

Role of Thyroxine in the Development of Keratoconus

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Purpose: Keratoconus (KC) is a corneal ectasia whose pathophysiology is still mostly unknown. We investigated whether thyroid gland dysfunction (TGD) is associated with the development of KC.

Methods: We first conducted an epidemiological study, examining the prevalence of TGD among patients with KC. Then, we compared tear thyroxine (T4) in TGD and immunohistochemical staining of its receptors (T4Rs) between patients with KC and controls. The significance of T4 for corneal metabolism was studied in organotypic tissue cultures from monkey corneas.

Results: We found that TGD prevalence among patients with KC is 13.6%, which is higher than its prevalence in the general population (about 2%). Tear T4 was higher in KC, and keratocyte T4Rs were elevated in KC compared with controls. Furthermore, core proteins such as collagen and cytokeratins were equally altered both in KC and in the cultured corneas substituted with T4.

Conclusions: Our data implicate a crucial role of T4 in KC pathophysiology, which is most likely mediated by T4Rs.

Key Words: cornea, thyroid hormones, thyroid hormone receptors, immunoassay, immunohistochemistry, keratoconus

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Keratoconus (KC) is one of the most important corneal ectatic disorders, with onset at puberty. Major features of KC are thinning and conical ectasia of the cornea; its incidence rate is 1 per 50,000 among the general population.^{1,2} No causal pharmacological treatment of KC exists, although collagen cross-linking using riboflavin and UV-A light has been introduced recently and may alter the course of the disease when it is progressing.^{2–4}

Despite extensive clinical investigations and basic research, the pathophysiological processes underlying KC have not been fully elucidated with proposed mechanisms to include proteolytic degradation in the corneal stroma, oxidative damage, epithelial mechanical injury, immunological factors, and genetic factors.^{1,5} Hormonal imbalances are likely to affect the corneal metabolism and may be also associated with KC. Among the various endocrinologic dysfunctions assumed so far, thyroid gland dysfunction (TGD) (hypo- or hyperthyroidism; comprised as TGD) is frequently associated with eye diseases such as Graves disease. Patients with KC exhibiting symptoms of TGD were first observed in the 1930s, when Appelbaum reported a series of patients with KC with a high prevalence of hypothyroidism symptoms.⁶ Later, King reported KC after thyroidectomy.⁷ Thyroxine (T4) is crucial for corneal dehydration and transparency during embryonic development and regulates the synthesis of keratin sulfate proteoglycan in the chicken cornea.^{8,9} T4 receptors (T4Rs) have been found in the lacrimal gland, confirming that the tear-producing gland is a target organ of T4.¹⁰ The T4 level was found to be elevated in the tears of patients with KC.¹¹ It is also interesting that KC usually begins at childhood and puberty, when the level of T4-binding globulin is lowest.¹² The initial clinical signs of KC are irregular astigmatism and irregularities in the corneal topography.¹³ All these data represent evidence of a clinical association between KC and abnormal thyroid gland function, but the significance of these observations remains to be established. In this study, we examined this clinical association and the role of T4 in KC pathophysiology.

MATERIALS AND METHODS

Study Population and Data Collection

Anonymous human samples were examined according to the guidelines of the Ethics Commission of the Deutsche Ärztekammer. The ethics committee of the University approved the use of probes. Written informed consent was obtained for the use of tissue samples from the cornea bank, and the methods adhered to the tenets of the Declaration of Helsinki, which involved that all tissue material used in this study was handled anonymously at all times.

We studied the demographic, ophthalmologic, and endocrinologic profiles of 154 patients with KC treated in our clinic. Our screening included evaluation of the demographic data, and complete ophthalmologic examination. Corneal

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topography maps were examined using the Rabinowitz–McDonnell criteria for the clinical diagnosis of KC, based on the central corneal power, the inferosuperior asymmetry value, and the difference in central corneal power between both eyes of a given subject.¹³

The endocrinologic examination included measuring the serum concentrations of free T4 (fT4) and thyroid-stimulating hormone (TSH) using an immunoassay method (ADVIA Centaur; Bayer Diagnostics, Germany). The normal reference ranges for TSH and fT4 were determined to be 0.35 to 4.5 mIU/L and 0.7 to 1.65 ng/dL, respectively (Central University Laboratory of the Saarland University Medical Center). Clinical cases with fT4 serum concentrations beyond these reference ranges were defined as TGD. All examinations were conducted at the University Hospital Münster and Saarland University Medical Center and handled anonymously.

T4 Levels in the Tears

Tear samples were collected from all patients with KC and TGD, from a group of patients with KC but without TGD, and from a sex- and age-matched control group of healthy subjects who were free of ocular pathology and TGD. The normal subjects were classified as euthyroid after serum TSH and T4 levels were measured using a previously described immunoassay method used for patients with KC (ADVIA Centaur; Bayer Diagnostics).

Tear T4 levels were measured using 50 μ L of tear fluid that was obtained with the aid of microcapillaries from all groups and then stored at -80°C for up to 2 weeks. Multiple samples were taken from each patient during a number of visits. All samples were analyzed using an ELISA T4 Kit (ADVIA Centaur; Bayer Diagnostics). Standard curves were prepared using diluted serum samples with known T4 concentrations. Then, 40- μ L aliquots of each tear sample were used for T4 determination, and the assays were repeated 3 times.

Immunohistochemistry

The control group consisted of sections obtained from 20 normal corneoscleral rings (10 of male origin and 10 of female origin) and 20 central corneal buttons from patients with KC (10 of male origin and 10 of female origin) who underwent penetrating keratoplasty (PK). The mean age of the donors was 32 years (range: 25–56 yrs), and the mean age of the patients with KC was 48.6 years (range: 22–66 yrs). All tissues were obtained from the local cornea bank at the University Eye Hospital, Münster, Germany.

Scleral tissue was removed from the corneoscleral rings with a razor blade under a dissecting microscope, and the central corneal buttons were then cut into 2 equal segments. These small tissue blocks were frozen in liquid nitrogen to prepare 12- μ m-thick cryostat sections (Jung CM 1500; Leica Microsystems, Wetzlar, Germany). Sections were collected on clean gelatin-coated glass slides and dried for 2 hours at room temperature. All sections were fixed in cold 99% methanol for 10 minutes at -20°C , washed 3 times for 5 minutes each in phosphate-buffered saline, and blocked

with 10% fetal calf serum (FCS) for 30 minutes. The primary antibody to T4Rs was a mouse anti-human monoclonal antibody (V10283, Biomedica; Biozol, Eching, Germany) used at a dilution of 1:100. For immunohistochemistry (IHC), primary antibodies were diluted in 10% FCS (1:100); then, the sections were incubated overnight at 4°C and processed using standardized laboratory protocols. Specific binding for T4Rs was detected by a donkey anti-goat Cy2-conjugated secondary antibody, a goat anti-mouse Cy2-conjugated secondary antibody, and a goat anti-rabbit Cy2-conjugated secondary antibody (all 3 from Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the probes were mounted in antifading solution for fluorescence microscopy (Mowiol; Merck, Darmstadt, Germany) containing bisBenzimide H 33258 (4',6-diamidino-2-phenylindole [DAPI]; Sigma, St. Louis, MO) and observed with under a fluorescence microscope (Axiophot; Carl Zeiss Meditec, Oberkochen, Germany) using appropriate filters (395–440 nm for DAPI and 450–490 nm for Cy2). Photographs were taken with a digital camera attached to the microscope (AxioCam, Carl Zeiss Meditec).

Animal Tissue

All animal research has been conducted according to relevant national and international guidelines. No monkey was killed for the purpose of this study. All tissues (eyes) were obtained from monkeys after the animals were killed within the frame of experiments in the Institute of Regenerative Medicine (ethics committee approval number: LANUV: 39.32.7.1/39.32.7.2.1 and LANUV: 8.87-50.10.46.09.018).

Corneal Isolation and Culture

Corneas were obtained from 44 cadaver eyes of 22 adult monkeys (*Macaca fascicularis*) of both sexes from the breeding colony of the Institute for Regenerative Medicine, Münster within 30 minutes after death and enucleation (approval: 8.87-50.10.46.09.018). The breeding and use of the monkeys was approved by the state's ethics within the frame of research by the Institute of Regenerative Medicine (approval: 39.32.7.1/39.32.7.2.1). The corneas were removed by trephination (with a 6-mm trephine) and transferred to sterile Hanks balanced salt solution (pH 7.4). The corneas were divided into 3 groups as follows: for IHC ($n = 5$), Western blotting (WB; $n = 5$), and organ cultures ($n = 34$). Monkey corneas were used as controls. Before culturing, the epithelium was mechanically removed using a surgical scalpel and cultured separately from the stroma and endothelium, to selectively study the T4 effects on each layer ($n = 30$). In a control group, the total corneas were cultured without previous epithelial removal. Thirty corneas were divided into 5 subgroups ($n = 6$ corneas each) in which culturing was performed with concentrations of T4 ranging from 0 to 5000 mg/L. The medium (Dulbecco's modified Eagle's medium + 10% FCS) was exchanged every 3 days with fresh medium plus the respective hormone concentration in each group. Cell growth was monitored by phase contrast microscopy for 14 days, after which all tissues were

collected and harvested for either IHC or WB analysis. The aforementioned antibodies were used for the sections and WB analysis. Before use, each antibody was checked for whether it cross-reacted with its antigen in noncultured monkey corneal sections.

Statistical Analysis

The Mann–Whitney test was used for statistical comparisons between the study groups. *P* < 0.05 was considered statistically significant. Otherwise, the *z*-test was used except where stated otherwise. Statistical analysis was performed with SPSS statistics software (SPSS for Windows, version 11.0; SPSS, Chicago, IL).

RESULTS

Increased Prevalence of TGD in the Study Population of KC

In the population of 154 patients with KC and TGD examined (Table 1), 21 patients (6 men and 15 women; 13.6%) had TGD, of which 19 (5 men and 14 women; 12.3%) had hypothyroidism and 2 (1 man and 1 woman; 1.3%) had hyperthyroidism. There were 15 patients with severe KC and 6 patients with moderate KC. The age at diagnosis was 25.9 ± 7.1 years (mean ± SD) in patients without TGD (non-TGD) and 36.8 ± 10.8 years in TGD patients. Mean visual acuity without best correction at the last follow-up in the right and left eyes was 0.30 ± 0.32 and

0.05 ± 0.10, respectively, in the TGD group, and 0.49 ± 0.36 and 0.44 ± 0.34 in the non-TGD group. Corneal hydrops was present in 9 (5 men and 4 women; 5.8%) of the 154 patients. All 9 cases affected the right eye. Three (14.3%) of the 21 TGD patients developed acute KC.

A total of 117 (75.9%) patients (76 men and 80.8%; 41 women, 66.6%) received PK, comprising 19 patients (4 men and 15 women; 86.3%) of the TGD group and 91 patients (62 men and 29 women; 69.9%) of the non-TGD group. The mean age at the operation was 43.4 ± 14.1 years for the TGD group and 31.8 ± 10.4 for the non-TGD group. The indication for PK was unacceptable visual acuity in 98 patients (57 men and 41 women; 83.7%) and contact lens intolerance in 19 patients (14 men and 5 women; 16.2%).

Thyroxine Levels Increased in the Tears of KC Patients With and Without TGD

The apparently high prevalence of TGD in patients with KC prompted us to examine the T4 level within the tear fluid. The T4 tear concentration was significantly higher in all 21 patients with KC and TGD (11.8 ± 6.2 nM/L) than in the normal controls (3.0 ± 1.7 nM/L) (*P* < 0.0001, Mann–Whitney test), and higher in patients with KC but without manifest clinical TGD (*n* = 21; 10.9 ± 5.1 nM/L) than in controls (*P* < 0.0001, Mann–Whitney test). The T4 tear concentration did not differ significantly between patients with KC and TGD and those with KC but without TGD (*P* < 0.0001, Mann–Whitney test). The immunoassay results are summarized in Figure 1. The data indicate that even in nonmanifested TGD, KC is accompanied by disturbances in the amount of T4 in the tears.

T4R Expression Upregulated in Stromal Keratocytes of KC Corneas

T4 circulating within either the blood or the tears binds and activate cellular receptors. We therefore sought to localize T4Rs within the cornea. Twelve corneas without ectasia and 20 KC corneas were processed for immunostaining. Control sections processed without the first antibody remained unstained. T4R expression was apparent in the normal cornea (Fig. 2) and was localized within the epithelium, absent in stromal keratocytes, and reappearing within the endothelium. The distribution of T4R staining was uniform both along the depth of the epithelium (Figs. 2B, C) and along the eccentricity of the cornea (data not shown). Individual stromal keratocytes were only faintly stained for T4Rs (Figs. 2E, F). The endothelium showed strong expression of T4Rs (Figs. 2H, I). Figures 2A, D, and G represent DAPI stain. T4Rs were also detected in all KC corneas, showing a similar localization within the entire depth of the epithelium (Figs. 3B, C). In addition, the stromal keratocytes were clearly immunostained (Figs. 3E, F) and showed brighter positive immunostaining for T4Rs than in normal corneas. The receptor expression in the endothelium (Figs. 3H, I) was similar to that for normal keratocytes. T4Rs in epithelium was similar in KC and normal corneas. In stromal keratocytes, the T4R expression was significantly higher in KC corneas (*n* = 20) than in normal corneas (*n* = 12). These data

TABLE 1. Demographic Data of Patients With KC

Demographic Data	Patients with KC			<i>P</i>
	Men (N = 94), Mean ± SD or N (%)	Women (N = 60), Mean ± SD or N (%)	Total (N = 154), Mean ± SD or N (%)	
Race				
White	75 (79.78)	43 (71.66)	118 (76.62)	0.03
Black	19 (20.22)	17 (28.34)	36 (23.38)	0.42
Age at diagnosis, yrs	26.0 ± 7.7	28.6 ± 7.9	26.8 ± 7.8	0.24
Topography				
Right eye	41 (43.61)	22 (36.66)	63 (40.90)	0.02
Left eye	35 (37.23)	21 (35.00)	56 (36.36)	0.04
Both eyes	18 (19.14)	17 (28.33)	35 (22.72)	0.32
Contact lens wear	78 (82.97)	50 (83.33)	128 (83.11)	0.18
Operation				
Right eye	35 (37.23)	19 (31.66)	54 (35.06)	0.16
Left eye	41 (43.61)	22 (36.66)	63 (40.90)	0.03
Both eyes	76 (80.85)	41 (68.33)	117 (75.97)	0.11
Age at operation, yrs	31.3 ± 9.5	35.2 ± 12.9	32.7 ± 11.1	0.14
Total time of follow-up, yrs	10.57 ± 7.74	9.63 ± 7.77	10.27 ± 7.72	0.21

The Student test was applied for mean ± SD comparison. The chi-squared test was applied for frequencies comparison. *P* < 0.05 was considered statistically significant.

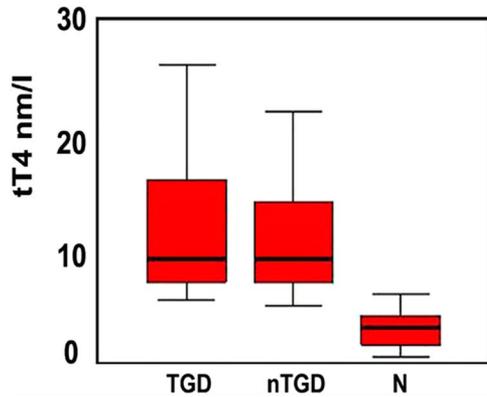


FIGURE 1. Tear concentration of thyroxine (T4) in patients with KC and normal subjects. T4 concentration in tears as analyzed by an immunoassay method (tT4; in nmol/L) in patients with KC and TGD (TGD, n = 21), patients with KC but without TGD (nTGD, n = 21), and euthyroidic normal subjects free of ocular pathology (N, n = 21). tT4 values in the TGD, nTGD, and N groups were 11.8 ± 6.2 , 10.9 ± 5.1 , and 3.0 ± 1.7 nmol/L, respectively. tT4 was significantly higher in TGD and nTGD groups than in the N group (both $P < 0.0001$, Mann–Whitney test).

show that expression of T4Rs is altered within the corneal tissue, especially in keratocytes, in KC.

In addition to IHC, the results from which were barely quantifiable, WB was performed with stroma and epithelium obtained from controls and KC corneas. It seemed that expression of T4Rs (55- and 52-kDa bands) was similar within the epithelium, whereas both bands were upregulated in the stroma of KC corneas (Figs. 3J, K).

To ensure that the antibodies provide similar staining in the monkey cornea, IHC was performed with sections from the normal monkey cornea. Both antibodies tested positive in the monkey cornea (see Figure 1A–D, Supplemental Digital Content 1, <http://links.lww.com/ICO/A444>), and their pattern of staining was very similar to that described in the human cornea, being localized within the epithelium and endothelium while sparing stromal keratocytes.

T4R Expression Depends on T4 Concentration

To examine whether regulation of the T4Rs depends on T4, healthy corneas obtained from adult monkey eyes (n = 44) were cultured for 2 weeks in media containing T4 at various concentrations. As known from human eye banking, thinning of the epithelium to a monolayer over the Bowman membrane (BM) was observed throughout culturing. Checking the cultures by routine microscopy revealed that the cell proliferation was higher in T4-treated cultures than in control cultures (data not shown). Sectioning and immunostaining for T4R expression in cultures without T4 in the medium revealed that the staining was localized within the thinned and superficial stroma (Figs. 4B, C). Treatment with 1000 mg/L T4 resulted in comparable T4R staining within the epithelium, but increased staining within the stromal keratocytes (Figs. 4E, F). Analogously, the endothelium showed similar expression of T4Rs in corneas lacking T4 (Figs. 4H, I). However, substitution with 1000 mg/l thyroxine increased T4R staining in the stromal keratocytes (Figs. 4K, L). Figures 4A, D, G, and J represent DAPI stain. Other concentrations of T4 ranging between 0 and 5000 mg/L resulted in similar staining (data not shown).

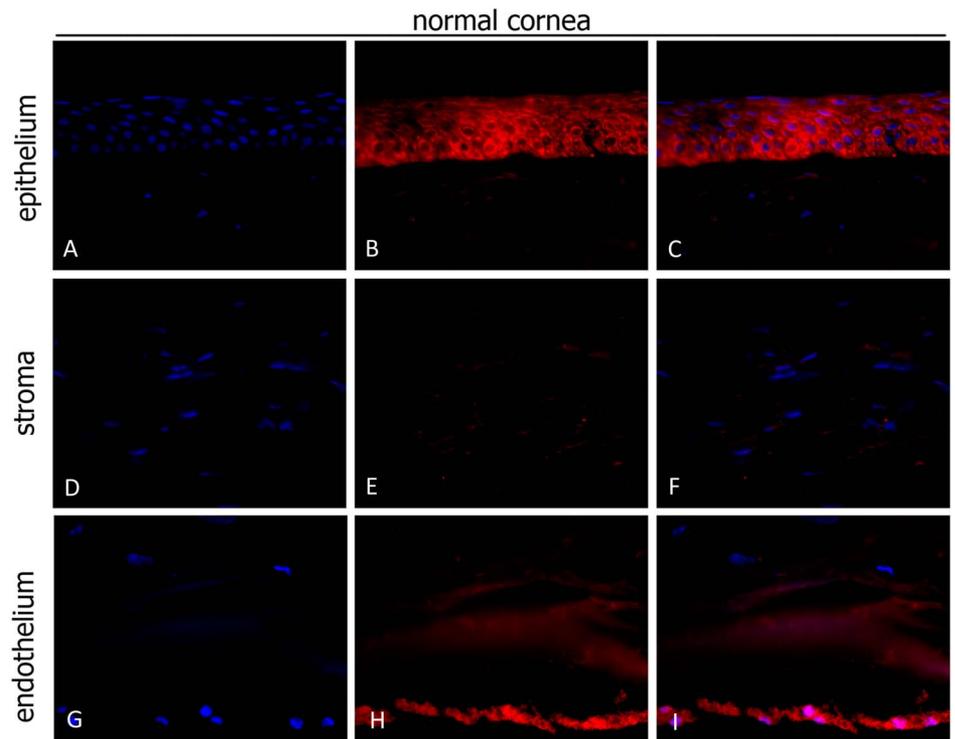


FIGURE 2. Expression of thyroxine receptors (T4Rs) in the normal human cornea. Nuclei of corneal epithelial cells were stained blue (DAPI). Positive immunostaining appeared red (Cy3). Immunostaining for T4Rs was positive throughout the epithelium (A–C) and endothelium (G–I) in the control corneas. There was no immunostaining in the stroma (D–F). Scale bar: 100 μ m.

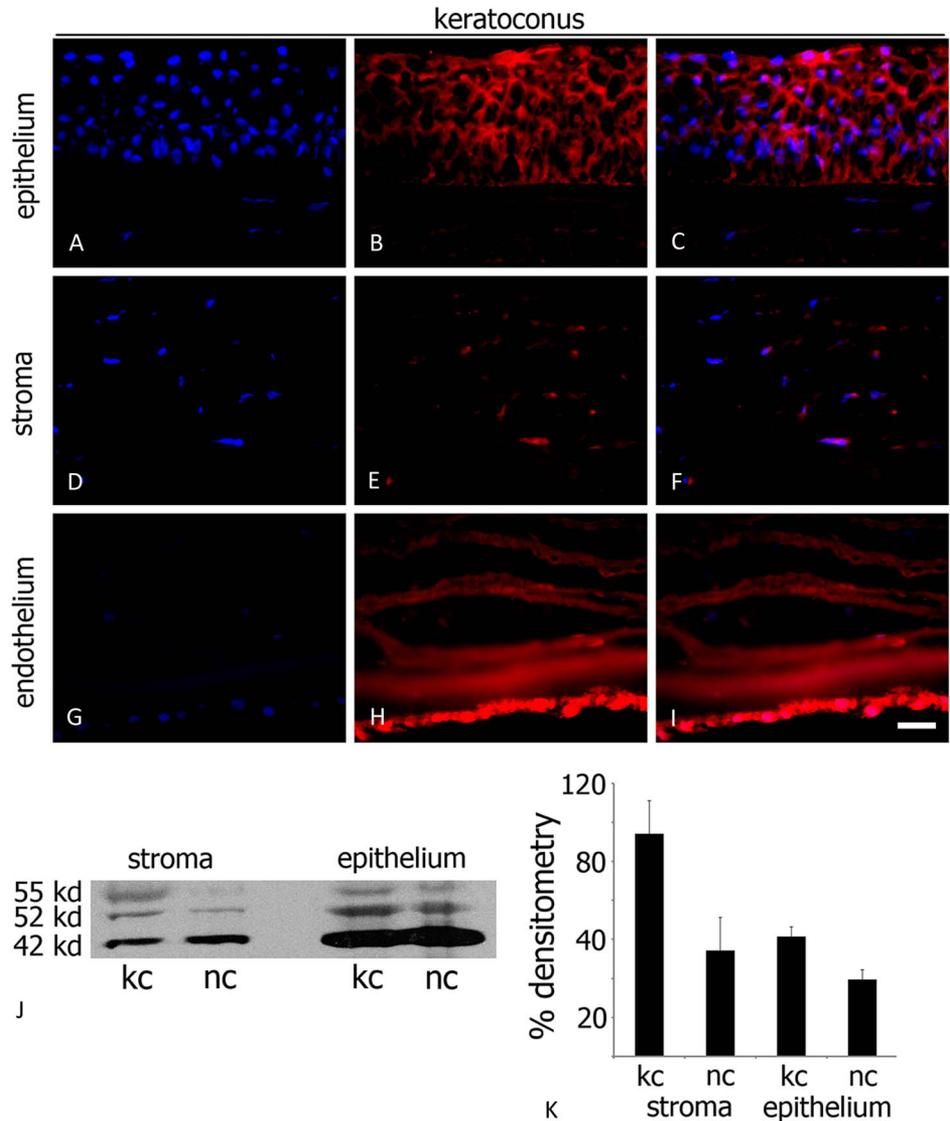


FIGURE 3. Expression of thyroxine receptors (T4Rs) in the KC cornea. Dense immunostaining was evident in the epithelium (B–C). The stromal keratocytes were clearly positive for T4Rs (E–F), whereas the endothelium also showed clear staining (H–I). J, WB confirming the expression of T4Rs both in normal (nc) and KC (kc) tissue. The staining also showed 2 bands corresponding to alpha and beta subunits at 52 and 55 kDa, respectively, whereas a 42-kDa strong band was the ladder control (actin). Although expression in the epithelium was comparable in diseased (kc) and normal (nc) tissue, staining for both bands in the stroma was stronger in the diseased cornea. K, Bars showing relative expression measured by densitometry. Controls showed no signal as expected (A, D, G).

To examine whether this higher staining intensity was caused by upregulation of T4Rs, WB was performed with both stroma and epithelium being cultured with T4 at different concentrations. It seemed that T4 increased the T4R bands compared with controls in the stromal tissue (Figs. 4M, N), whereas the T4R expression remained comparable to that in control tissue in the epithelium (Figs. 4M, N). These data indicate a regulatory effect of T4 on stromal monkey keratocyte T4R expression and support the data obtained on human probes showing that the regulation of T4Rs differs between epithelium and stroma.

Different Concentrations of the T4 Alter the Expression of Collagens Within the Cornea

Next, we examined staining of monkey corneal sections for different extracellular matrix (ECM) proteins. It seemed that collagen types 1 to 7 were expressed in layers of different

intensities in the cornea (see Figure 2, Supplemental Digital Content 2, <http://links.lww.com/ICO/A445>). In addition, cyokeratin 13 was clearly stained before culturing (see Figure 2, Supplemental Digital Content 2, <http://links.lww.com/ICO/A445>). Increasing the concentration of T4 in monkey cornea cultures enhanced the staining for collagen types 1, 6, and 7 (Fig. 5) but had no effect on the staining for collagen 5 (Figs. 5C, D). Collagen 1 was especially strongly expressed (Fig. 5B) compared with the T4-free cultured cornea (Fig. 5A). Collagen 6 was expressed within the stroma when cultured in 1000 mg/L T4 (Fig. 5F), with the staining being stronger than in the culture without T4 (Fig. 5E). Finally, collagen 7, which showed BM localization in the noncultured cornea (compare with see Figure 2J, Supplemental Digital Content 2, <http://links.lww.com/ICO/A445>), was also located in the BM in corneas cultured without T4 (Fig. 5G) and exhibited clearly enhanced staining in the cornea cultures with higher concentrations of T4 (Fig. 5H).

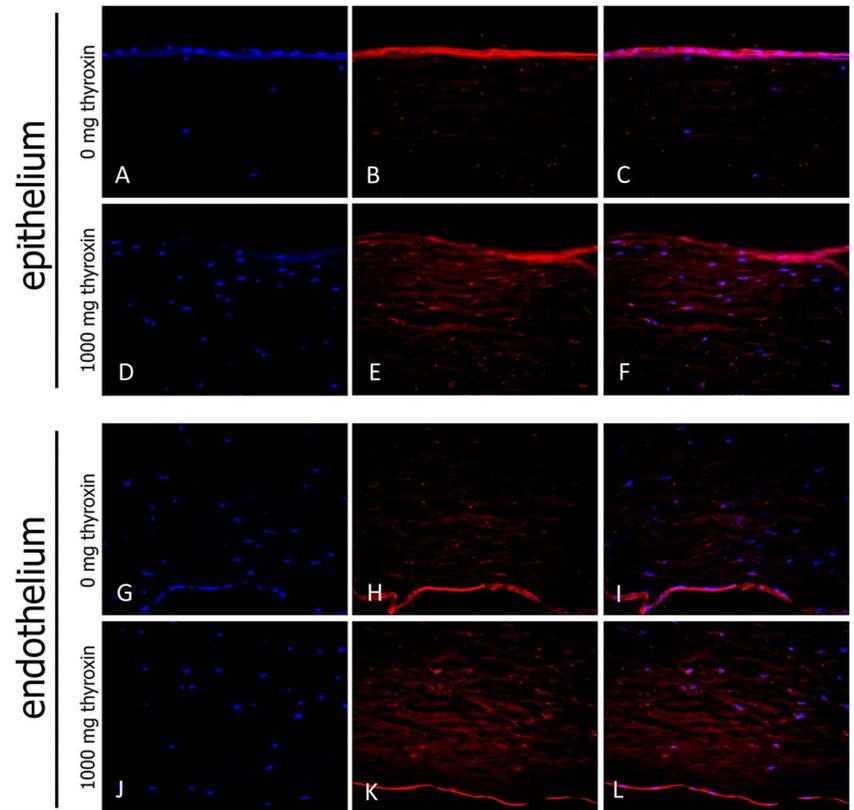
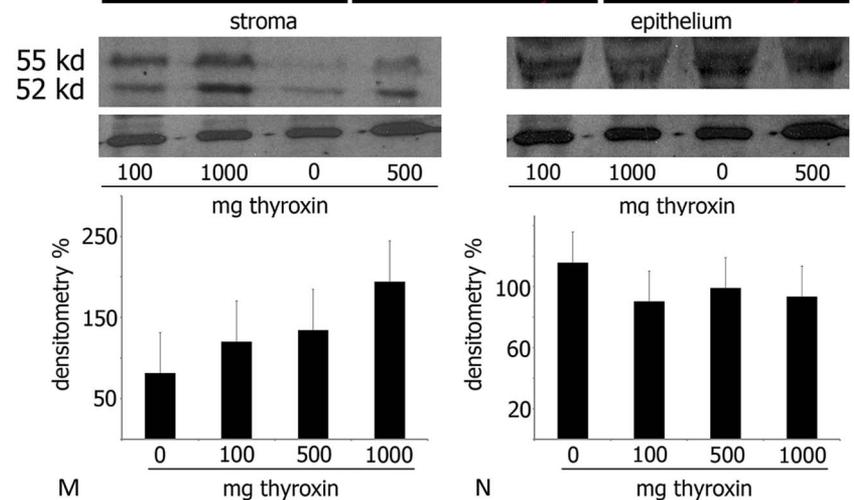


FIGURE 4. Expression of thyroxine receptors (T4Rs) in the cultured monkey cornea. Nuclei of corneal cells were stained blue (DAPI). A–C, Control section cultured for 14 days without T4. The epithelium and stromal keratocytes were stained. D–F, Section from T4-substituted culture (1000 mg/L T4) showing a similar expression of T4Rs within the epithelium but brighter fluorescence in keratocytes. G–I, Control section showing staining of the endothelium and inner keratocytes. J–L, Section from a T4-treated cornea (1000 mg/L) showing similar staining in the endothelium but brighter fluorescence in keratocytes. M and N, Immunoblotting of stroma and epithelium showing a selective upregulation of T4Rs (bands 52 and 55 kDa) in the stroma cultures exposed to 100, 500, and 1000 mg/L T4. Expression within the epithelium was not detectably changed (N) and remained high in all probes.



Cytokeratin 13 in the Cornea

Cytokeratin 13 is localized within the epithelium and BM in the monkey (see Figure 2K, Supplemental Digital Content 2, <http://links.lww.com/ICO/A445>). In the cultured monkey cornea, cytokeratin 13 staining in the BM increased with the T4 concentration (see Figure 3A–D, Supplemental Digital Content 3, <http://links.lww.com/ICO/A446>). In WB, the antibody formed 2 bands at 48 and 54 kDa, both of which were enhanced with higher concentrations of T4 (see Figures 3E, F, Supplemental Digital Content 3, <http://links.lww.com/ICO/A446>).

In human normal corneal sections, cytokeratin 13 was uniformly localized within the epithelium and BM, including

the superficial stroma (Figs. 6A, B). Because of the different degrees of KC disease state (expected in the diseased tissue obtained from different eyes), we decided to stain several KC corneas with cytokeratin 13 antibodies (n = 8). Figures 6C–F show representative sections from 6 KC corneas. The protein was localized within the entire epithelium, including the superficial cells, showing abnormal distribution compared with the healthy corneas. In particular, cytokeratin 13 was found in basal microlesions (Figs. 6C–F) and epithelial detachments from the BM. These data show that cytokeratin 13 is associated with anatomical detachment sites between the basal epithelium and BM.

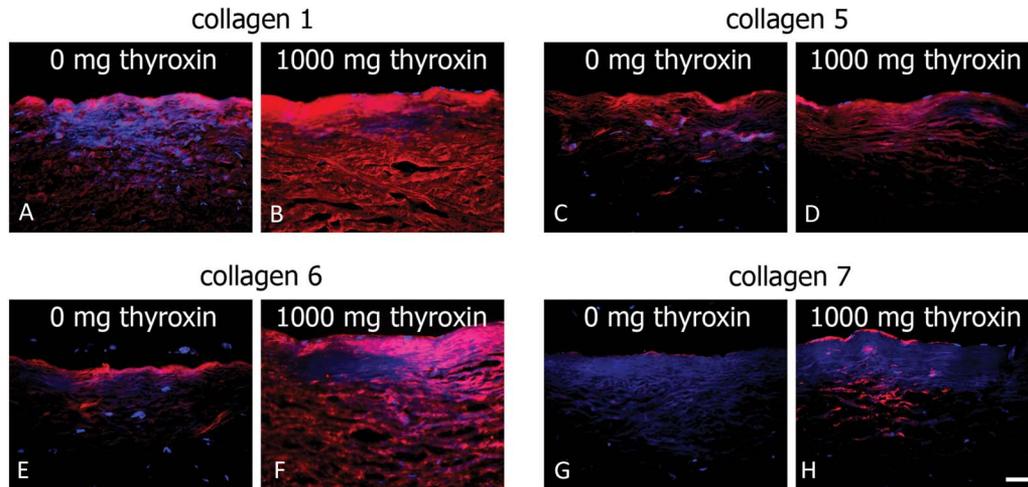


FIGURE 5. IHC in the thyroxine (T4)-incubated monkey cornea. Staining for selected collagens in sections through cultured stroma without T4 and with 1000 mg/L T4. Collagen types 1, 5, 6, and 7 seemed upregulated with T4 substitution. Scale bar: 100 μ m.

DISCUSSION

The epidemiological part of our study revealed that the prevalence of TGD was significantly higher in patients with KC than in the general population; the prevalence of hypothyroidism was 23.3% in @women (14/60) and 5.3% in men (5/94) in the KC group, whereas it is 2% in women and 0.2% in men in the general population.¹⁴ This finding is consistent with clinical observations suggesting an association between KC and TGD of hypothyroidism,¹⁴⁻¹⁶ the iatrogenic induction of KC after thyroidectomy,⁷ and of Hashimoto autoimmune thyroiditis.¹⁷ Interestingly, we found

significantly higher concentrations of T4 in tear samples of patients with KC and TGD and those with KC but without TGD, compared with age-matched controls without ocular pathology. This indicates that next to the high prevalence of systemic TGD in our KC population, T4 is specifically altered in the tear milieu of all patients with KC.

The important role of T4 in corneal^{9,18,19} and bone development²⁰⁻²³ might be related to its role in collagen synthesis, because the corneal stroma consists predominantly of collagens organized into collagenous fibrils. It is therefore not surprising that T4Rs are also localized within collagen-

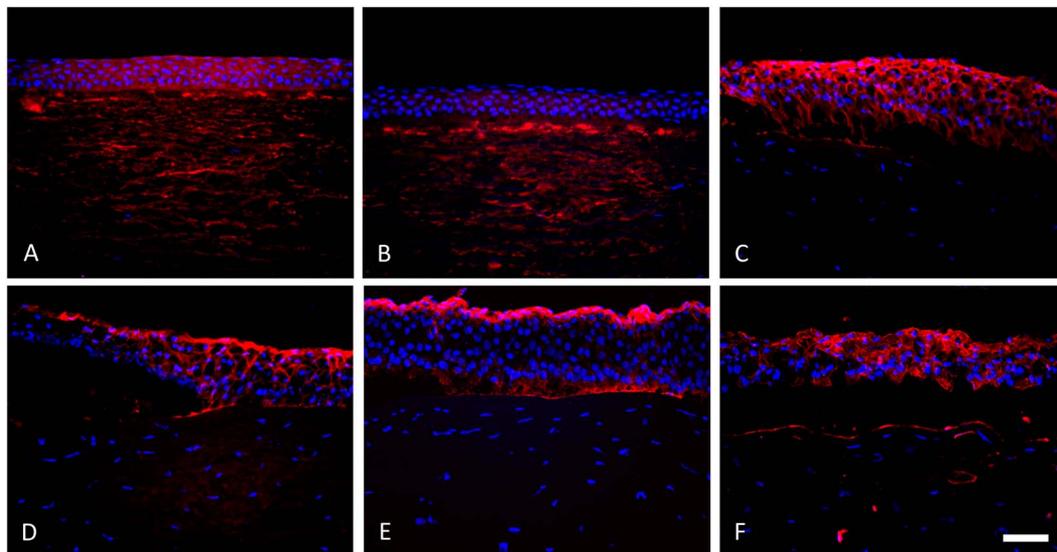


FIGURE 6. IHC for cytokeratin 13 in the normal human cornea and KC cornea. A and B, Staining for cytokeratin 13 in the normal human cornea with strong staining along the BM and superficial stroma. Diffuse staining was also observed within the epithelium. C-F, Examples of KC corneas showing a translocation of the cytokeratin staining toward the superficial epithelium, and stronger expression within the basal epithelium. In addition, the BM showed strong staining, with microlesions and detachments of the epithelium from the BM being clearly outlined by cytokeratin 13. Scale bar: 100 μ m.

producing stromal keratocytes both in human and monkey corneas. We found that different collagens were altered within cultured monkey corneas as the concentration of T4 was increased. Collagenases or other proteases within the stroma can change to induce thinning and ectasia, and it may be that T4-T4R is one of the regulators of the homeostasis between collagen degradation and synthesis in the tissue.⁸ In addition, T4Rs may influence other components of the ECM.⁹ These data suggest a role for T4s in collagen metabolism in the cornea, as in other tissues characterized by high collagen content. The direct T4R-regulatory effects of T4 on cultured monkey corneal stroma that we found are likely a line of evidence that this hormone can influence keratocyte metabolism.

KC usually begins at puberty when the thyroid binding globulin level is lowest, and therefore alters T4 homeostasis in this age group.¹² The increased T4 concentration in the tear fluid of patients with KC, which we found, is independent of the manifestation of TGD. These results are consistent with T4 being elevated 2- to 50-fold in KC tears, especially during the progressive phase of the disease.¹¹ This excess T4 might be attributable to the inadequate use of tear T4 by the cornea, ineffective binding of T4 to epithelial hormone receptors, or depressed collagen synthesis in corneal stroma, and therefore a low metabolic demand for T4. Interestingly, the T4 tear concentration was significantly higher in patients with KC than in normal subjects without ocular pathology. Analogously, T4Rs were increased in the keratocytes of patients with KC but not in the epithelium, which supports the hypothesis that KC is primarily a stromal disease. The tear T4 concentrations were elevated even in the hypothyroid patients with KC with low serum T4 concentrations, which suggests that T4 accumulates in tears because of inadequate use of tear T4. IHC staining and WB of T4Rs revealed that this incidence was higher in the stromal keratocytes in KC than in the normal corneas. This is supported by the culture experiments with monkey corneas also showing a T4-dependent stromal keratocyte-targeted upregulation of T4Rs. It is not surprising therefore that we found an altered expression of various collagens in corneal cultures with different doses of T4 in the medium.

ECM components other than collagens also seem to respond to T4. As an example, we demonstrated that cytokeratin 13 changed its site of expression in KC corneas, with diffuse staining throughout the epithelium depth. Interestingly, cytokeratin staining was strong at basal epithelium breaks and at sites of detachment from the underlying BM. These data may point to either primary pathophysiological involvement in the detachment or to secondary expression because of the preceding detachment. Data from IHC alone are insufficient to decide on either mechanism. However, the direct relationship between cytokeratin 13 expression and T4 was demonstrated in the cultured monkey cornea, together with enhanced expression of T4Rs. These data support the view that thyroxine-driven T4R upregulation in keratocytes probably causes the cytokeratin 13 increase, at least in the cultured cornea. A similar mechanism may operate in the manifestation of KC with high TH in the tears to induce T4R upregulation, and subsequently changes in the expression of cytokeratin 13.

A limitation of this study is that it remains to be shown which molecular cell-signaling mechanisms operate to produce progressive ectasia of the corneal tissue. The origin of KC was assumed to be the epithelium or stroma, with stromal proteases and their inhibitors.²⁴ No correlation has been reported between the stromal proteases and hormone receptors. However, the clinical, IHC, and experimental results reported in this study implicate T4 in KC pathophysiology, thereby defining a certain endocrinologic aspect of the probable multifactorial etiology of KC. Independent of the underlying mechanisms, early treatment of TGD might positively influence the progression of KC. Therefore, it is crucially important to design further studies aimed at elucidating the roles of T4 and T4Rs in KC to unravel the signal transduction pathways mediating the onset and progression of the disease. In addition, studies involving T3 supplementation at least in patients with KC suffering from evident systemic hypothyroidism may shed light into further aspects of KC development; such studies are currently ongoing in our laboratory. Moreover, factors such as contact lens wear, corneal hypesthesia, dry eye disease, etc might have influenced the T4 concentration in tears. However, it was not possible to match the study and control groups for all these factors that could potentially affect the T4 concentration in tears. This is another limitation of our study.

In summary, we were able to demonstrate a direct association between thyroxine and KC. The data obtained on (1) clinical and epidemiological findings of KC, (2) histopathological images from KC sections, and (3) laboratory experiments of the monkey cornea suggest that TGD changes could have a role in the development of KC, thereby requiring further investigation.

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REFERENCES

- Rabinowitz YS. Keratoconus. *Surv Ophthalmol*. 1998;42:297-319.
- Kennedy RH, Bourne WM, Dyer JA. A 48-year clinical and epidemiologic study of keratoconus. *Am J Ophthalmol*. 1986;101:267-273.
- Smiddy WE, Hamburg TR, Kracher GP, et al. Keratoconus. Contact lens or keratoplasty? *Ophthalmology*. 1988;95:487-492.
- Raiskup-Wolf F, Hoyer A, Spoerl E, et al. Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: long-term results. *J Cataract Refract Surg*. 2008;34:796-801.
- Bochert A, Berlau J, Koczan D, et al. Gene expression in keratoconus. Initial results using DNA microarrays [in German]. *Ophthalmologe*. 2003;100:545-549.
- Appelbaum A. Keratoconus. *Arch Ophthalmol*. 1936;15:900-921.
- King ET. Keratoconus following thyroidectomy. *Trans Ophthalm Soc UK*. 1953;73:31-39.
- Conrad AH, Zhang Y, Walker AR, et al. Thyroxine affects expression of KSPG-related genes, the carbonic anhydrase II gene, and KS sulfation in the embryonic chicken cornea. *Invest Ophthalmol Vis Sci*. 2006;47:120-132.

9. Coulombre AJ, Coulombre JL. Corneal development. 3. The role of the thyroid in dehydration and the development of transparency. *Exp Eye Res.* 1964;3:105–114.
10. Dias AC, Módulo CM, Jorge AG, et al. Influence of thyroid hormone on thyroid hormone receptor beta-1 expression and lacrimal gland and ocular surface morphology. *Invest Ophthalmol Vis Sci.* 2007;48:3038–3042.
11. Káhn IL, Varsányi-Nagy M, Toth M, et al. The possible role of tear fluid thyroxine in keratoconus development. *Exp Eye Res.* 1990;50:339–343.
12. Fisher DA, Sack J, Oddie TH, et al. Serum T4, TBG, T3 uptake, T3, reverse T3, and TSH concentrations in children 1 to 15 years of age. *J Clin Endocrinol Metab.* 1977;45:191–198.
13. Rabinowitz YS, McDonnell PJ. Computer-assisted corneal topography in keratoconus. *Refract Corneal Surg.* 1989;5:400–408.
14. Tunbridge WM, Evered DC, Hall R, et al. The spectrum of thyroid disease in a community: the Whickham survey. *Clin Endocrinol (oxf).* 1977;7:481–493.
15. Gatziofias Z, Thanos S. Acute keratoconus induced by hypothyroxinemia during pregnancy. *J Endocrinol Invest.* 2008;31:262–266.
16. Lang GE, Naumann GO. Keratoconus in Alagille syndrome [in German]. *Klin Monbl Augenheilkd.* 1991;98:555–557.
17. Koçak Altintas AG, Gül U, Duman S. Bilateral keratoconus associated with Hashimoto's disease, alopecia areata and atopic keratoconjunctivitis. *Eur J Ophthalmol.* 1999;9:130–133.
18. Beckenhauer DM, Conrad GW. The effect of thyroxine on transparency and PAPS synthesis in the avian cornea. *Dev Biol.* 1981;84:225–229.
19. Masterson E, Edelhauser HF, Van Horn DL. The role of thyroid hormone in the development of the chick corneal endothelium and epithelium. *Invest Ophthalmol Vis Sci.* 1977;16:105–115.
20. Bassett JH, Williams GR. The molecular actions of thyroid hormone in bone. *Trends Endocrinol Metab.* 2003;14:356–364.
21. Robson H, Siebler T, Stevens DA, et al. Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation. *Endocrinology.* 2000;141:3887–3897.
22. Siragusa V, Terenghi A, Rondanini GF, et al. Congenital hypothyroidism: auxological retrospective study during the first six years of age. *J Endocrinol Invest.* 1996;19:224–229.
23. Pereira RC, Jorgetti V, Canalis E. Triiodothyronine induces collagenase-3 and gelatinase B expression in murine osteoblasts. *Am J Physiol.* 1999;277: E496–E504.
24. Collier SA. Is the corneal degradation in keratoconus caused by matrix-metalloproteinases? *Clin Exp Ophthalmol.* 2001;29:340–344.