

The presence of kynurenine aminotransferases in the human cornea: Evidence from bioinformatics analysis of gene expression and immunohistochemical staining

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Purpose: Kynurenine aminotransferases (KATs) catalyze the synthesis of kynurenic acid (KYNA), a compound of significant biological activity. The aim of this study is to investigate the presence and distribution of KAT immunoreactivity in the healthy human cornea.

Methods: Data on gene expression in human eye structures were extracted from public microarray experiments using Genevestigator software. Immunohistochemistry was conducted using polyclonal antibodies against KAT I, II, and III on sections of eight enucleated eyes from patients with choroidal melanoma.

Results: Bioinformatics analysis showed that all four KAT isoforms were actively transcribed in the cornea and the conjunctiva. Immunohistochemical analysis revealed the presence of KAT I, II, and III in all examined corneal sections. The corneal endothelium showed the strongest reactivity for all three KAT isoforms. There was a slight positive staining of the corneal stroma for KAT I and II. KAT III immunoreactivity was found only in the stroma of the limbal region. In the corneal epithelium, the expression of all three KAT isoforms showed a specific pattern of the stain with fine squatter granules throughout the cytoplasm. This reactivity was more pronounced in the basal cell layers. The intermediate cell layers showed only faint immunoreactivity, and occasionally, there was no staining. KAT I, II, and III were also present in the adjacent limbal conjunctiva.

Conclusions: The results indicate that kynurenine can be metabolized to KYNA in the corneal epithelium, stroma, and endothelium.

Ocular surface diseases rank high among the leading causes of blindness worldwide. The cornea as a part of the ocular surface is vulnerable to physical and chemical injuries, infections, and other harmful conditions. The cornea is a complex structure consisting of the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium. The functions of the corneal epithelium are regulated by humoral factors derived from tear fluid, the stroma, and the conjunctiva, as well as by neural factors released from sensory nerves. Limbal corneal stem cells that continuously renew epithelial cells are derived from the neural ectoderm and express some neuronal properties [1,2]. The viability of the stroma and the endothelium is dependent, for the most part, on the transportation and diffusion of aqueous humor,

glucose, amino acids, and growth factors across the endothelium [3].

Tryptophan is an essential amino acid and a component of body fluids, including tear fluid, aqueous humor, and serum [4]. Tryptophan deficiency causes corneal neovascularization and cataracts in rats [5]. Relatively high tryptophan content in corneal proteins contributes to the absorption of ultraviolet (UV) light [6]. About 95% of tryptophan is metabolized via the kynurenine pathway [7]. Enzymes involved in the metabolism of tryptophan along this pathway are located throughout the body and the brain [8]. Interestingly, several enzymes in the pathway are under tight control of inflammatory mediators [9]. The first enzymes of the kynurenine pathway are indole-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), which convert tryptophan to N-formylkynurenine [10,11]. N-formylkynurenine is then metabolized to kynurenine (KYN) by kynurenine formamidase. At this point, the pathway bifurcates into at least two

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distinct branches regulated by kynurenine monooxygenase (KMO) and kynurenine aminotransferases (KATs) I–IV. KATs display transamination activity toward KYN leading to the formation of kynurenic acid (KYNA) [9]. All four KATs are multifunctional enzymes and share many amino acid and α -ketoacid substrates. Therefore, the four KATs most likely have overlapping biologic functions. All four KATs belong to the alpha family of pyridoxal 5'-phosphate (PLP)-dependent enzymes, where they have been assigned to the fold type I group. The biochemical properties of all four KATs were reviewed by Han and coworkers [12,13]. Previously, the presence of KAT I, II, and III has been demonstrated in the animal and human brain, retina, and vitreous body and in cataractous lenses [14-20]. However, little is known about the role of KATs in the physiology and pathology of the ocular surface.

KYNA, the product of KATs-catalyzed reactions, shows significant biological activity. KYNA possesses neuroprotective, anti-inflammatory, antioxidant, and antiproliferative properties [9,21-24]. KYNA is a well-described antagonist of endogenous glutamate receptors and is preferentially active at the N-methyl-D-aspartate (NMDA) receptor glycine-binding site [22,25]. KYNA is also a potent, noncompetitive antagonist of the alpha-7 nicotinic acetylcholine receptor [26]. As far as we know, the role of KAT I, II, and III and KYNA in the physiology and pathophysiology of the ocular surface has not been studied yet. It is tempting to speculate that tryptophan and its metabolites, especially KYNA, may protect the cornea from UV radiation, oxidative stress, corneal neovascularization, and infections. Moreover, KYNA as a neuroprotectant can prevent neurodegeneration of the corneal nerves, which may eventually help to keep the cornea clear. This wide spectrum of potential physiologic properties of KYNA made us interested in confirming the expression of KATs (KYNA-producing enzymes) in corneal tissue. Thus, the purpose of this study was to investigate the presence and distribution of KAT I, II, and III in healthy human corneas.

METHODS

Bioinformatics analysis of gene expression: Data on gene expression in eye layers were extracted from public microarray experiments with Genevestigator v5.11.05 (Nebion AG, Zurich, Switzerland) using "Anatomy search tool" [27]. Only data sets with at least three data points available were included in the analysis. Only data from healthy patients were used. Absolute expression values were plotted using Prism 6 (GraphPad Software, La Jolla, CA). Differences in gene expression between the cornea, conjunctiva, and retina were assessed statistically using the Kruskal–Wallis test followed

by Dunn's multiple comparisons test. A p value of less than 0.05 was considered statistically significant.

Immunohistochemical study: Eight human eyes from eight patients (four women, four men, age range: 27–85 years) enucleated due to primary choroidal malignant melanoma were used for this study. There were no other ocular diseases. The eyes with choroidal melanoma were used in previously published papers concerning ocular immunohistochemical studies [28,29]. Following enucleation, all globes were fixed immediately in a solution of 4% formaldehyde and 1% glutaraldehyde in 0.1% phosphate buffer (pH 7.2), dehydrated, and embedded in paraffin. Pupil-optic (P-O) sections (5 μ m) including the center of the disc, the pupil, the central and peripheral cornea were subjected to immunohistochemistry. We used anti-KAT I, II, and III polyclonal antibodies (1:50). The antibodies were validated using the western blotting technique. Each antibody showed a single band toward the crude extract [30,31]. The antibodies were used at least twice for staining, using the streptavidin-biotin method, as described previously [32]. Subsequently, after deparaffinization and rehydration, the sections were digested for 10 min with proteinase K (Dako, Glostrup, Denmark) before incubation with peroxidase. Afterward, they were incubated with primary antibody (30 min) and horseradish peroxidase (HRP)-conjugated secondary antibody using EnVision+ System – HRP Labeled Polymer Anti-Rabbit kit (Dako) according to the manufacturer's instructions. Then, the sections were developed with the 3-amino-9-ethylcarbazole (AEC)⁺ substrate (red reaction product). We used the AEC Substrate Chromogen Ready-to-Use kit (Dako) according to the manufacturer's instructions. In the final stage, the sections were counterstained with Mayer's hemalum (Chroma, Münster, Germany) and mounted in an aqueous-based medium (Faramount; Dako). Preimmune serum was included as the negative control, and no staining of the cornea was observed. Finally, the sections were photographed with a microscope (Axiophot; Carl Zeiss, Oberkochen, Germany) using color film (Ektachrome 64 T; Eastman Kodak, Rochester, NY). This study adhered to the ARVO statement on human subjects. The methods used in this study followed the tenets of the Declaration of Helsinki. The study protocol was approved by the Human Ethics Committee of University of Erlangen-Nuernberg (Germany).

RESULTS

Bioinformatics analysis: Initial data mining revealed that the genes encoding KATs are all expressed across the eye layers (Figure 1). This includes the *KYAT1* gene coding for KAT I (Gene ID 883, OMIM ID 600547), the *AADAT/KYAT2* gene

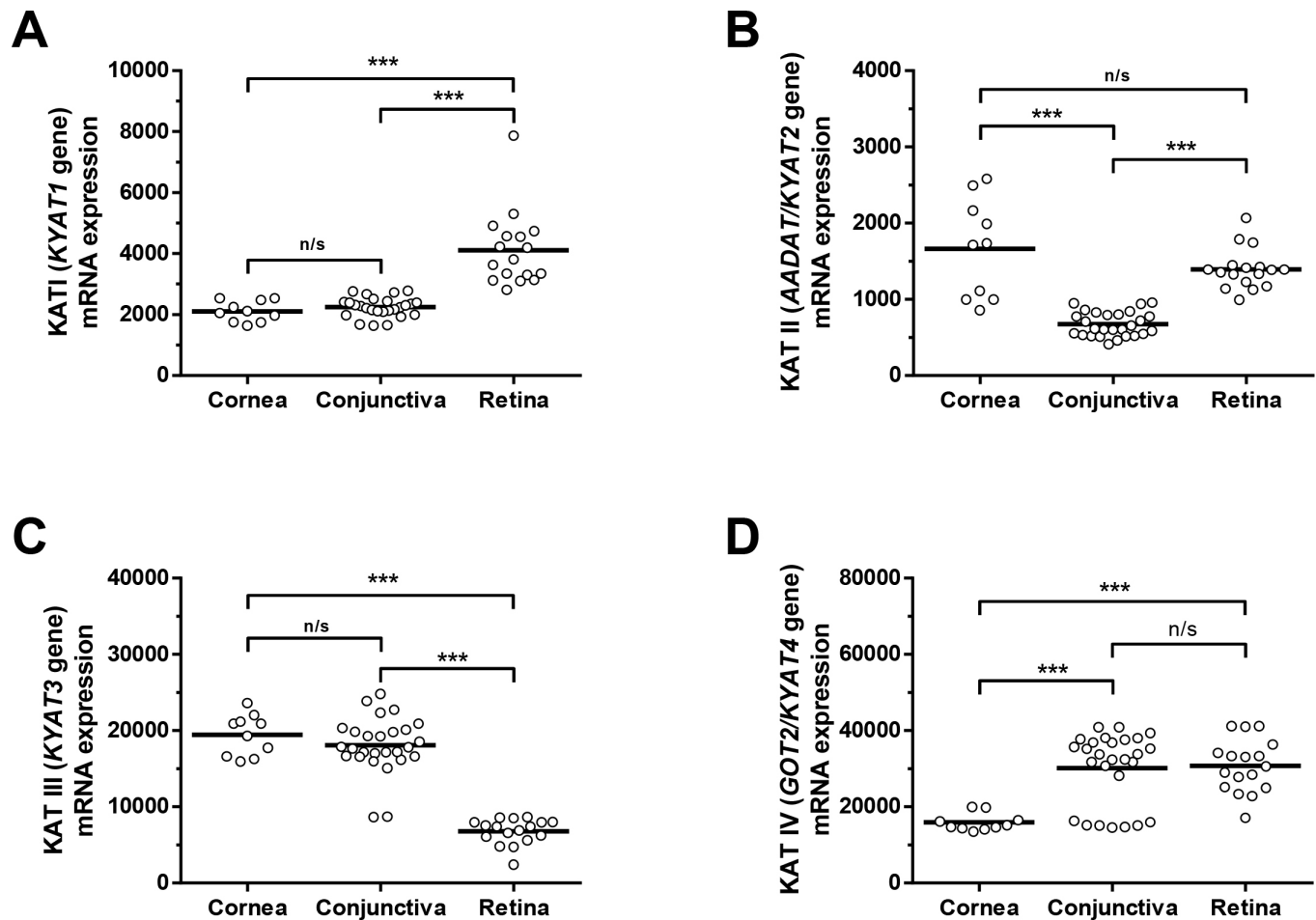


Figure 1. mRNA expression of kynurenine aminotransferases in the cornea, conjunctiva, and retina. Expression of (A) KAT I (the *KYAT1* gene), (B) KAT II (the *AADAT/KYAT2* gene), (C) KAT III (the *KYAT3* gene), and (D) KAT IV (the *GOT2* gene) in the cornea (n = 10), conjunctiva (n = 27), and retina (n = 17). Previously described [44-46] microarray data were obtained from the Gene Expression Omnibus (GEO) database (accession numbers: GSE29402, GSE38190, GSE28133, and GSE20436) using Genevestigator software. The Kruskal-Wallis test and Dunn's post hoc test were used to statistically evaluate the differences in gene expression between the tissues of interest. *** p<0.001; n/s., not significant.

coding for KAT II (Gene ID 51166, OMIM ID 611754), the *KYAT3* gene coding for KAT III (Gene ID 56267, OMIM ID 610656), and the *GOT2/KYAT4* gene coding for KAT IV (Gene ID 2806, OMIM ID 138150). The expression of the *KAT I* gene was statistically significantly lower in the cornea and the conjunctiva when compared with the retina (Figure 1A). The *KAT II* gene displayed a different pattern of expression, with relatively high mRNA levels in the cornea and the retina (Figure 1B). However, the retina was found to express lower levels of KAT III when compared with the cornea and the conjunctiva (Figure 1C). There was no statistically significant difference between the conjunctiva and the retina in terms of the expression of the *KAT IV* gene. The cornea expressed the lowest level of KAT IV among the tissues of interest (Figure 1D). Thus, although with some variability,

all four KATs were actively transcribed in the cornea and the conjunctiva.

Immunohistochemical study: Immunohistochemistry showed the presence of KAT I, II, and III immunoreactivity in all cornea sections studied (Figure 2, Figure 3). The immunohistochemical analysis revealed, that KAT I, II and III were present in the corneal epithelial cells of all studied corneas and showed a specific pattern of staining. The expression of KAT was characterized by fine squatter granules throughout the cytoplasm of the epithelial cells. This reactivity was more pronounced in the basal cell layers. The intermediate cell layers showed only faint KAT immunoreactivity, and occasionally, there was no staining. In addition, some focally more pronounced immunoreactivity was found in the superficial epithelial cell layers for all three KAT isoforms (Figure

2A,C,E; Figure 3A,C,E). There was a slight positive staining of the corneal stroma for KAT I and II (Figure 2A–D; Figure 3A–D). Immunoreactivity for KAT III was found only in the corneal limbal stroma (Figure 2E,F; Figure 3E,F). KAT I, II, and III were present in the adjacent limbal conjunctiva (Figure 3A,C,E). The corneal endothelium showed the strongest reactivity for all three KAT isoforms. The more pronounced staining of KAT I, II, and III was present in all endothelial cells of the sections (Figure 2, Figure 3).

DISCUSSION

The database search for the expression profiles of the genes encoding KATs in the cornea, conjunctiva, and retina was performed based on the records deposited in the public [Gene Expression Omnibus \(GEO\)](#) repository. We found that all four KATs were actively transcribed in the cornea and the conjunctiva. The data derived from the bioinformatics analysis were confirmed with immunohistochemical analysis. The presence of KAT I, II, and III in the human cornea and

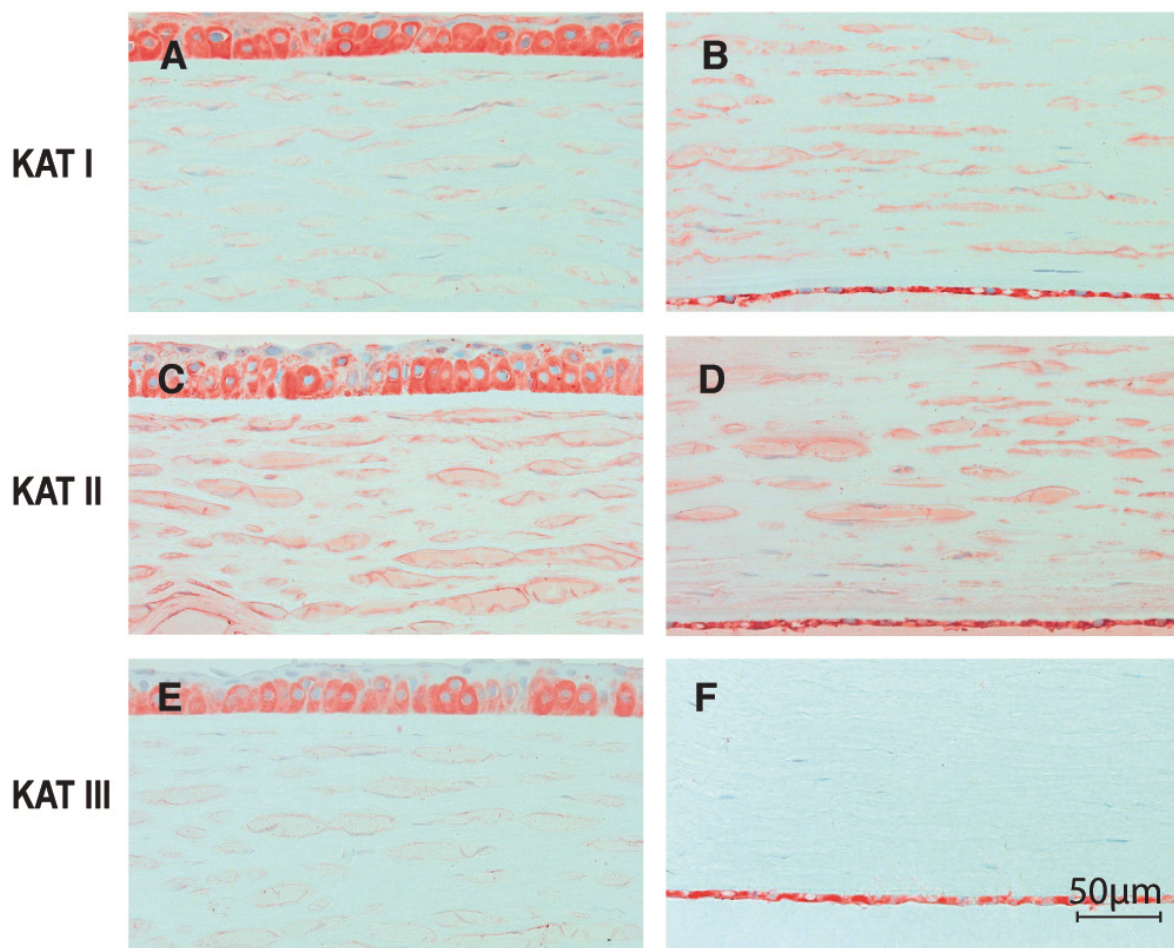


Figure 2. Immunoreactivity of kynurenine aminotransferases KAT I, KAT II, and KAT III in a healthy human central cornea. All three KAT isoforms show a positive reaction in the epithelium and the endothelium (red staining of the cytoplasm around the nucleus). There is a slight positive staining of the corneal stroma for KAT I and II. No KAT III immunoreactivity was found in the corneal stroma. **A:** KAT I (epithelium + stroma). **B:** KAT I (stroma + endothelium). **C:** KAT II (epithelium + stroma). **D:** KAT II (stroma + endothelium). **E:** KAT III (epithelium + stroma). **F:** KAT III (stroma + endothelium; magnification 200X). Horizontal scale bar = 50 μm presented in panel **F** refers to all panels **A-F**.

the limbal conjunctiva was demonstrated using anti-KAT I, II, and III polyclonal antibodies. According to our results, immunoreactivity of KAT I, II, and III was found in all the examined eyes of patients at different ages. Immunohistochemical analysis revealed the presence of KAT I, II, and III in the corneal epithelium and the endothelium. Only a slight staining of the stroma for KAT I and KAT II was detected. KAT III immunoreactivity was found only in the stroma of the limbal region. Strongly marked staining for all three KAT isoforms was present in all endothelial cells of the sections.

In the epithelium, the reactivity was more definite in the basal cell layers while the intermediate cell layers showed only faint KAT immunoreactivity, and occasionally, there was no staining. In the superficial epithelial cell layers, some focally more pronounced immunoreactivity for all three KAT isoforms was found.

The presence of KATs in the cornea suggests that KYN can be metabolized to KYNA in the corneal epithelium, stroma, and endothelium. Notably, KYNA possesses many important biological properties, including neuroprotective,

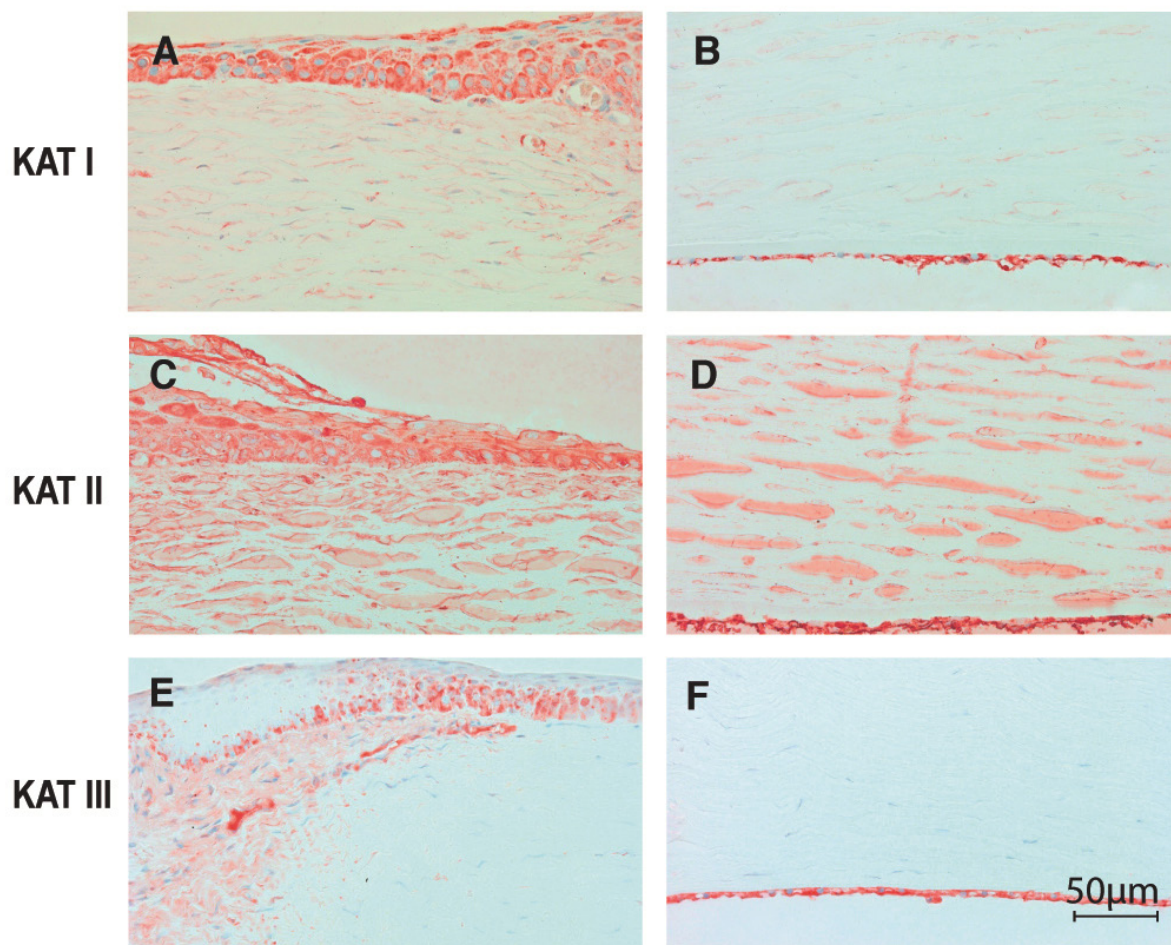


Figure 3. Immunoreactivity of kynurenine aminotransferase KAT I, KAT II, and KAT III in the healthy human limbal cornea. All three KAT isoforms show a positive reaction in the epithelium, stroma, and endothelium (red staining of the cytoplasm around the nucleus). **A:** KAT I (epithelium + stroma). **B:** KAT I (stroma + endothelium). **C:** KAT II (epithelium + stroma). **D:** KAT II (stroma + endothelium). **E:** KAT III (epithelium + stroma). **F:** KAT III (stroma + endothelium; magnification 200X). Horizontal scale bar = 50 µm presented in panel F refers to all panels A-F.

antioxidant, anti-inflammatory, antiscarring, and antifibrotic activity [9,23-25]. KYN, a precursor of KYNA, is formed by IDO and kynurenine formamidase from tryptophan. The activity of IDO was found in the corneal endothelium and the stroma [33-35]. The activity of IDO in the corneal epithelium has not been studied yet. As expected, in the present study the distribution of KAT isoforms on the corneal sections is similar to the distribution of IDO in the endothelium and the stroma [34,35]. The physiological significance of such a pattern of distribution of these enzymes in the stroma is unknown. This study cannot contribute to the reasons of variable expression, but it can be speculated that KYNA is delivered to the stroma from the basal cell layers of the epithelium and the endothelium where more pronounced reactivity for all three KATs was found. Another reason for the physiological importance of the slight reactivity for KAT I and II and the lack of KAT III in the stroma may be the need to filter UV radiation and protect the cornea from oxidative stress and apoptosis. Tryptophan, IDO, and kynurenines are considered to play a role in UV radiation filtering [6,33,36]. KYN may be accumulated in the stroma over time and play a crucial role in UV protection as the concentration of tryptophan within the corneal epithelium and the stroma decreases with age [36]. KYNA as the only known naturally occurring endogenous glutamate receptor antagonist may play a certain role in corneal homeostasis. Glutamate is known for its neurotoxicity and can be involved in neurotrophic diseases of the cornea and the remaining ocular surface structures. KYNA in micromolar concentrations antagonizes the glycine site of the NMDA receptor complex and acts as a neuroprotectant. It was shown that epithelial cells express NMDA receptors that belong to the glutamate receptor family [37,38]. According to these authors, the corneal epithelium and trigeminal neurons communicate via the purinergic and NMDA receptors. This seems to be important in terms of wound healing [37,38]. Furthermore, KYNA as a potent, noncompetitive antagonist of the alpha-7 nicotinic acetylcholine receptor may influence healing of epithelial erosions. It was demonstrated that the murine corneal epithelium expresses the acetylcholine (ACh)-synthesizing enzyme choline acetyltransferase, the ACh-degrading enzyme acetylcholinesterase, two muscarinic ACh receptors (mAChRs), namely, M3 and M4, and several nicotinic ACh receptors (nAChRs), including $\alpha 7$ - and $\alpha 9$ -made homomeric nAChRs and predominantly the $\alpha 3\beta 2$ \pm $\alpha 5$ subtype of heteromeric nAChRs [39].

The kynurenine pathway also participates in the immunological processes in the cornea. Proinflammatory cytokines interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) induce major histocompatibility complex (MHC) classes I and II on antigen presenting cells (APCs) for T-cell

activation while they upregulate IDO expression in human corneal endothelial cells and keratocytes, which strongly inhibits functionality of cytotoxic T lymphocyte proliferation and responses [35,40]. The products of IDO activity (KYN and the other tryptophan metabolites (3-hydroxykynurenine and 3-hydroxyanthranilic acid) express proapoptotic properties aimed mainly at T lymphocytes that may promote tolerance in eyes [40,41]. Other data support the proposition that adequate levels of endothelium-derived KYN might contribute to the maintenance of a relative immune privilege in the ocular anterior chamber and the preservation of corneal allogenic cells after corneal transplantation [34,40]. Similarly, the IDO activity in the stroma is considered to be a factor of ocular immune privilege in corneal keratocytes [35]. The expression of IDO significantly increased following corneal transplantation [34,40] while the unstimulated, normal murine cornea showed low levels of IDO [40]. Moreover, the pattern of IDO expression varied between rejected (high IDO expression) and non-rejected corneal transplants (absent or only scanty expressed) [34]. The role of KYNA in corneal transplantation has not been studied yet. It seems that KYNA may regulate peripheral cellular responses by antagonism on the NMDA receptors and activation of the G protein-coupled receptor 35 (GPR35) that is predominantly detected on immune cells [42]. As the kynurenine pathway is activated by proinflammatory stimuli, the anti-inflammatory effect of KYNA provides an additional feedback mechanism in modulating the immune responses [43]. In this study, we showed the presence of KAT I, II, and III in the structures of the healthy human limbal conjunctiva and the cornea. These findings support the hypothesis that tryptophan can be metabolized to KYNA in the corneal epithelium, endothelium, and stroma. The intensive staining for KAT I, II, and III localized in specific layers seems to confirm the particular role of KATs and KYNA in the cornea. However, before making a definitive conclusion about the role of KYNA, the distribution and activity of KATs need to be studied in corneal diseases. The data presented here may contribute to a better understanding the role of tryptophan and its metabolites in the physiology and pathology of the ocular surface and the cornea.

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