxidative stress early in life, and showed ultrastructural defects. These defects were prevented by daily administration of N-acetylcysteine (NAC) a free radical scavenger to $Cyp1b1^{-/-}$ pups from postnatal day 2. In addition, the abnormal cellular functions of $Cyp1b1^{-/-}$ TM cells including altered TM cell adhesion, that would influence the proper expansion of intercellular spaces and allowance of the trabecular outflow, were restored by treating the cells with the antioxidant NAC. Increased oxidative stress due to Cyp1b1 deficiency was also responsible for the decreased production of Periostin (Postn), an extracellular matrix protein revealed to be critical for the structural development and functions of TM.

**PERIOSTIN AND DEVELOPMENT OF TM TISSUE**

Periostin (Postn) is an extracellular matrix (ECM) protein essential for collagen fibril assembly and maturation. $Cyp1b1^{-/-}$ TM tissue was found to express less Postn than normal $Cyp1b1^{+/+}$ TM tissue and contained irregularly distributed collagens. Likewise, human glaucomatous TM tissues expressed decreased levels of Postn as compared to the levels expressed in normal human TM.

The present study was first to demonstrate a significant role for Postn in the structural integrity of TM tissues. The collagen fibers in Postn$^{-/-}$ TM were significantly disrupted, with large empty spaces representing abnormal distribution or even loss of collagen fibers. In addition, due to lack of support from regular collagen fibers, the trabecular cytoplasm became uneven and presented severe distortions. Overall, these results substantiate a role for Postn in dictating mechanical strength and structural integrity of TM tissue. Importantly, antioxidant NAC restored the expression of Postn in $Cyp1b1^{-/-}$ TM cells. In general, Postn expression is regulated by Cyp1b1 and cellular oxidative state, and its appropriate expression is essential for ultra-structural development and integrity of TM tissue. Uncovering how Cyp1b1 deficiency negatively impacts Postn expression in TM cells is the subject of ongoing investigation and its results will provide deeper insight into TM development in which Postn participates and Postn is a marker for assessment of therapy.

**Cyp1b1 AND VASCULAR FUNCTION**

Cytochrome P450s are now recognized as important modulators of vascular function through synthesis and metabolism of various vaso-regulators. However, there are limited known facts regarding the expression and function of Cyp1b1 in vascular cells. Our laboratory was the first to show expression of Cyp1b1 in endothelial cells and perivascular supporting cells prepared from vascular bed of various tissues, and its expression can be induced using an AhR agonist. We have found Cyp1b1 expression is critical in maintaining the function of retinal vascular cells including retinal endothelial cells and pericytes (PC), which are essential components of retinal blood vessels. Cyp1b1 is constitutively expressed in retinal endothelial cells and pericytes.

Retinas from Cyp1b1$^{-/-}$ mice exhibited reduced vascular density and failed to undergo neovascularization during oxygen-induced ischemic retinopathy. Cyp1b1-deficiency in retinal endothelial cells led to decreased migration, attenuated endothelial nitric oxide synthase expression and/or nitric oxide bioavailability and compromised their capillary morphogenesis. Cyp1b1-deficiency in retinal pericytes resulted in altered signaling pathways including sustained activation of NF-κB p65, and compromised endothelial cell and pericytes interaction in vitro. A summary of Cyp1b1-deficient cell functions is presented in Table 1. These defects were mainly attributed to increased intracellular oxidative stress in endothelial cell and pericytes, and could be relieved by decreased oxygen level (2%) or addition of NAC.

Our studies suggest that increased oxidative stress associated with Cyp1b1 deficiency results in increased expression of thrombospondin-2, a member of the thrombospondin family of matricellular protein with antiangiogenic activity, in both endothelial cells and...
pericytes. We have recently shown that expression of thrombospondin-2 is responsible for sustained activation of NF-kB and production of inflammatory mediators in retinal pericytes, with adverse effects on pericytes and endothelial cells function. Thus, modulation of vascular cell oxidative state by Cyp1b1 plays a key role in modulation of vascular function. Together our studies establish an important role for Cyp1b1 in modulation of intracellular oxidative state.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Our studies on the vascular and TM cells from Cyp1b1−/− mice established a significant role for Cyp1b1 expression in modulation of oxidative state. Although the exact identity of ROS remains elusive, we believe these are oxygenated products of polyunsaturated fatty acids, which are normally metabolized by Cyp1b1 relieving intracellular oxidative stress. While Cyp1b1−/− tissues neutralize the endogenous generation of ROS to some degrees, Cyp1b1 deficiency may deprive tissues from its ability to counteract ROS, rendering them less protected and much more susceptible to the onset of oxidative stress-related diseases such as PCG. Our data has suggested that the contribution of NADPH-oxidase, xanthine-oxidase, lipoxygenases, mitochondria or glutathione levels to ROS generation and oxidative stress in these cells is minimal. The nature of these ROS generated with Cyp1b1 deficiency and how they are regulated by oxygen levels is another important area of future research.

Many studies have revealed Cyp1b1 as a critical candidate gene in PCG, which is directly related to TM integrity. The detailed mechanism of how lack of Cyp1b1 expression and/or activity contributes to PCG has remained under debate. The most recent study from our group has shown that Cyp1b1 is an essential modulator of cellular oxidative state in the absence of which, increased oxidative stress results in decreased Postn production and TM maldevelopment. However, how increased oxidative stress leads to reduced production of Postn remains elusive. The novel finding of Postn as an important regulator of TM tissue development, and the inter-relationship between Cyp1b1, oxidative stress and Postn expression, may provide an innovative biomarker for detection and treatment of PCG. This knowledge also provides the rationale for using genetic counseling and antioxidants as an adjunct therapy along with conventional treatments to tackle this disease.

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