Age-Related Decrease in Human Corneal γ-Glutamyltranspeptidase Activity

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Purpose: To investigate age-related effects on human corneal γ-glutamyltranspeptidase (GGT) (ectoenzyme important to maintaining corneal hydration and antioxidant potential via glutathione recapture).

Methods: Age-related differences between total, endothelial, and epithelial GGT activity and endothelial cell density were determined for corneas from 29 donors (mean age, 53 ± 17 years; age range, 13–83 years). GGT activity was determined using a standard colorimetric assay based on the transpeptidation reaction. Corneal GGT localization and expression was determined by immunohistochemistry.

Results: Total corneal, endothelial, and epithelial GGT activities in the young (<50 years) donor corneas were 37% (P = 0.02), 44% (P = 0.001), and 36% (P = 0.06) higher, respectively, than in the senior (≥50 years) corneas. The age-related rates of decline for GGT activity were 1.0 unit per year for total cornea, 0.4 to 0.5 unit per year for endothelium, and 0.3 to 0.4 unit per year for epithelium. Notably, endothelial cell density in the young corneas was 14% (P = 0.001) higher than in the senior corneas declining about 100 cells per square millimeter per decade (0.3% per year). GGT activity per 10^6 endothelial cells decreased at about 0.2 units per year and GGT activity per 10^6 endothelial cells in the young corneas was 41% higher (P = 0.01) than in the senior corneas. Fewer immunoreactive GGT-positive epithelial cells were detected in senior cornea.

Conclusion: The age-related loss of human corneal GGT activity was associated with reductions in endothelial and epithelial GGT activity, being because of reduced number of GGT-positive endothelial and epithelial cells and reduced GGT activity per endothelial cell.

Key Words: age, cornea, endothelium, epithelium, eye, gamma-glutamyltranspeptidase

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Age-related changes in human corneal physiology,[1] structure, and biochemistry include increased thickness, decreased endothelial cell density, increased epithelial basement membrane thickness, decreased elasticity, and increased stromal glycation and advanced glycation end-products.[2–7] Similar age-related changes with regard to corneal thickness have been reported in rabbit and canine corneas.[8] In addition, age-related decreases in glutathione (GSH) synthesis enzymes and enzyme scavengers of reactive oxygen species (ROS) have been reported in rabbit cornea.[9]

The cornea is exposed constantly to exogenous ROS because of its position in the eye and endogenous ROS produced by the high metabolic activity in the mitochondria-rich endothelial and epithelial cells. Depletion of glutathione (GSH, a potent intracellular antioxidant) and GSH-related synthesis enzymes renders the cornea less capable of removing ROS, maintaining barrier function, hydration, cell membrane integrity, and detoxification of xenobiotic agents.[9–14] GSH depletion; increased ROS; and cell death play a pathologic role with cataracts, diabetes, and aging.[14–16] Differences in epithelial and endothelial GSH uptake, synthesis, and efflux pathways have been reported recently for the rat cornea.[17]

γ-Glutamyltranspeptidase (GGT) is an important enzyme in the γ-glutamyl cycle[18] acting to maintain cell viability and antioxidant potential via the recapture of extracellular GSH (tripeptide of glutamate, glycine, and cystine).[19,20] Briefly, extracellular GSH is cleaved enzymatically by GGT providing transportable cystine essential for intracellular GSH synthesis.

Intracameral GGT is distributed uniquely to barrier epithelium within the retina, ciliary body, lens, and cornea.[21] Within the human cornea, GSH and mitochondria-rich endothelial and basal columnar epithelial cells express GGT.[17,22] GGT regulation and functions in human corneal endothelium and epithelium are largely unknown. However, inhibition of rabbit endothelial cell GGT activity has been shown to regulate corneal thickness (hydration) and transparency[11,23] consistent with the idea that loss of GGT-positive corneal endothelial cells[3,4] may account for some of the age-related functional, structural, and biochemical changes observed in the human cornea as in the lens.[24] Age-related effects on GGT activity in human cornea have not been reported. The present study investigates GGT activity in corneas from 29 human donors 13 to 83 years of age. The results support a decrease in corneal GGT activity with increased donor age.

MATERIALS AND METHODS

Human Donor Corneas

Corneas (with medical history for most donors) were obtained through the auspices of the Northwest Louisiana Eye

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Bank, Shreveport, LA, and the Southern Eye Bank, New Orleans, LA. The donor globes and/or corneal buttons (deemed for research only) were frozen in corneal transport media (<72 hours post enucleation) and transported on ice. Before analysis, the globes/corneas were quickly thawed to room temperature and uniform corneal buttons were produced using 11.0-mm trephination (Hampton Medical Devices, LLC, Valley Park, MO). The corneal buttons were rinsed gently 3 times with 1 mL phosphate-buffered saline (pH 7.2) to remove the transport medium. The whole cornea GGT activity was determined as described below and rinsed to remove GGT assay medium. The anterior epithelial and posterior endothelial layers were peeled using iris forceps, surgical blades, and binocular 10× magnification. Epithelium and endothelium layers were stored at 4°C in phosphate-buffered saline until assayed for GGT activity as described below. [Note that only corneal layers with intact endothelial and epithelial layers were assayed.]. In additional studies, paraffinized tissue sections from 2 enucleated eyes (9-year-old female post failed corneal transplant pursuant to glass in the eye and 69 year-old male with chronic hypertensive blind painful eye) were obtained through the auspices of the Department of Pathology (LSU Health, Shreveport, LA). This investigation was performed in accordance with the principles expressed in the Declaration of Helsinki.

GGT Assay

GGT activity was determined using standard methods described previously.[25] Briefly, whole corneal, endothelial, and epithelial layers were incubated for 3 to 5 hours in 1.0 mL γ-glutamyltranspeptidase assay medium (composed of 1.8 mM γ-glutamyl-p-nitroanilide with 20 nM glycylglycine in Tris–HCl buffer, pH 8.2). The corneas were removed from the γ-glutamyltranspeptidase assay medium, and the reaction terminated by the addition 1.0 mL of 2 N acetic acid.

TABLE 1. Donor Demographics, Medical History, and Corneal Endothelial Cell Density

			ECD/mm²*		
Donor	**Age**	**COD**	**Medical History**	**OD**	**OS**
1	13	MVA	—	3008	2989
2	16	Boating accident	Closed head injury, splenectomy	—	3067
3	22	Hanging	Anoxic brain injury	2652	2728
4	32	Drowning	—	—	2936
5	36	Myeloma	Seizures, myeloma, brain Sx, liver Sx, herpes infection	3120	NCA
6	43	Shock	ARDS, head trauma, ovarian CA, pelvic Fx	3394	3132
7	44	MVA	—	2715	2759
8	48	Head trauma	Gout	3380	3179
9	48	SAH	Hernia Sx, ventriculostomy, cerebral angiogram	2720	—
10	51	Testicular CA	Seizures, renal failure	1846	1836
11	51	GI bleed	Lung CA, thrombocytosis, gallbladder Sx, GI bleed	—	2951
12	52	Throat CA	Appendectomy, back Sx	2866	2797
13	53	CAD	HTN, CHF	2701	—
14	54	Probable MI	HTN, angina, hyperlipidemia	2787	—
15	55	Head trauma	Dermatitis, possible osteoarthitris, heel spurs	2292	—
16	56	COPD	Prostate CA, heart Sx	2913	2813
17	56	CPA	HTN, COPD, cirrhosis, alcohol abuse, chronic bronchitis	2811	2793
18	57	MI	HTN, asthma, hysterectomy, hernia Sx	2636	2672
19	57	MI	—	NCA	—
20	61	MI	CHF, CAD	2550	—
21	62	HTN crisis	CHF, asthma, gallbladder Sx, pulmonary edema	2658	—
22	65	Throat CA	CHF, HTN, cardiomegaly, pneumonia, renal failure	2727	2694
23	65	CHF	CHF, COPD, HTN, arm Sx	2423	2659
24	66	Lung CA	COPD, pneumonia, right knee Sx, gallbladder Sx	2427	2401
25	68	Probable MI	HTN, CHF, pacemaker, gallbladder Sx, leg ulcers	2527	2587
26	68	MI	Nephrectomy, kidney CA, MI, lymphosarcoma	2603	2523
27	71	CPA	—	NCA	NCA
28	74	MI	—	NCA	NCA
29	83	CPA	—	NCA	—
Av ± SD	52 ± 16	—	—	2732	±323

*Endothelial cell density (cells/mm²) provided by Southern Eye Bank, New Orleans, LA.

ARDS, acute respiratory distress syndrome; AV, atrioventricular; CA, cancer; CAD, coronary artery disease; CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; CPA, cardiopulmonary arrest; GI, gastrointestine; HTN, hypertension; MI, myocardial infarct; MVA, motor vehicle accident; NCA, no endothelial cell count available; SAH, subarachnoid hemorrhage; Sx, surgery.

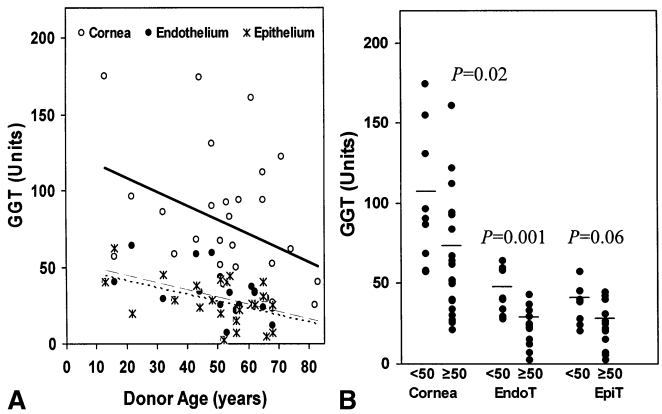

FIGURE 1. Corneal GGT activity. A, Scatter graph with a linear regression trend lines for GGT levels in whole corneal (—), endothelial (—), and epithelial (····) versus donor age. B, Scatter graph of whole corneal, endothelial, and epithelial GGT activity for donor corneas <50 years and ≥50 years (bar = mean).

To compare endothelial and epithelial GGT activity, paired endothelial and epithelial sections were assayed for GGT activity. One unit of GGT activity equals the conversion of 1 nmol of L-γ-glutamyl-p-nitroanilide to p-nitroaniline. The GGT activities per human cornea, endothelium, and epithelium per hour were calculated. GGT activity per 10^6 endothelial cells per 11.0-mm trephined corneal button was calculated by dividing the units of GGT activity by the total number of endothelial cells per 11.0 mm diameter endothelial corneal button (ie, average number of endothelial cells/mm^2 multiplied by the estimated square millimeter endothelial surface area) divided by 10^6.

Immunohistochemistry

Immunoreactive GGT was detected in deparaffinized human corneal tissue sections after reaction with a 1:100 dilution of rabbit anti-GGT (a gift from Dr. David Castle, Department of Cell Biology, University of Virginia Health System School of Medicine, Charlottesville, VA)[26] using standard methods (Vector Laboratories, Burlingame, CA). Digitalized images were captured with a Photometrics Cool SNAPfx monochrome CCD camera controlled with Scanalytics IPLab software.

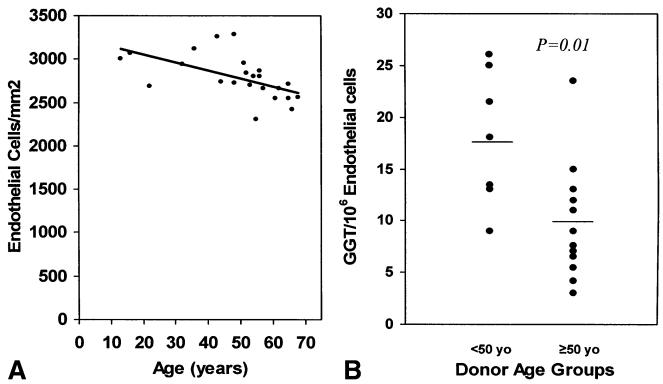

FIGURE 2. Endothelial cells and GGT activity. A, Scatter plot and trend line for corneal endothelial cell density versus donor age. B, Scatter graph of the GGT activity per 10^6 endothelial cells in the young (<50 years) and senior (≥50 years) donor corneas (bar = mean).

Statistical Analysis

The mean data from eye pairs with data from each single were analyzed, and linear regression analysis trend lines were plotted to assess relationships between GGT activities and age groups using Microsoft Excel (Richmond, VA) and SigmaPlot software (Systat Software, San Jose, CA). The linear relationship between 2 data sets was determined by Pearson correlation coefficient (r). Statistical inferences for age-related differences were determined using nonparametric Mann–Whitney U test (P) and the unpaired Student t test (P). Differences were statistically significant at $P < 0.05$.

RESULTS

Corneal Donors

The average age (±SD) of the 29 corneal donors was 52 ± 16 years and ranged between 13 and 83 years (Table 1). The causes of death (COD) for the eye donors included heart disease (39%, 11 of 29), accidental death (24%, 7 of 29), cancer (17%, 5 of 29), lung disease (10%, 3 of 29), and miscellaneous (10%, 3 of 29). Notably, accidents accounted for 6 of 9 deaths in the young (<50 years) donors. The average corneal endothelial cell density for all corneas was 2732 ± 323 cells per square millimeter. Corneas with the lowest endothelial cell densities were from donor 10 who died from testicular cancer and had a medical history significant for seizures and renal failure. Gross evaluation of the corneal donor data pointed toward a general decrease in endothelial cell density with age.

Age-Related Change in Corneal GGT Activity

The average GGT activity for the corneas from 29 donors was 77 ± 43 units per cornea. The scatter graph and linear regression analysis ($R = 0.381$, $t = -2.14$, $P = 0.04$) of whole corneal GGT activity versus donor age supported a loss (Pearson correlation, $r = -0.932$) of activity with increasing donor age (Fig. 1A). The results suggest that corneal GGT activity decreases at an approximate rate of 10 units per cornea per decade (ie, 1.0 unit per year). Accordingly, GGT activity was 37% higher in the corneas of the 9 young (<50 years) donors (104 ± 46 units/cornea) than in corneas of the 20 senior (≥50 years) donors (65 ± 37 units/cornea; $P = 0.02$) (Fig. 1B). Linear regression analysis ($R = 0.456$, $t = -2.35$, $P = 0.03$) of endothelial GGT activity versus donor age supported an age-related decrease ($r = -0.441$) in GGT activity at a rate of 4 to 5 units per decade (0.4–0.5 unit/year) (Fig. 1A). Corneal endothelial GGT activity was 44% higher in the young (44 ± 14 units) than in the senior (24.5 ± 12 units, $P = 0.001$) donor eyes (Fig. 1B). In addition, linear regression analysis ($R = 0.486$, $t = -2.546$, $P = 0.02$, $r = -0.449$) estimated an age-related loss of epithelial GGT activity at a rate

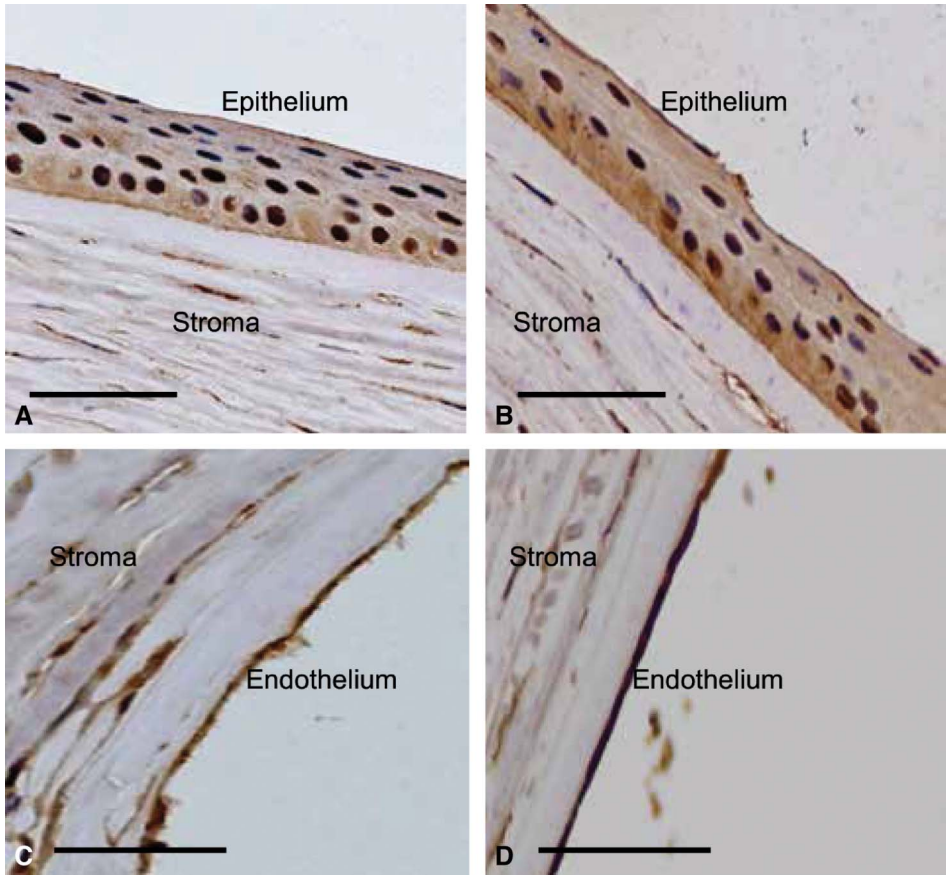

FIGURE 3. Immunohistochemical staining of GGT in the corneal epithelium (A, B) and endothelium (C, D) of the enucleated eyes from a 9-year-old female and a 69-year-old male, respectively (bar = 50 μm). Note the reddish brown GGT-positive nuclei and cytoplasm of the columnar cells and the blue GGT-negative nuclei of the wing cells.

of 3 to 4 units per epithelium per decade (0.3–0.4 units/year) (Fig. 1A). The epithelial GGT activity in corneas of the young (36 ± 14 units) was 36% higher ($P = 0.06$) than in the corneas of the senior donors (23 ± 14 units) (Fig. 1B). These results support age-related decline in corneal GGT activity and suggest that the reduction is most likely because of loss of epithelial and endothelial GGT activity.

Donor Age, Endothelial Cell Density, and GGT Activity

The following analyses were performed to determine if corneal endothelial cell density in our corneas decreased with donor age as previously reported by others.[3,4] The scatter plot and linear regression analysis ($R = 0.577$, $t = -3.315$, $P = 0.003$) of average corneal endothelial cell density for each donor eye pair and each single eye suggested that corneal endothelial cell density decreases with donor age ($r = -0.664$; Fig. 2A). Concomitantly, endothelial cell density was 14% higher ($P = 0.001$) in the corneas of the young (2984 ± 245 cells/mm^2) than in the corneas of the senior (2564 ± 110 cells/mm^2) donors (not shown). These results support age-related loss of endothelial cells in our donor corneas at an approximate rate of 100 cells per square millimeter per decade (0.3%/year) and suggest that senescent loss of GGT-positive endothelial cells could contribute to the lower GGT activity in the senior donor corneas.

Because loss of GGT activity could be due to reduced expression per cell, we next calculated the level of the GGT activity per corneal endothelial cell per donor cornea to determine if the GGT activity per endothelial cell was the same for each cornea. The results of the scatter graph and linear regression analyses ($R = 0.402$, $t = -2.011$, $P = 0.057$) point toward a decrease in GGT activity per 10^6 endothelial cells (Pearson correlation, $r = -0.449$) with increasing donor age (not shown). Accordingly, the GGT activity per 10^6 endothelial cells in the young donor corneas (16.9 ± 6.8 units/10^6 cells) was 41% higher ($P = 0.01$) than GGT activity per 10^6 endothelial cells in the senior nondiabetic donor corneas (9.9 ± 4.9 units/10^6 cells; Fig. 2B). These results support an age-related loss of GGT activity per 10^6 endothelial cells at a rate of 0.2 to 0.3 unit per year and suggest that the corneas of the older donors expressed less GGT activity per endothelial cells than the younger donor corneas.

Distribution of Corneal GGT

The localization and expression of immunoreactive GGT within the corneal epithelium and endothelium in enucleated eyes from a 9-year-old female and a 69-year-old male were assessed in parallel by immunohistochemistry. High levels of immunoreactive GGT signal were associated with the columnar epithelial cell nuclei and cytoplasm and

superficial flat epithelial cells at the corneal surface with lower levels of immunoreactive GGT in wing cells of both corneas (Fig. 3). Moreover, the levels of cytoplasmic and nuclear-associated immunoreactive GGT were not markedly different in the young and aged enucleated eyes. However, fewer epithelial cells were noted in the older cornea consistent with the idea that lower GGT activity in the aged cornea could be because of fewer GGT-positive epithelial cells.

DISCUSSION

The results suggest that total corneal GGT activity declines with age. Accordingly, the corneal GGT activity of the young donors (<50 years) was significantly higher than the corneal GGT activity of the senior donors (≥50 years). Endothelial and epithelial GGT activities were significantly higher in the corneas from young donors than from the senior donors, in parallel with the age-related decline in whole corneal GGT activity. The combined decreases in endothelial corneal GGT activity (in the range of 0.4–0.5 unit/year) and epithelial cell GGT activity (in the range of 0.3–0.4 unit/year) were consistent with the idea that the age-related loss of corneal GGT activity (1.0 unit/year) was primarily because of reductions in endothelial and epithelial cell GGT activity. The senescent loss of weakly GGT-positive[22] stromal keratocytes[4] cannot be ruled out. Senescent loss of corneal endothelial cells in our donor group was about 0.3% per year in agreement with previously reported confocal microscopy results of 0.5% per year.[4] In addition, GGT activity per endothelium declined by an estimated annual rate of 0.2 unit per 10^6 endothelial cells per year, suggesting that an age-related reduction in extrinsic GGT expression per endothelial cell may also contribute to the lower levels of GGT activity in corneas of the senior donors.

The age-related reduction in GGT activity in the corneal epithelium seemed to be because of fewer GGT-positive epithelial cells. That is, little differences were detected in the distribution of immunoreactive GGT in the epithelium and endothelium of young and aged corneas, but fewer wing cells were noted in the aged cornea. This result is consistent with the absence of detectable age-related differences in the basal epithelial cell layer.[4] However, the possibility that age-related changes such as basement membrane thickness[5] may affect GGT expression/distribution/activity within the epithelial layers cannot be ruled out.

The results are consistent with the idea that corneal GGT activity decreases with age and is likely because of senescent loss of GGT-positive cells and reduced GGT activity per endothelial cell. The results are taken to suggest that other γ-glutamyl cycle enzymes important to maintaining corneal GSH and health may be reduced in aged humans as reported in rabbits and canines.[8,9] Given the protective roles that endogenous and exogenous GSH and GSH-related enzymes exert in ocular tissues,[14] the localization of GGT in endothelium and epithelium,[22] the differential distribution of GSH and GSH pathways within the corneal epithelium and endothelium,[17] and the putative importance of GGT to maintaining corneal GSH, one may predict that oxidative stress associated with diabetes, cataract, irradiation, and administration of some drugs[4] may exacerbate the age-related loss of corneal GGT activity and GSH.

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