Article

Quantification of Double Stranded DNA Breaks and Telomere Length as Proxies for Corneal Damage and Replicative Stress in Human Keratoconus Corneas

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Purpose: The pathogenesis of keratoconus (KC) is multifactorial, and associated with oxidative stress and subsequent DNA damage. We investigate differences in DNA damage and replicative stress in patients with KC, and in healthy and diseased controls.

Methods: We obtained 64 corneal buttons from 27 patients with KC after corneal transplant surgery, 21 with a decompensated graft (DG), and 16 healthy controls (HC). The amount of intact Alu elements per genome copy as measured by quantitative polymerase chain reaction (qPCR) was used to quantify intact DNA. Telomere length was measured as a proxy for replicative stress. In addition, telomerase reverse transcriptase (*hTERT*) gene expression level was assessed.

Results: Mean ($\pm$ standard deviation [SD]) DNA damage was similar between the KC (5.56 $\pm$ 14.08), DG (3.16 $\pm$ 8.22), and HC (3.51 $\pm$ 6.66) groups ($P = 0.807$). No associations were found between DNA damage and patient age ($P = 0.523$), atopic constitution ($P = 0.240$), or contact lens wear ($P = 0.393$). Telomere length differed ($P = 0.034$), most notably in the KC group, and *hTERT* was not detected in any corneal sample. Three cross-linked (CXL) KC corneas did not contain significantly more DNA damage ($\times 2.6$, $P = 0.750$).

Conclusions: Based on these findings, differences in actual corneal DNA damage in KC could not be identified, and the longer telomere length in KC did not support replicative stress as a major etiologic factor in the pathogenesis of KC. Future longitudinal investigations on KC etiology should assess progressively early cases to better comprehend the cellular and molecular processes preceding the archetypical morphologic changes.

Translational Relevance: The standard treatment for progressive keratoconus promotes the crosslinking of collagen fibers through ultraviolet radiation and the subsequent formation of reactive oxygen species. Our study helps to underline the safety of this treatment approach.

Introduction

Keratoconus (KC) is a corneal condition that can lead to refractive myopia, irregular astigmatism, corneal thinning, and poor visual acuity due to the hallmark "cone-like" shape of the cornea and, in advanced cases corneal scarring.[1] Environmental (e.g., eye-rubbing, atopic constitution, and so forth) and genetic factors have been linked to hypersensitive oxidative stress responses at the ocular surface.[2–4] Currently, crosslinking (CXL; a combination of local

ultraviolet (UV) radiation and riboflavin photosensitizing eye drops) is regarded as an established treatment to prevent disease progression and much research is devoted to study UV-related treatment effects.[5] However, DNA damage induced by UV radiation also has been suggested as a possible causative factor in the development of KC.[6,7] UV radiation can damage DNA, leading to breaks in the double-stranded DNA, and the ocular surface is heavily exposed to the potentially detrimental effects of UV radiation on DNA integrity.[7,8] Consequently, the cornea has several robust intrinsic defense systems against UV-induced damage and reactive oxidative species (ROS) in particular. Indeed, studies have found altered activity of several enzymes in the corneas of patients with KC, including the enzymes superoxide dismutase,[9] aldehyde dehydrogenase,[10] catalase,[11] cathepsin,[11] glutathione reductase, transferase, and peroxidases.[10] Therefore, this altered enzyme activity may contribute to oxidative stress and accumulation of damage.

When DNA damage accumulates, cells usually enter apoptosis. Hence, when there is increased exposure to, for instance, UV light, cells will accumulate DNA breaks rapidly. When DNA repair mechanisms are not able to cope with these breaks, the cells will enter apoptosis, subsequently leading to an increased cell turnover. In keratoconus, one can hypothesize that an increased epithelial apoptosis influences the local homeostasis of the cornea, leading to the loss of stromal collagen fibrils fundamental in the disruption of the normal corneal architecture. In addition, increased cellular turnover could be a sign of increased damage to corneal cells, forcing increased cellular replication to replace damaged cells. A well-known measure for the rate of cellular turnover and replicative senescence is telomere length (TL). Telomeres function as a noncoding protective end region of chromosomes. Due to the DNA end replication inefficiency of polymerases, chromosomes shorten every cell division. After a certain number of divisions, the threshold of attrition is reached, and cells go into apoptosis or enter a senescence state. Therefore, telomeres are indicative of the number of divisions the cell lineage has undergone and are a measure of replicative stress. Repair systems exist (i.e., human telomere reverse transcriptase [hTERT]) and sometimes are upregulated in cells that divide rapidly, such as stem cells.[12]

To answer the question whether corneal cells present in the cornea of keratoconus patients consist of cells refractive to DNA damage–induced apoptosis, we quantified the amount of DNA breaks in these cells by measurement of intact *Alu* elements, a proxy for the amount of DNA breaks.[13–17] The amount of DNA breaks represents a composite measure of the balance between DNA repair and DNA damage. An increase in DNA breaks in keratoconus corneas compared to healthy or decompensated graft corneas could highlight a role for either increased DNA damage, or decreased damage response in this disease.

Secondly, we investigated whether these corneal cells show signs of increased replicative cell turnover by measuring TLs and *hTERT* gene expression as additional parameters of replicative function. Hypothetically, short telomeres in corneal cells could indicate a constitutional increased replicative rate, preceding the disrupted morphologic structure of a keratoconus cornea.

To answer these questions we assessed DNA breaks, TL, and *hTERT* expression in 64 human corneal buttons from 27 patients with KC who underwent corneal transplant surgery, 21 with a decompensated graft (diseased controls; DG) not related to KC, and 16 unaffected (healthy) postmortem donor corneas (HC).

Material and Methods

Corneal Samples

This study was approved by the medical research ethics committee (MREC) of the UMC Utrecht. It reviews research protocols in accordance with the Medical Research Involving Human Subjects Act (WMO). The MREC of the UMC Utrecht is accredited by the Central Committee on Research Involving Human Subjects (CCMO) since November 1999. The MREC of the UMC Utrecht also is member of the Dutch union of MRECs (NVMETC). No donor was from a vulnerable population and all donors or next of kin provided freely-given written informed consent.

Twenty-seven cornea samples were obtained from 27 patients who received a corneal transplant for severe KC and one with pellucid marginal degeneration (the KC group). A second group of 21 corneal samples was obtained from 21 patients who underwent a regrafting procedure due to a decompensated corneal graft in which the indication for the primary graft was not KC (the DG group). The corneal buttons in the KC and DG groups were embedded in Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA) immediately after resection and stored at −80°C.

Corneal samples also were obtained from 10 healthy controls (HC group); these samples were obtained from the Euro Cornea Bank (Beverwijk, The Netherlands) and the Department of Anatomy, University Medical Center Utrecht, Utrecht, The Netherlands. Within 24 hours of death, the corneas were prepared from postmortem tissue obtained from 16 unrelated donors, each of whom had no documented history of KC, ocular inflammation, or vitreoretinal disease. All patients provided written informed consent after explanation of the procedure. Informed consent for the postmortem donation of ocular tissue was provided under the auspices of the head of the Department of Anatomy, University Medical Center Utrecht, The Netherlands. All tissues were acquired in compliance with Dutch law (*Wet op de lijkbezorging*, Art 18, lid 1/ 18–06–2013) and the institutional guidelines established by the University Medical Center Utrecht.

Clinical Data Extraction

Additional data were extracted from patient records and included patient history and the preoperative assessment, which included the results of a slit-lamp evaluation, Schirmer's test, and Scheimpflug corneal tomography (Pentacam HR; Oculus GmbH, Wetzlar, Germany). The data available for the HC group were limited to age, sex, and cause of death. Each KC cornea was graded as clear, mildly hazy, or clouded based on slit-lamp biomicroscopy. All decompensated grafts were considered clouded and all healthy corneas were considered clear.

Assessment of DNA Damage

DNA was isolated from the corneal buttons using TRIzol (Life Technologies, Thermo Fisher Scientific, Grand Island, NY); this approach allows for the isolation of small DNA molecules, which can be lost when using column-based isolation techniques. After dissolving the cornea in TRIzol reagent, the RNA was removed and the original tubes containing the TRIzol reagent and DNA were stored at −20°C. After isolation, double-stranded DNA was measured using a Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific). The number of intact Alu elements in the DNA was measured using quantitative polymerase chain reaction (qPCR) and was used as a proxy for quantifying intact DNA per genome copy, as indicated by the single genome marker 36B4.[13] DNA damage was assessed in the first 33 consecutive samples and validated in the subsequent 31 samples.

Since baseline characteristics and study outcomes varied only marginally, these were reported as one pooled cohort.

Measurement of TL

Corneal TL was measured by the Bio-Rad cfx-96 real-time qPCR detection system in duplicates in two separate experiments. Briefly, the length of telomeres (long repetitive hexamer [TTAGGG] sequences) can be accurately determined by using a calibration curve based on linear serial dilution of a synthetic 84-mer (14 consecutive TTAGGG sequences) oligonucleotide (Geneworks, Adelaide, Australia) with a predetermined molecular weight per reaction (60×10^{-12} gr of telomere oligomer or 1.36×10^9 oligomers). The total number of base pairs in the highest standard can be calculated as ($[1.36 \times 10^9$ molecules of oligomer] $\times$ [84 oligomer length] = 1.18×10^8 kilo base pairs). The relative TL per sample is extrapolated from serial dilutions of the synthetic standard in each qPCR measurement. Similarly, a synthetic standard also was designed for the single copy housekeeping gene *36B4*. Absolute telomere base pairs per genome are quantified by subdividing the total number of telomere base pairs from *36B4* (which has only one copy per gene) following; TL/house-keeping gene = TL per genome.[18] TL quantification was performed in the first 33 consecutive corneal samples.

hTERT Gene Expression

hTERT gene expression level was quantified using synthesized cDNA (Biorad iScript kit) from RNA that was extracted from the corneal cells, in the first 33 consecutive cornea samples. Quantstudio qPCR apparatus with TaqMan assay (Applied Biosystems, Thermo Fisher Scientific) was used to perform qPCR under conditions as specified by the manufacturer. To normalize the *hTERT*-gene expression, the housekeeping *GUSB* and *GAPDH* genes were included.

Statistical Analysis

The level of DNA damage (measured using the number of amplified Alu elements) was corrected for input DNA and graphed in a box plot; the mean levels of DNA damage and TL were calculated for each study group. Statistical analyses were performed using SPSS 21.0 (IBM, Armonk, NY). Outlier analysis was performed by removing samples with DNA damage that exceeded >3 standard deviations (SD); these values were analyzed separately. Differences in DNA damage, TL, and *hTERT* expression

were tested using the 1-way independent analysis of variance (ANOVA) or Kruskal-Wallis test. Multiple comparisons were tested using the post hoc Tukey's test.

Results

Study Population

The mean ($\pm$SD) ages of subjects in the KC, DG, and HC groups were 42.8 $\pm$ 14.8, 67.6 $\pm$ 12.0, and 83.6 $\pm$ 8.8 years, respectively. Males were overrepresented (63.0%), underrepresented (33.3%), and equally distributed (56.3%) in the three groups, respectively. As expected, concurrent atopic disease and contact lens wear were more prevalent in the KC group (63.0% and 77.8%, respectively) compared to the DG group (9.5% and 57.1%, respectively). The KG and DG eyes were similar with respect to the Schirmer's test results. Four samples in the DG group, five in the HC group and one in the KC group did not yield sufficient DNA for analysis; thus, reducing the effective sample size from 64 corneas to 54. The characteristics of the four DG patients with nonviable samples did not differ from the mean group (data not shown). The donors of the nonviable HC samples were among the oldest samples (mean age of these four subjects was 87 years). Mean age of the 33 samples used for TL/*hTERT* assessment did not differ materially from the pooled group (KC 40.8 $\pm$ 15.2, DG 66.7 $\pm$ 11.9, HC 81.5 $\pm$ 9.8 years).

DNA Damage

Mean levels of DNA damage in the pooled KC ($n = 26$), DG ($n = 17$), and HC ($n = 11$) groups were 5.56 $\pm$ 14.08, 3.16 $\pm$ 8.22, and 3.51 $\pm$ 6.66, respectively. Log^{10} normalized values were -0.66 ± 1.63, -0.59 ± 1.54, and -1.01 ± 1.60, respectively ($P = 0.807$; Fig. 1).

The aforementioned values represent the total number of breaks in the DNA double strands of all Alu elements per genome copy; thus, considerable variability was observed with respect to the total number of DNA breaks per sample (which ranged from 8.653×10^{-5} to 70.2 in this study). Therefore, we used outlier analysis to normalize the groups; using this approach, we excluded three KC, one DG, and two HC samples from analysis (see Table 1). Removing these outliers reduced the average DNA damage in the KC, DG, and HC groups to 1.74 $\pm$ 2.71, 1.19 $\pm$ 1.24, and 0.71 $\pm$ 1.61 respectively, but did not affect the mean differences between the

groups ($P = 0.407$). Similar results were obtained when the results were adjusted for age (data not shown), and we found no significant correlation between age and DNA damage (Spearman's $\rho = -0.095$, $P = 0.523$).

Atopy and contact lens wear are known risk factors for development of keratoconus. In the KC group, 17 patients had atopy and 21 patients wore contact lenses compared to two and 12, respectively, in the DG group. Therefore, we next investigated the extend of DNA damage among these subgroups. The amount of DNA damage did not differ significantly between the atopic (2.26 $\pm$ 2.97) and nonatopic patients (1.26 $\pm$ 1.30; $P = 0.240$) or between the patients who did and did not wear contact lenses preoperatively ($P = 0.393$). We found that the level of DNA damage did not differ significantly from the clear to hazy corneas ($P = 0.418$). Clear corneas showed a comparable amount of DNA damage (1.18 $\pm$ 2.47) to the mildly hazy (1.80 $\pm$ 2.57) and clouded (1.18 $\pm$ 1.16) corneas. Three KC cases underwent CXL before a grafting procedure. DNA-damage increased 2-fold compared to overall outcomes, though this difference was not significant ($P = 0.750$).

TL and hTERT Expression

TL is represented as total number of base pairs per genome. Four samples did not yield sufficient DNA for analysis. Mean TL in KC cases ($n = 11$) was $3.92 \times 10^9 \pm 6.61 \times 10^9$ (log^{10} 8.88 $\pm$ 0.945), $2.09 \times 10^9 \pm 5.78 \times 10^9$ (log^{10} 7.22 $\pm$ 1.83) in decompensated grafts ($n = 10$), and $5.83 \times 10^9 \pm 1.46 \times 10^{10}$ (log^{10} 8.02 $\pm$ 1.49) base pairs in healthy controls ($n = 8$). These differences were statistically significant ($P = 0.034$). Correction for age and sex did not affect these findings. TL was log^{10} normalized to perform these analyses. *hTERT* did not reach detectable levels in any corneal sample.

Discussion

We hypothesized that increased DNA damage could be a feature of keratoconus corneal cells, caused either by increased DNA damage or decreased DNA damage responses. In addition, we hypothesized that increased damage could lead to increased replicative senescence or that increased replication of corneal cells on itself could underlie the epithelial remodeling in keratoconus. In this particular large sample, we found that the level of DNA damage and TL in the corneas of patients with keratoconus was similar to
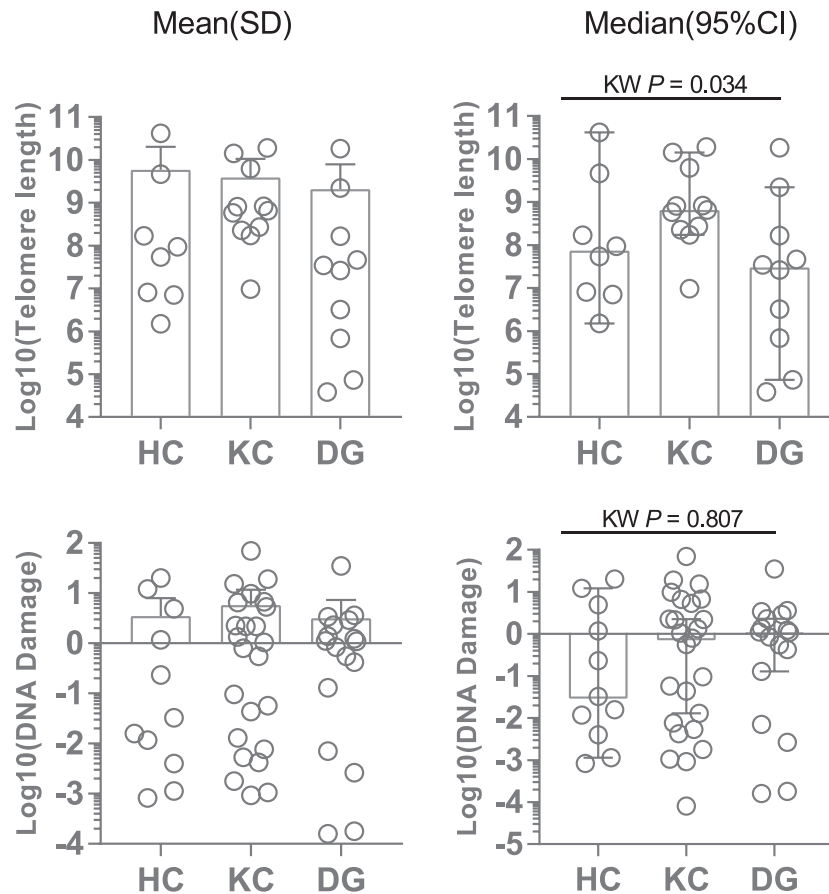
Figure 1. Box plots with overlying scatter plots summarizing the mean and median DNA damage and TL measured in the three study groups. DNA damage was measured as the number of intact *Alu* elements per genome copy. TL is represented as total number of base pairs per genome. *Top*: TL. *Bottom*: DNA damage.

two control groups (patients with a decompensated graft not related to KC and healthy donor subjects). *hTERT* was not expressed in any corneal sample. In addition, we found no significant correlation between DNA damage, TL and either age, sex, atopic constitution, or the use of contact lenses. TL and *hTERT* expression levels have not been assessed previously in corneal tissue.

Several lines of evidence support the notion that keratoconic eyes have altered antioxidant function and/or an inadequate DNA repair system.[19] DNA breaks are a feature of severe DNA damage and can be caused by various agents, such as UV light, ROS, or chemicals. When these breaks occur, cells initiate DNA repair responses. After this response there are three possible outcomes; the break is repaired, there is too much damage and the cell goes into apoptosis, or, as recently discovered, the break persists and the cell goes into a senescent state.[20] Keeping this in mind, the DNA breaks assessed in our study represent recent nonrepaired breaks as well as accumulated unrepaired

breaks that did not lead to apoptosis. Following our results, we did not have an indication of increased DNA damage in corneal cells of keratoconus patients compared to healthy and diseased controls.

This does not rule out a contribution to KC development by more specific mechanisms. For example, Atilano et al.[6,21] reported increased damage to mitochondrial DNA (mtDNA) in the corneas of patients with KC. However in another study, the corneal epithelium of KC patients did not appear to have increased DNA damage.

The majority of DNA sampled in our specimens should be regarded of epithelial origin and these short-lived mitotic highly active cells are replenished from the stem cell niche, located at the corneal limbus. The limbal region of the cornea is not explanted in routine corneal transplant surgery, though the epithelial cells should represent the genomic make-up of their progenitor stem cells.[22] This has been well studied and established for telomere attrition; due to the DNA end-replication

Table 1. Summary of DNA Damage in the Three Study Groups ($N = 54^a$)

	N	Mean Age	Mean ± SD (All Samples)	Multiple Comparison[b]	Mean ± SD (Outliers Removed)[c]	Multiple Comparison[b]
Keratoconus (KC)	26	42.7	5.56 ± 14.08		1.74 ± 2.71	
Decompensated grafts (DG)	17[a]	71.4	3.16 ± 8.22	P = 0.807	1.19 ± 1.24	P = 0.407
Healthy controls (HC)	11[a]	81.4	3.51 ± 6.66		0.71 ±1.61	

[a] Four samples in the DG group and five in the HC group did not yield sufficient DNA for analysis.
[b] Independent samples Kruskal-Wallis test.
[c] Three outliers were removed from the KC group, one from the DG group, and two from the HC group.

inefficiency of polymerases, chromosomes shorten every cell division. In line with this, the epithelial cells will reflect the number of cell divisions made by their progenitor cells. Since the differences we found between TLs point towards longer telomeres in KC patients, we assumed that there was not a clear indication of increased regeneration of corneal epithelial cells in keratoconus. Naturally, biologically younger samples were expected to have longer telomeres, and statistical correction for age effects was hampered by the poor age distribution among the three sample sets. Since to our knowledge this is the first report on TL in corneal tissue, no comparison with younger healthy subjects was available. Mallet et al.[23] demonstrated that corneal epithelial cells have very efficient DNA repair mechanisms, and are less prone to UV-induced apoptosis. Possibly the need to remove highly damaged cells is hereby reduced.[23] Overall, it seems that the morphologic characteristics of keratoconus are not mediated through accumulated DNA damage, aberrant DNA repair systems, or aberrant telomerase systems. Should the peculiar preclinical epithelial remodeling rather be attributed to inflammation in the corneal microenvironment?[24,25]

Interestingly, three patients with KC in our study underwent an epithelium-off corneal crosslinking procedure with UV-A irradiation years before the grafting procedure, and these patients only showed ×2.6 higher level of DNA damage compared to the other samples. Therefore our data did not support a putative link between induced UV-A exposure and DNA damage, which was suggested in a recent report on a case in which crosslinking was associated with intraepithelial neoplasia.[26]

Finally, our study investigated samples derived from patients who generally suffered from more advanced stages of corneal disease, which are inherently more eligible for corneal transplant surgery. To circumvent this, further investigations should preferably take into account early stages of KC and a longitudinal study design. This could be facilitated by studying corneal epithelium harvested at a CXL procedure, a promising tissue that we are currently exploiting in our laboratory to further explore the dynamics of KC pathology.

Conclusions

To our knowledge, this study is the first to quantify DNA damage using intact *Alu* elements and assess TL and *hTERT* expression in corneal tissues. In summary, we found no signs of increased DNA damage or replicative stress in corneal samples obtained from patients with keratoconus compared to healthy controls and those with decompensated grafts. Thus, the link between DNA repair system dysfunction or accumulated genetic changes due to oxidative stress or UV radiation in the development of progressive KC is unclear. Future longitudinal research should be aimed at younger samples of progressive KC. A thorough analysis of local and systemic autoinflammatory changes should further elucidate our understanding of KC development.

Acknowledgments

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