Original Article
Effects of thyroxine and hydrocortisone on normal human keratocytes and keratoconic keratocytes in vitro

Xuefei Song1,2, Zisis Gatziofas1,3, Jiong Wang4, Tanja Stachon1, David O’Brart5, Nora Szentmary4, Valerie Saw3, Achim Langenbicher6, Berthold Seitz1

1Department of Ophthalmology, Saarland University Medical Center, Homburg, Saar, Germany; 2Department of Ophthalmology, Shanghai Ninth People’s Hospital, Shanghai, China; 3Moorfields Eye Hospital, London, United Kingdom; 4Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; 5Department of Ophthalmology, St Thomas Hospital, London, United Kingdom; 6Experimental Ophthalmology, Saarland University, Homburg, Saar, Germany

Received March 9, 2016; Accepted June 3, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Purpose: To investigate the effects of thyroxine and hydrocortisone on the viability, proliferation, apoptosis, growth factor and interleukin secretion of human keratocytes in vitro in normal and keratoconic corneas. Methods: Seven normal corneas, not meeting the criteria for corneal transplantation, as well as five corneal buttons from patients with keratoconus who underwent keratoplasty were used to isolate normal human primary keratocytes (NHPK) and keratoconus human primary keratocytes (KHPK). NHPK and KHPK were treated with thyroxine and hydrocortisone in concentrations of 0, 0.01, 0.1, 1 and 10 µg/ml for 24 h. Cell viability was determined by AlamarBlue® assay and cell proliferation by the cell proliferation ELISA BrdU kit, while cell apoptosis was evaluated with the APO-DIRECT™ kit. Five and 24 hours after hormone treatment, FGFb, HGF, TGFβ1, VEGF, KGF, IL-1β, IL-6 and IL-8 secretion was measured using enzyme-linked-immunosorbent assay (ELISA). Results: Twenty-four hours after hormone treatment, we did not observe any changes neither in cell viability or apoptosis of NHPK and KHPK, nor in proliferation of KHPK. Cell proliferation of NHPK increased significantly 24 h after treatment with thyroxine in all performed concentrations (P<0.01), but there were no changes with hydrocortisone. In NHPK, thyroxine down-regulated IL-6 at 5 h and up-regulated HGF at 24 h, while hydrocortisone down-regulated IL-6, IL-8 and FGFb at 5 h, as well as IL-6, IL-8, EGF and TGFβ1 at 24 h (P<0.05). In KHPK, thyroxine up-regulated the secretion of IL-8 and TNFα at 5 h, as well as HGF at 24 h, whereas hydrocortisone down-regulated IL-6, IL-8 and TNFα at 5 h, down-regulated IL-6 and TGFβ1 at 24 h and up-regulated HGF at 24 h (P<0.05). Conclusions: Thyroxine and hydrocortisone appear to have no significant impact on cell viability and apoptosis of human keratocytes in vitro. Treatment with thyroxine triggers the proliferation of NHPK, but has no influence on the proliferation of KHPK. Hydrocortisone does not affect significantly cell proliferation of NHPK and KHPK. Thyroxine significantly up-regulates the secretion of certain growth factors and interleukins by KHPK, although it does not affect their secretion by NHPK, while hydrocortisone predominantly down-regulates them by both NHPK and KHPK. It appears that hormonal influences have differential effects on the secretion of these factors by human keratocytes, thereby modulating to some extent the corneal homeostasis.

Keywords: Thyroxine, hydrocortisone, keratocytes, keratoconus, proliferation, viability, apoptosis, growth factor, interleukin

Introduction

Keratoconus is a multi-factorial disease causing corneal degeneration, thinning and secondary ectasia, which can severely degrade visual performance. Its pathophysiology is unknown but suggested aetiological factors include oxidative damage, repetitive mechanical injury, immunological and genetic influences [1]. In addition, hormonal factors have been shown to have possible role [2-4]. A number of investigators, including our group, have reported that hypothyroidism may play a function in the exacerbation or even initiation of keratoconus [2, 3]. Others have demonstrated a possible role of tear fluid thyroxine in keratoconus development [4]. Certainly, thyroid hormones are known to be important in corneal dehydration and transpar-
Effect of thyroxine and hydrocortisone on keratocytes in vitro

From Spoerl’s report, high dose cortisol was also confirmed to impact on biomechanics of incubated porcine corneal strips [7].

Furthermore, female sex hormones are also known to affect ocular physiology and pathophysiology, particularly during pregnancy [8, 9]. A number of investigators have reported the exacerbation of iatrogenic ectasia after laser refractive surgery in pregnancy [10, 11], whilst Tachibana et al. have described an androgen-dependent mouse model of keratoconus, identifying the genes Cyp21a1 and Slp in the major histocompatibility complex (MHC) region as responsible [12].

Although keratoconus has been primarily defined as a non-inflammatory corneal disease, it has been reported that inflammatory molecules such as interleukin 1α and tumor necrosis factor α are over-expressed in keratoconus corneas as well as in the tear film of patients with keratoconus [13-15].

Within this context, we evaluated the levels of the pre-inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor α (TNFα), the growth factors fibroblast growth factor basic (FGFβ), hepatocyte growth factor (HGF), transforming growth factor β1 (TGFβ1), epidermal growth factor (EGF), the chemokine macrophage inflammatory protein 1α (MIP1α) and the matrix metalloproteinase 9 (MMP-9), after incubation of human corneal keratocytes with thyroxine and hydrocortisone in vitro.

Such studies support an important role of hormonal influences in the maintenance of corneal structure and biomechanics. The aim of this present study was to investigate the effect of thyroxine and hydrocortisone on human keratocytes in vitro and evaluate potential differences between keratocytes from normal corneas, normal human primary keratocytes (NHPK), and those from keratoconic eyes, keratoconus human primary keratocytes (KHPK), and whether hormonal influences may modulate the secretion of certain cytokines, chemokines, growth factors and matrix metalloproteinases by human normal NHPK and KHPK in vitro.

Materials and methods

Seven normal corneas, which did not meet the criteria for corneal transplantation, were obtained from the LIONS-Cornea Bank Saar-Lor-Lux, Trier/Westpfalz. Five corneal buttons were excised from patients with keratoconus who were subjected to penetrating keratoplasty in the Department of Ophthalmology, University of Saarland, Medical Center UKS, Homburg/Saar, Germany. All samples were studied separately and all following treatments were performed individually.

All experiments were conducted with respect to the tenets of Helsinki. The ethics committee of the University of Saarland approved our study entitled: “Effects of Thyroxine and Hydrocortisone on Normal Human Keratocytes and Keratoconic Keratocytes in vitro”. Written informed consent was obtained by all participants in this study. The ethics committee of the University of Saarland approved this consent procedure.

Keratocyte isolation and cell cultures

After removing the corneal endothelium with sterile surgical disposable scalpels, the Eye Bank corneas and the keratoconic corneal buttons were washed repeatedly with Dulbecco’s PBS without Ca²⁺ and Mg²⁺ (PAA, H15-002). The corneoscleral rims of the Eye Bank corneas were trimmed off and a corneal button was cut by a 8.0 mm diameter trephine. Following this initial preparation, the corneal buttons were moved to a 24-well culture plate with 1 ml Dispase (Roche 04942 078 001) (2.4 U/ml in medium). After incubation with Dispase in a humidified 37°C incubator with 5% CO₂ for 4 hours, the epithelium of cornea was removed with sterile surgical disposable scalpel.

The stoma lacking an epithelial layer, was washed for times with PBS and medium and then wholly digested with 1 mg/ml collagenase type I (Sigma, C0773). Digestion was carried out at 37°C for 10 hours after which the cells were washed with medium containing 5% fetal bovine serum (FBS) (PAA, 16076). The particulate material from each of our twelve study corneas were individually seeded into one well of a 6 well culture plate containing 2 ml of medium.

Cells were grown in DEME/F-12 HAM (Sigma, D6421) supplemented with 10% FBS, 1% penicillin (100 iu/ml)/streptomycin (100 μg/ml) (PAA, 07500) at 37°C in a 5% CO₂/air incubator.
Effect of thyroxine and hydrocortisone on keratocytes in vitro

and passaged by standard trypsinisation using 1× trypsin (2.5 M)/EDTA (0.38 M) solution (PAA, L11-004). After confluence was reached in a 75 cm² flask, the cultures were split 1:3. In this research, cells were established in 96 well culture plates seeding at 4,000 cells/well for cell viability and cell proliferation studies after the third passage.

Cell treatment with thyroxine and hydrocortisone in vitro

Hydrocortisone (Sigma, H0135) was dissolved in absolute ethanol. Final dilution was carried out in DMEM/F-12 HAM obtaining a solution containing 20 μg/ml. L-Thyroxine (Sigma, T1-775) was diluted with DMEM/F-12 HAM obtaining a solution containing 50 μg/ml. These were divided into aliquots containing the volume needed daily, kept frozen, and thawed only once on the day of use. The doses of both hormones used in this experiment were 10 μg/ml, 1 μg/ml, 0.1 μg/ml and 0.01 μg/ml, respectively. In order to avoid the effect of ethanol in cell culture among different groups, contents of ethanol in each concentration of hydrocortisone were kept the same.

Before starting hormone treatment, culture medium was replaced with DMEM/F-12 HAM with 10% FBS. Different concentrations of hormones were added, as mentioned above, as well as cells seeded for 24 hours, and cultures were returned to the incubator for 24 hours.

BrdU proliferation assay

Cell Proliferation (CP) of keratocytes was determined in vitro using a BrdU proliferation ELISA kit (Roche, 11647229001) before and after 24 hours of hormone treatment according to the manufacturer’s instructions. Briefly, 10 μl of BrdU labeling solution were added to each well and reincubate for additional 4 hours at 37°C. Cells were then dried at room temperature for 15 minutes, fixed, and the DNA denatured in order to make the incorporated BrdU more accessible for detection by the antibody. The monoclonal anti-BrdU peroxidase conjugated antibody was added to the cultures and incubated for 90 min at room temperature. After three washing steps the bound peroxidase was detected by subsequent substrate reaction. This reaction was stopped by adding 1 M H2SO4 and quantified by measuring the optical density (OD) of the yellow reaction product at a wavelength of 450 nm and a reference wavelength of 655 nm using an ELISA reader.

Viability assay

Cell viability (CV) was evaluated by the Alamar Blue assay (Invitrogen, DAL1025). AB was added directly into culture media at a final concentration of 10% and the plate was returned to the incubator at 37°C with 95% air/5% CO2. Viability was measured when the medium in control wells turned from blue to pink, typically 3 h after adding AB. Optical density of the plate was measured at 540 and 630 nm with a standard spectrophotometer. As a negative control, AB was added to medium without cells.

Cell apoptosis

Cell apoptosis was evaluated using the APO-DIRECT™ kit. Cells were digested and centrifuged in tubes for FACS. Afterwards the supernatant was removed and the cells were suspended in 1% paraformaldehyde (1 mL for 1 million cells, 1% paraformaldehyd = 0.1 g powder/10 mL PBS). Cells were then placed on ice for 60min and afterwards they were washed once with PBS (1 mL PBS/tube). Subsequently they were resuspended in the residual PBS and 1 mL 70% ice cold ethanol was added for at least 30 min at -20°C. Afterwards the control cells were suspended and 0.5 mL was placed in the tubes, respectively. After centrifugation of the control cells and the probes, ethanol was carefully removed by aspiration. Each tube was resuspended with 1 mL wash-buffer. Centrifuge and removal of the supernatant by aspiration. Control cells and probes were resuspended in 50 μL DNA-labeling-solution and incubated at least for 30 min at 37°C. Washing with 1 mL rinse buffer, centrifugation and removal of the supernatant. The cellpellet was resuspended in 500 μL PI/RNA-buffer and stored at 4°C over night. After incubation for at least 30 min at room temperature in the dark, cells were analyzed by FACS.

Measurement of IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα

Five hours and 24 hours after the hormone treatment, the concentration of IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα in each well was measured by taking a 100 μl aliquot of the supernatant of the wells. Measurements were performed by ELISA.
Effect of thyroxine and hydrocortisone on keratocytes in vitro

(KOMABIOTECH, Seoul, Korea) with the following measurement ranges:

- EGF: 250-7.8 pg/mL
- FGFb: 1000-8 pg/mL
- HGF: 8000-60 pg/mL
- IL-6: 600-10 pg/mL
- TGFβ1: 2000-16 pg/mL
- IL-1β: 1000-8 pg/mL
- MMP9: 2000-16 pg/mL
- TNFα: 1000-8 pg/mL
- MIP1α: 500-4 pg/mL

Measured concentrations below the above values were considered as zero. The cytokine concentrations were quantified by using a human recombinant IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EG, MIP1α, MMP-9 and TNFα as standard. The measurements were performed exactly following the manufactures’ ELISA-protocols. In each well, the concentration of the cytokines in the supernatant was standardized to the cell protein concentration of the respective well. The absorbance was measured at 450 nm (Model 550 Bio-Rad Laboratories GmbH, München, Germany). The experiments were repeated five times using keratocyte cultures of five different donors/patients.

Figure 1. Proliferation, viability and apoptosis of NHPK and KHPK 24 h after treatment with L-thyroxine and hydrocortisone in vitro. A. Proliferation of NHPK and KHPK 24 h after treatment with L-thyroxine in vitro. *Twenty-four hours after hormone treatment, the proliferation of normal human primary keratocytes (NHPK) significantly increased using 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, and 10 µg/ml of L-thyroxine (P<0.01) compared to control. B. Proliferation of NHPK and KHPK 24 h after treatment with hydrocortisone in vitro. No changes were detected in the proliferation of NHPK or KHPK 24 h after treatment with hydrocortisone (P>0.05). C. Viability of NHPK and KHPK 24 h after treatment with L-thyroxine and hydrocortisone in vitro. Twenty-four hours after hormone treatment, no changes were observed in cell viability of NHPK and KHPK for both thyroxine and hydrocortisone (P>0.05). D. Apoptosis of NHPK and KHPK 24 h after treatment with L-thyroxine and hydrocortisone in vitro. There were no changes observed in cell apoptosis of NHPK and KHPK after treatment of thyroxine and hydrocortisone (P>0.05).

Protein measurement

After taking the supernatant for ELISA, the total protein concentration of each well was measured following detachment of the cells with 150 µl CellLyticCHM M (Sigma, Deisenhofen, Germany). Protein quantity was determined according to the method of Bradford, which is based on the formation of a complex between the dye, Brilliant blue G and proteins in solution. The absorbance was measured at 595 nm and the concentrations were quantified using bovine serum albumin (BSA) as standard protein.

Statistical analysis

The SPSS statistical software package for Windows (version 17.0, SPSS, Inc) was used for statistical analysis. Normality of the measured data was assessed with the Shapiro-Wilk test. Student’s t-test was applied for statistical comparisons. P values below 0.05 were considered to be statistically significant.
Results

Cell proliferation of NHPK and KHPK 24 h after thyroxine treatment

Cell proliferation of NHPK increased significantly 24 h after treatment with thyroxine from concentration of 0.01 µg/ml and higher (P<0.01), but there were no changes in KHPK with thyroxine (P>0.05). Specifically, cell proliferation of NHPK increased by 49%, 48%, 51% and 46% at a concentration of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml, respectively (Figure 1A).

Figure 2. Cytokines secretion of NHPK 5 h after hormone treatment.
Effect of thyroxine and hydrocortisone on keratocytes in vitro

Cell proliferation of NHPK and KHPK 24 h after hydrocortisone treatment

We could not detect any changes in the proliferation of NHPK or KHPK 24 h after treatment with hydrocortisone (P>0.05) (Figure 1B).

Cell viability of NHPK and KHPK 24 h after hormone treatment

Twenty-four hours after hormone treatment, we did not observe any changes in cell viability of NHPK and KHPK for both thyroxine and hydrocortisone (P>0.05) (Figure 1C).

Figure 3. Cytokines secretion of KHPK 5 h after hormone treatment.
Cell viability of NHPK and KHPK 24 h after hormone treatment

Similarly, there were no changes observed in cell apoptosis of NHPK and KHPK after treatment of thyroxine and hydrocortisone (P>0.05) (Figure 1D).

Cytokine secretion of NHPK 5 h after hormone treatment

The mean concentration of IL-6 5 h after hormone treatment, using 10 μg/mL L-Thyroxine or 10 μg/mL hydrocortisone was significantly lower than that in the untreated group (P=0.042 and P<0.001, respectively).

The mean IL-8 concentration after incubation with 10 μg/mL hydrocortisone was significantly higher than that in the untreated group (P<0.001). However, we could not detect any changes following treatment with 10 μg/mL L-Thyroxine (P=0.336).

The mean FGFβ concentration decreased after treatment with 10 μg/mL hydrocortisone (P=0.0019), whereas there were no changes detected following treatment with 10 μg/mL L-Thyroxine (P=0.22).

We could not detect any changes in the secretion of HGF or TGFβ1 at any of the examined groups 5 h following hormone treatment, compared to untreated controls (P>0.119).

The secretion of IL-1β, EGF, MIP1α, MMP-9 and TNFα was below the detection limit in the treated and untreated cell cultures 5 h after treatment.

The concentrations of IL-1β, IL-6, IL-8, FGFβ, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by normal keratocytes 5 h after hormone treatment are summarized in Figure 2.

Cytokine secretion of KHPK 5 h after hormone treatment

The mean IL-6 concentration significantly increased following treatment with 10 μg/mL hydrocortisone (P<0.001). However, we could not detect any changes following treatment with 10 μg/mL L-Thyroxine (P=0.949).

We could not detect changes in the secretion of MIP1α or TGFβ1 at any of the examined groups 5 h following hormone treatment, compared to untreated controls (P>0.183).

The secretion of IL-1β, EGF, FGFβ, MMP-9 and HGF was below the detection limit in the treated and untreated cell cultures 5 h after treatment.

The concentration of IL-1β, IL-6, IL-8, FGFβ, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by keratoconus keratocytes 5 h after hormone treatment are summarized in Figure 3.

Cytokine secretion of NHPK 24 h after hormone treatment

The mean IL-6, IL-8, EGF and TGFβ1 concentration was significantly decreased after treatment with 10 μg/mL hydrocortisone (P<0.001, P=0.017, P=0.02 and P=0.019, respectively), whereas no changes were observed following treatment with 10 μg/mL L-Thyroxine (P=0.736, P=0.301, P=0.297 and P=0.651, respectively).

The mean HGF concentration after treatment with 10 μg/mL L-Thyroxine, was significantly increased (P=0.038), while there were no changes following treatment with 10 μg/mL hydrocortisone (P=0.783).

We could not detect changes in the secretion of TNFα at any of the examined groups 24 h following hormone treatment, compared to untreated controls (P>0.534).

The secretion of IL-1β, MIP1α, MMP-9 and FGFβ was below the detection limit in the treated and untreated cell cultures 24 h after treatment.

The concentrations of IL-1β, IL-6, IL-8, FGFβ, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by keratoconus keratocytes 24 h after hormone treatment are summarized in Figure 4.

Cytokine secretion of KHPK 24 h after hormone treatment

The mean IL-6 and TGFβ1 concentration was significantly decreased 24 h following treat-
Effect of thyroxine and hydrocortisone on keratocytes in vitro

Figure 4. Cytokines secretion of NHPK 24 h after hormone treatment.

The mean HGF concentration was significantly increased after treatment with 10 μg/mL L-Thyroxine (P=0.005), and decreased after treatment with 10 μg/mL hydrocortisone (P<0.001).

We could not detect changes in the secretion of IL-8 or TNFα at any of the examined groups 24 h following hormone treatment, compared to untreated controls (P>0.417).
The secretion of IL-1β, MIP1α, MMP-9, EGF and FGFβ was below the detection limit in the treated and untreated cell cultures 24 h after treatment.

The concentrations of IL-1β, IL-6, IL-8, FGFβ, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by keratoconus keratocytes 24 h after hormone treatment are summarized in Figure 5.

Discussion

The potential clinical association between corneal ectatic disease and thyroid gland dysfunction has known for several decades. Clinical
observations of hypothyroidism in patients with keratoconus were first reported by Appelbaum in mid-1930’s [16]. King subsequently reported the appearance of keratoconus following thyroidectomy [3] Lang et al. described a case of Alagille syndrome with keratoconus and secondary hypothyroidism [17]. More recently, Kocak et al. reported a case of bilateral keratoconus associated with Hashimoto’s disease [18].

The implication of thyroid hormones in corneal embryogenesis and physiology is of paramount importance, since it is well-established that thyroid hormones play a crucial role in corneal dehydration and transparency during embryonic development [5]. Conrad et al. have reported the presence of thyroxine receptors alpha and beta in the chicken cornea [6]. Moreover, thyroid hormonal changes and female sex hormones affect distinctly ocular physiology and pathophysiology during pregnancy [8-11, 19-23]. This has been highlighted by recent case reports of keratoconus progression and development of acute corneal hydrops, induced by thyroxinemia during pregnancy [2] and pregnancy-associated exacerbation of iatrogenic ectasia [10, 11].

Kahan et al. were the first to show that tear thyroxine levels in patients with keratoconus, independently of their thyroid function, were 2-50 times higher than that of normal subjects [4]. They found that tear thyroxine levels were higher during the progression of keratoconus and declined once cornea 1 curvature reached a new steady value.

At a molecular level, thyroxine functions as a transcription regulator, binding to nuclear thyroxine receptors α (THRα) and β (THRβ), which bind DNA thyroxine receptor response elements (TREs) and recruit either corepressor or coactivator complexes that subsequently repress or activate target gene transcription. Conrad et al. documented that all corneal layers of the chick cornea express THRα mRNA, while keratocytes and endothelial cells also express THRβ mRNA [6]. During normal development, THRβ expression increases 20-fold from embryonic day 12 to embryonic day 20, whereas THRα expression remains constant [6]. Exposure of chicken embryonic corneas to thyroxine in vitro affected significantly the expression of corneal transparency-associated genes, which control the keratan sulfate proteoglycan (KSPG) synthesis and the production of crystallins [5].

Xie et al. demonstrated that the functional expression of Toll-like receptors-2 and -4 in human corneal epithelial cells is enhanced in vitro by low-concentration hydrocortisone [24]. Toll-like receptors (TLRs) play an essential role in triggering the innate immune response by recognizing pathogen-associated molecular patterns and stimulating the activity of host immune cells against several microbial products [25]. It has been well established that that TLR are expressed on human corneal epithelium and play an important role in cornea protection and defense against microbial infection [25].

In our study, we investigated the effect of thyroxine and hydrocortisone on normal and keratoconus human corneal keratocytes in vitro. Exposure to thyroxine significantly increased the proliferation of NHPK, but not that of KHPK. Treatment with hydrocortisone in vitro did not significantly affect the cell proliferation of both NHPK and KHPK. Neither of the two hormones induced any significant changes in cell viability and/or cell apoptosis in NHPK or KHPK. These results suggest that thyroxine may play an important role in the cell metabolism of human corneal keratocytes in the normal cornea, thereby explaining to some extent the reported corneal biomechanical variations induced by thyroid hormone changes.

Although keratoconus has primarily been described as a non-inflammatory disease, there is increasing evidence that immunological and inflammatory processes are playing a role in its pathophysiology [13-15]. The association between keratoconus and allergy [26, 27], as well as the role played by eye rubbing in the development of corneal ectatic disease, is well established [28, 29]. Eye rubbing may contribute to the development of keratoconus by activating inflammatory mediators, more so than by the physical pressure applied to the eyeball [30]. Indeed, IL-1 has been implicated as a mediator of keratoconus in eye patients presenting excessive eye rubbing [14]. Moreover, patients with keratoconus demonstrate increased levels of inflammatory molecules in the tear film and the concentration of these
agents has been associated with the severity of keratoconus [15, 32].

Keratocytes are the major cell type of the corneal stroma, and they contribute to the local inflammatory response by releasing various chemokines, such as IL-1β, IL-6, and IL-8 [33, 34]. IL-1β plays a crucial role in corneal wound healing and it is able to induce the expression of keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) by corneal keratocytes, thereby regulating the proliferation, motility, and differentiation of corneal epithelial cells following corneal surface injury [35] IL-6 and IL-8 are important mediators of inflammatory cell response in the cornea, and are secreted in response to inflammatory stimulus [36]. IL-6 secretion by corneal keratocytes is induced following trauma or infection by IL-1α and TNFα, secreted from corneal epithelial cells [37] IL-8 is a pro-inflammatory cytokine, produced by corneal keratocytes, which plays an important role in inflammation and wound healing [38, 39] IL-8 has the capacity to recruit T-cells as well as nonspecific inflammatory cells into sites of inflammation by activating neutrophils [40]. Furthermore, IL-8 is chemotactic for fibroblasts and accelerates their migration and can stimulate deposition of tenascin, fibronectin, and collagen I during wound healing in vivo [41]. Both NHPK and KHPK have been shown to synthesize and release IL-8 following cytokine stimulation and/or infection [42, 43].

On the other hand, fibroblast growth factor basic (FGFb), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGFβ1) represent important mediators of corneal stromal-epithelial interactions and they are involved in corneal wound healing [44-47]. Furthermore, TGFβ1 is involved in inflammatory reaction and induces myofibroblast differentiation, while FGFb promotes angiogenesis, cell proliferation and migration, inducing the differentiation of keratocytes into a fibroblastic phenotype [48]. HGF delays the process of re-epithelialization, induces myofibroblastic transformation of keratocytes, while it also has an inhibitory effect on cell apoptosis [49, 50].

It is has been reported that all corneal layers in chicken cornea express thyroxine receptor alpha (TRA) mRNA, while keratocytes and endothelial cells express thyroxine receptors beta (TRB) mRNA [40]. During normal development TRA expression increases 20-fold from embryonic day 12 to embryonic day 20, whereas TRB expression remains stable [51]. Interestingly, treatment with thyroxine modifies the synthesis of keratan sulfate proteoglycan and modulates the expression of carbonic anhydrase genes in chicken cornea [51]. We have also shown that patients with Grave’s disease have significantly altered corneal biomechanical properties, and that hypothyroidism may induce corneal topographical and biomechanical variations or even exacerbate keratoconus and cause acute corneal hydrops [53, 54]. Moreover, it has been documented that hydrocortisone down-regulates the expression of Toll-like receptor-2 and -4 by corneal keratocytes in vitro, [55] thereby modulating the innate corneal immune system, since Toll-like receptors stimulate the pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6, IL-8, IL-18, and monocyte chemotactic protein-1 [56, 57]. The release of IL-6 and IL-8 is inhibited by hydrocortisone in vitro [58].

In our study, we have shown that treatment with thyroxine significantly down-regulates the secretion of IL-6 by normal keratocytes in vitro, but it up-regulates the secretion of IL-8 and TNFα by keratoconus keratocytes in vitro. These results suggest that thyroxine inhibits the inflammatory response in normal cornea in vitro, while it promotes the inflammation process in keratoconus cornea in vitro. In this study, we have discovered that thyroxine stimulates the proliferation of NHPK in vitro, while the proliferation potential of KHPK is significantly reduced. Hereby, we assume that modified expression of the corneal thyroxine receptors occurring in keratoconus may account for the observed differences in keratocyte proliferation. Differentiated expression of thyroxine receptors by corneal keratocytes as well as alteration in corneal homeostasis may also contribute to the different effect of thyroxine on interleukins in normal and keratoconus cornea in vitro.

On the other hand, treatment with hydrocortisone significantly down-regulates the secretion of IL-6, IL-8, FGFb, EGF and TGFβ1 by NHPK in vitro, while it also down-regulates the secretion of IL-6, IL-8, TNFα and TGFβ1 by KHPK in vitro. These results are in agreement with other stud-
Effect of thyroxine and hydrocortisone on keratocytes in vitro

Studies reporting that hydrocortisone inhibits the release of IL-6 and IL-8, decreasing the impact of the inflammatory cascade [53-55]. Moreover, we observed that hydrocortisone down-regulates the production of growth factors which induce angiogenesis and differentiation of keratocytes into myofibroblasts, thereby playing a significant role in corneal wound healing.

These results were produced with concentrations approximately 10 times lower than the ones in the condition in vivo, since such high concentrations are not common in stimulation studies, because high hormone concentrations may induce cell apoptosis and influence the final results [59]. The in vivo concentrations could have a multiplying effect on these results.

Our results suggest that hormonal influences may modulate the secretion of interleukins and growth factors by human corneal keratocytes in vitro, thereby affecting the inflammatory response in corneal stroma. The effects are different in NHPK and KHPK, which may reflect the alteration of receptors expression by corneal keratocytes in keratoconus.

Further studies on presumably abnormal THRA and THRβ receptors on KHPK are required in order to delineate the reason why thyroxine did not cause proliferation of KHPK and also the complex mechanisms of hormonal influences on corneal cell physiology and homeostasis. Further investigation is also required in order to delineate the pathophysiological mechanisms underlying the hormonal modulation of inflammatory response mediated by human corneal keratocytes.

Disclosure of conflict of interest

None.

Address correspondence to: Xuefei Song, Department of Ophthalmology, Saarland University Medical Center, Kirberger Str 100, 66421, Homburg, Saar, Germany. Tel: 0049-6841-1622387; Fax: 0049-6841-1622400; E-mail: 89215667@qq.com

References


Effect of thyroxine and hydrocortisone on keratocytes in vitro


Effect of thyroxine and hydrocortisone on keratocytes in vitro


