



Research Article

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Cytokine secretion of human keratocytes following crosslinking/riboflavin-UVA photodynamic inactivation (PDI), *in vitro*

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## ABSTRACT

Crosslinking/Riboflavin-UVA photodynamic inactivation (PDI) is a potential treatment option in keratoconus and in therapy resistant infectious keratitis. We aimed to evaluate the impact of crosslinking/riboflavin-UVA-PDI on TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6 and IL-8 secretion of normal human keratocytes, *in vitro*. Primary human keratocytes were isolated by digestion in collagenase (1.0 mg/ml) from human corneal buttons, and cultured in DMEM/Ham's F12 medium supplemented with 10% FCS. Cell cultures underwent UVA-light illumination (8.0 mW/cm<sup>2</sup>) for 4.10 minutes during exposure to 0.1% concentration of riboflavin-5-phosphat. Five and 24 hours after crosslinking, cytokine concentration was determined in the culture supernatant using ELISA. Five hours after riboflavin-UVA-PDI, FGFb, VEGF and HGF secretion of keratocytes decreased significantly ( $p<0.01$ ;  $p<0.05$ ;  $p<0.01$ ) and TGF $\beta$ 1, IL-6 and IL-8 secretion remained unchanged. Twenty-four hours following riboflavin-UVA-PDI, FGFb and KGF secretion decreased ( $p<0.01$ ;  $p<0.01$ ) and IL-6 secretion increased significantly ( $p<0.01$ ), whereas TGF $\beta$ 1, VEGF and HGF secretion remained unchanged. Crosslinking/riboflavin-UVA-PDI decreases FGFb, VEGF and HGF secretion 5 hours and FGFb and KGF secretion 24 hours after treatment, while it increases IL-6 secretion of keratocytes after 24 hours. In the short term, riboflavin-UVA-PDI does not have an impact on TGF $\beta$ 1 and IL-8 secretion of keratocytes, *in vitro*.

**Key words:** UVA, human keratocytes, riboflavin, interleukins, growth factors

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## INTRODUCTION

In recent years corneal crosslinking (CXL) has been successfully used to stop the progression of keratoconus or as alternative antimicrobial therapy for therapy resistant infectious keratitis. In treatment of keratoconus, crosslinking between collagen fibers is supposed to stop the disease progression. In infectious keratitis, first excitation of the photosensitizer riboflavin through UVA light, then relaxation from the excited state results in free oxygen radicals and reactive oxygen species production, which leads to local damages of microorganisms but also of the surrounding tissue. As an antimicrobial treatment, crosslinking is one modality of so-called photodynamic therapy (PDT) or photodynamic inactivation (PDI).

Although studies verified that epithelial-off treatment and 30 minutes-long-illumination does stop keratoconus progression, up-to-date crosslinking is still an off-label therapy [1, 2]. We also do know that at least 400  $\mu$ m corneal

thickness is necessary in order to avoid corneal endothelial cell damage during treatment. The exact role of recently introduced riboflavin-solutions which support prompt intraoperative increase in corneal thickness still has to be clarified.

Cytokines play an important role in immunological and inflammatory processes of the ocular surface. During keratitis interleukin-6 (IL-6) and interleukin-8 (IL-8) are secreted as response to inflammatory stimulus [3]. Transforming growth factor  $\beta 1$  (TGF $\beta 1$ ) is also involved in inflammatory reactions and induces myofibroblast differentiation [4]. Fibroblast growth factor basic (FGFb), vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF) are markers of angiogenesis [5]. Decisive factors for wound healing are FGFb, hepatocyte growth factor (HGF), TGF $\beta 1$  and KGF. KGF is also described as a mediator of stromal-epithelial interactions [6, 7, 8, 9]. To the best of our knowledge, growth factor and interleukin secretion of keratocytes has not been analysed following crosslinking, yet.

The purpose of our present study was to determine the impact of crosslinking/riboflavin-UVA PDI on growth factor and interleukin secretion of human keratocytes, *in vitro*.

## EXPERIMENTAL SECTION

### Materials

Dulbecco's Modified Eagle Medium: (Nutrient Mixture F-12 (DMEM/F12)); FCS (fetal calf serum 10%); P/S (1% of 10000 u penicillin/ 10 mg streptomycin per ml); 0.05% trypsin/ 0.02% ethylenediaminetetra-acetic acid (EDTA), Riboflavin-5-phosphate, Dextran and Bradford Reagent were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany), Collagenase A, and Dispase II were obtained from Roche Diagnostics (Mannheim, Germany). All ELISA assays were performed with the DuoSet ELISA Development System from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany).

### Isolation of primary human corneal keratocytes

Human corneas not suitable for corneal transplantation were obtained from the LIONS Cornea Bank Saar-Lor-Lux, Trier/Westpfalz. Keratocytes were isolated as described previously [10]. In short, the human corneoscleral buttons were aseptically rinsed in phosphate-buffered saline (PBS) before removal of the endothelium including Descemet's membrane by sterile surgical disposable scalpel. A central corneal button with epithelium was cut using a 8.0 mm Barron's trephine and thereafter incubated in culture medium containing 2.4 U/ml Dispase II for 4 hours at 37 °C. In the following, the corneal button was washed with PBS for several times and the already loose corneal epithelium was removed with surgical disposable scalpel. The remaining corneal stroma was incubated in culture medium with 1.0 mg/ml collagenase A for 8-10 hours at 37 °C. The digested tissue and cells were pipetted three times and centrifuged at 800 g for 7 minutes and finally resuspended in 1.0 ml culture medium, which consisted of basic medium (DMEM/F12) supplemented with 10% FCS and 1% P/S. The cell suspension was seeded in 6-well plates and the medium was changed 24 hours after seeding. Medium was changed every 2 to 3 days until keratocytes reached confluence. The cells were subcultured in 25 cm<sup>2</sup> culture flasks after 5 to 10 days following dispersal with 0.05% trypsin-EDTA for 3 to 5 minutes. The passage 4 to 8 of cells was used for experiments.

### Cell culture

Cultured keratocytes showed typical morphologic characteristics of corneal stromal cells. After the proliferation period had started, a confluent monolayer was generally reached between day 3 and 5. Keratocytes were used at this stage for further experiments.

### Crosslinking / Riboflavin-UVA photodynamic inactivation

Human keratocytes were seeded in 24-well tissue culture plates and were allowed to grow for 48 hours before photodynamic treatment. Cells were washed with PBS once before riboflavin-5-phosphate was added. The concentration of riboflavin-5-phosphate was 0.1%, diluted in 20% Dextran-PBS. Thereafter, the cells were exposed directly to UVA light (375 nm) for 4.10 minutes (2 J/cm<sup>2</sup>). Following illumination, cells were washed twice with PBS, fed with culture medium and cultivated at 37 °C for 5 hours or 24 hours before measurements. The supernatant was collected and stored until ELISA measurement at -80°C. In summary, the cells were treated with the following combinations: riboflavin-5-phosphate-UVA, riboflavin-5-phosphate only, UVA only. The control cells were incubated only in the dark for 4.10 minutes.

**Measurement of TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6 and IL-8**

Five hours and twenty-four hours after the photodynamic inactivation the concentration of TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6 and IL-8 in each well was measured by taking a 100  $\mu$ l aliquot of the supernatant of the wells. Measurements were performed by ELISA with the following measurement ranges: TGF $\beta$ 1: 16-2000 pg/ml, FGFb: 8-1000 pg/ml, VEGF: 16-2000 pg/ml, HGF: 60-8000 pg/ml, KGF: 16-2000 pg/ml, IL-6: 10-600 pg/ml and IL-8: 16-2000 pg/ml. Measured concentrations below the above values were considered as zero. The growth factor concentrations were quantified by using a human recombinant TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6 and IL-8 as standard. The measurements were performed following the manufacturers' ELISA-protocols. In each well, the concentration of the growth factors in the supernatant was standardized to the cell protein concentration of the respective well. The absorbance was measured at 450 nm (Model Infinite F50, Tecan GmbH, Crailsheim, Germany). The experiments were repeated four times using keratocyte cultures of 5 different patients regarding each well.

**Protein measurement**

After taking the supernatant for ELISA, the total protein concentration of each well was measured following detachment of the cells. 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**

For statistical analysis the GraphPad Prism 2.01 was used. Data are represented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using Wilcoxon-Mann-Whitney Test. P values below 0.05 were considered statistically significant.

**RESULTS**

The concentration of TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6, and IL-8 in the supernatant of keratocytes 5 hours after riboflavin-UVA-PDI is summarized at **Table 1** and **Figure 1**. The secretion of KGF was below the detection limit in the treated and untreated cell cultures 5 hours after PDI. We could not detect changes in the secretion of TGF $\beta$ 1 and IL-6 of keratocytes in any of the examined groups 5 hours following riboflavin-UVA treatment.

Five hours after PDI, using 0.1% riboflavin-5-phosphat and illumination, the mean FGFb concentration in the supernatant was  $0.43 \pm 0.1$  pg/ $\mu$ g protein. This was significantly lower than FGFb concentration in the supernatant of untreated keratocyte cultures ( $0.89 \pm 0.72$  pg/ $\mu$ g protein; p<0.01). The FGFb concentration was also significantly decreased for this time point using UVA-light only ( $0.46 \pm 0.15$  pg/ $\mu$ g protein; p<0.01), compared to controls.

The concentration of VEGF 5 hours after riboflavin-UVA-PDI ( $0.89 \pm 0.30$  pg/ $\mu$ g protein) was significantly lower than in control cells ( $1.33 \pm 0.68$  pg/ $\mu$ g protein; p<0.05). The VEGF concentration also decreased significantly using UVA-light illumination separately ( $0.94 \pm 0.61$  pg/ $\mu$ g protein; p<0.05). The HGF concentration was significantly lower following PDI ( $2.22 \pm 1.10$  pg/ $\mu$ g protein; p<0.01) compared to controls ( $4.13 \pm 1.65$  pg/ $\mu$ g protein).

Using riboflavin-5-phosphat without subsequent illumination, the concentration of IL-8 in the supernatant of keratocytes increased significantly ( $2.72 \pm 0.98$  pg/ $\mu$ g protein; p<0.01) compared to controls ( $1.87 \pm 0.42$  pg/ $\mu$ g protein).

The concentration of TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6 and IL-8 24 hours following riboflavin-UVA-PDI is summarized at **Table 2** and **Figure 2**. TGF $\beta$ 1 and HGF secretion of keratocytes remained unchanged in all the examined groups 24 hours after treatment, compared to control cultures.

The secretion of FGFb was significantly lower ( $0.22 \pm 0.10$  pg/ $\mu$ g protein) compared to controls ( $0.64 \pm 0.43$  pg/ $\mu$ g protein; p<0.01), for this time point. FGFb concentration also decreased using UVA-light only ( $0.20 \pm 0.10$  pg/ $\mu$ g protein; p<0.01), compared to control cultures.

The secretion of KGF after riboflavin-UVA-PDI ( $0.27 \pm 0.15$  pg/ $\mu$ g protein), or using UVA-light only ( $0.46 \pm 0.28$  pg/ $\mu$ g protein), decreased significantly (p<0.01, p=0.04) compared to control cells ( $0.62 \pm 0.30$  pg/ $\mu$ g protein).

VEGF secretion of keratocytes decreased significantly using UVA-light illumination only ( $3.39 \pm 1.94$  pg/ $\mu$ g protein versus  $7.07 \pm 3.8$  pg/ $\mu$ g protein;  $p < 0.01$ ) and IL-8 secretion increased significantly following the use of riboflavin-5-phosphat without illumination ( $10.85 \pm 3.99$  pg/ $\mu$ g protein;  $p < 0.01$ ), compared to controls ( $7.44 \pm 2.84$  pg/ $\mu$ g protein).

Twenty-four hours following PDI, using 0.1% riboflavin-5-phosphat and UVA-light, the mean IL-6 concentration in the supernatant was  $2.59 \pm 1.08$  pg/ $\mu$ g protein. This was significantly higher than the IL-6 concentration in the supernatant of untreated cell cultures ( $0.81 \pm 0.31$  pg/ $\mu$ g protein;  $p < 0.01$ ).

## DISCUSSION

Several authors reported on occurrence of early bacterial or acanthamoeba keratitis following crosslinking therapy [11, 12, 13]. In contrast, case series describe the success of crosslinking in treatment of non-healing, therapy resistant bacterial or acanthamoeba ulcers or keratitis [14]. Cytokines and growth factors play an important role in wound healing and inflammatory processes in the cornea. To the best of our knowledge, this is the first study illustrating the growth factor and interleukin secretion of primary human keratocytes following crosslinking. A limitation of our study is that using ELISA, only secreted growth factors were measured and total protein or mRNA analysis was not performed.

We demonstrated that crosslinking using the therapeutical concentration of riboflavin-5-phosphat decreases FGFb, VEGF and HGF secretion 5 hours after treatment. Twenty-four hours following riboflavin-UVA-PDI, FGFb secretion is still decreased, whereas VEGF and HGF secretion normalizes in culture. In the short term, crosslinking does not have an impact on TGF $\beta$ 1 secretion of keratocytes, *in vitro*.

FGF promotes angiogenesis, cell proliferation and migration. With reduced FGFb secretion 5 and 24 hours after CXL, angiogenesis and keratocyte proliferation may be reduced in the cornea. In accordance with that, our previous study verified decreased proliferation of keratocytes 24 hours after CXL [15]. FGFb also induces the differentiation of keratocytes into a fibroblastic phenotype. Therefore, with reduced FGFb secretion after CXL, a reduced percentage of differentiated keratocytes (into fibroblastic phenotype) are expected. In accordance with that we have shown in our previous studies a promoted myofibroblastic transformation and an increased expression of the hemopoietic stem cell marker CD34 24 hours after crosslinking therapy using the same concentration of riboflavin [15]. It is known that HGF and TGF $\beta$ 1 induce myofibroblastic transformation of keratocytes. In accordance with decreased HGF and unchanged TGF $\beta$ 1 secretion, we detected an increased myofibroblastic transformation after treatment [15]. We relate the increased myofibroblastic transformation to the impact of the decreased FGFb secretion. However, to the best of our knowledge the interaction of different cytokines in the cornea has not been analyzed, yet.

HGF is secreted through the stromal cells of the cornea and are known to inhibit the process of epithelialization [16]. In contrast, KGF supports epithelial wound healing [17]. Our findings suggest that the decreased secretion of HGF 5 hours after CXL supports epithelial healing and the decreased secretion of KGF 24 hours after treatment inhibits the epithelial wound healing. The exact role of crosslinking on epithelial proliferation and migration has to be further analyzed.

Interestingly, in our previous studies with the photosensitizer chlorine e6 and 670 nm wavelength illumination, the impact on growth factor secretion of keratocytes was in part similar; HGF secretion decreased 5 hours and KGF secretion decreased 24 hours after treatment, however FGFb secretion *increased* after 5 hours [18]. We correlate the increased FGF secretion to the decreased keratocyte viability and increased apoptotic keratocyte death following chlorine e6 photodynamic therapy, with a photosensitizer with higher photosensitizing efficacy (more free oxygen radical production). The increased FGFb secretion may be a response to the induced cell death. In accordance with our hypothesis, we could not observe induced apoptosis in normal keratocytes, following riboflavin-UVA-PDI.

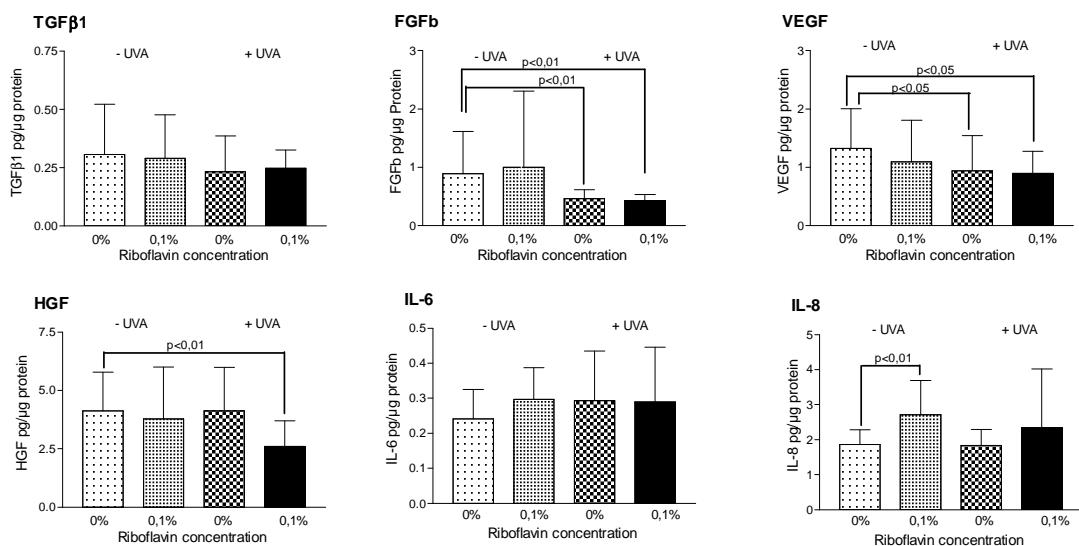
Lee *et al.* reported on the inhibitory effect of HGF on apoptosis of the cells. Using the photosensitizer chlorine e6 the HGF secretion was decreased, but obviously not enough to prevent keratocyte apoptosis. In contrast, using riboflavin-UVA-PDI, HGF secretion of keratocytes was at higher levels than following chlorine e6-PDI: it could still prevent apoptosis of keratocytes [19].

Interestingly, 5 hours after CXL VEGF secretion was significantly decreased, which indicates that riboflavin-UVA-PDI may inhibit hemangiogenesis and lymphangiogenesis in the short term. In contrast, using the photosensitizer chlorine e6, only a slight increase of VEGF secretion could be measured after 24 hours, which could result in promotion of hem- and lymphangiogenesis using this photosensitizer. The exact molecular mechanism here still has to be clarified.

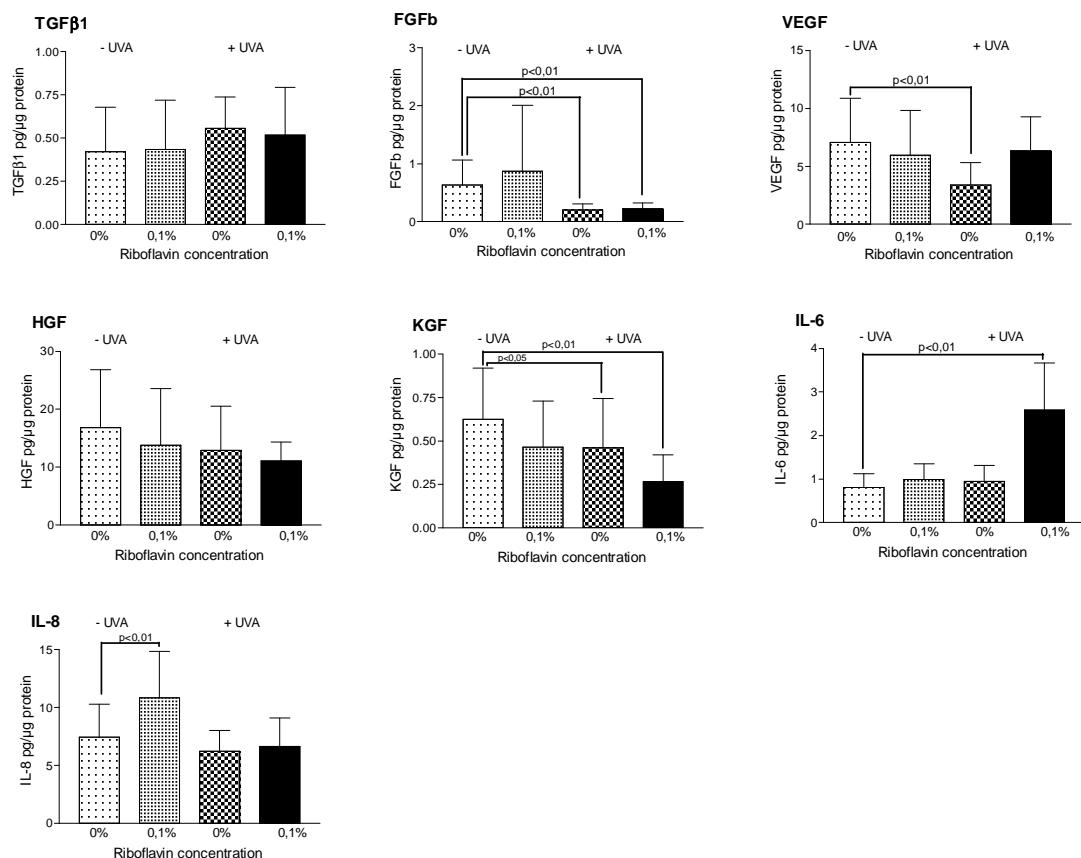
Konstantopoulos [20] et al. reported of IL-6, IL-8 and IL-1 $\beta$  as the key cytokines in corneal inflammation. They are shown to be chemotactic for PMNs in infected corneal tissue which can cause corneal destruction. We could detect an increase of IL-6 but no effect on IL-8 secretion in keratocytes 24 hours after treatment, *in vitro*. Increased levels of IL-6 in early stages of keratitis are able to recruit polymorphonuclear neutrophils (PMN) into the cornea [21], which is a favorable effect in the short term. Interestingly, these results differ from our previous measurements using the photosensitizer chlorine e6: using this treatment IL-6 and IL-8 decreased significantly 5 hours after treatment and normalized 24h after PDI [22].

For keratoconus patients, Kolozsvári et al described that secretion of several interleukins is correlated with the stage of the keratoconus disease [23]. Response of keratoconus keratocytes on crosslinking treatment also has to be further analyzed.

In summary, crosslinking/riboflavin-UVA photodynamic inactivation decreases FGFb, VEGF and HGF secretion 5 hours and FGFb and KGF secretion 24 hours after treatment, whereas it increases IL-6 secretion of keratocytes after 24 hours. In the short term, riboflavin-UVA-PDI does not have an impact on TGF $\beta$ 1 and IL-8 secretion of keratocytes, *in vitro*. To determine the exact role of the secreted cytokines on corneal epithelial cells and keratocytes, further studies are needed.



**Figure 1.** TGF $\beta$ 1, FGFb, VEGF, HGF, IL-6 and IL-8 concentration (mean pg/μg protein ± SD) 5 hours following crosslinking/riboflavin-UVA photodynamic inactivation. Significant differences are indicated.



**Figure 2.** TGF $\beta$ 1, FGFb, VEGF, HGF, KGF, IL-6 and IL-8 concentration (mean pg/μg protein ± SD) 24 hours following crosslinking/riboflavin-UVA photodynamic inactivation. Significant differences are indicated.

**Table 1.** Concentration (pg/μg protein) of different growth factors and interleukins in the supernatant of keratocyte cultures five hours after crosslinking/riboflavin-UVA photodynamic inactivation. Values indicate mean ± SD.

	Keratocytes	Keratocytes + UVA	Keratocytes + riboflavin	Keratocytes + riboflavin + UVA	* p-value	† p-value	‡ p-value
<b>TGF<math>\beta</math>1</b>	0.31 ± 0.22	0.23 ± 0.15	0.29 ± 0.19	0.25 ± 0.17	0.29	0.94	0.47
<b>FGFb</b>	0.89 ± 0.72	0.46 ± 0.15	1.00 ± 1.30	0.43 ± 0.1	<b>&lt;0.01</b>	0.60	<b>&lt;0.01</b>
<b>VEGF</b>	1.33 ± 0.68	0.94 ± 0.61	1.10 ± 0.71	0.89 ± 0.38	<b>0.04</b>	0.24	<b>0.04</b>
<b>HGF</b>	4.13 ± 1.65	4.13 ± 1.87	3.79 ± 2.22	2.22 ± 1.10	0.76	0.24	<b>&lt;0.01</b>
<b>KGF</b>	n.d.	n.d.	n.d.	n.d.	n/a	n/a	n/a
<b>IL-6</b>	0.24 ± 0.08	0.29 ± 0.14	0.30 ± 0.09	0.29 ± 0.09	0.34	0.08	0.61
<b>IL-8</b>	1.87 ± 0.42	1.84 ± 0.45	2.72 ± 0.98	2.35 ± 1.67	0.86	<b>&lt;0.01</b>	0.80

\* p-values indicate the difference between "Keratocytes" versus "Keratocytes + UVA" groups (Wilcoxon-Mann-Whitney test).

† p-values indicate the differences between "Keratocytes" versus "Keratocytes + riboflavin" groups (Wilcoxon-Mann-Whitney test).

‡ p-values indicate the difference between "Keratocytes" versus "Keratocytes + riboflavin + UVA" groups (Wilcoxon-Mann-Whitney test).

Significant p-values are shown in bold.

n.d. = not detectable

n/a = not applicable

**Table 2.** Concentration (pg/μg protein) of different growth factors and interleukins in the supernatant of keratocyte cultures twenty-four hours after crosslinking/riboflavin-UVA photodynamic inactivation. Values indicate mean ± SD.

	Keratocytes	Keratocytes + UVA	Keratocytes + riboflavin	Keratocytes + riboflavin + UVA	* p-value	† p-value	‡ p-value
<b>TGFβ1</b>	0.42 ± 0.26	0.56 ± 0.18	0.43 ± 0.29	0.52 ± 0.28	<i>0,06</i>	<i>0,79</i>	<i>0,20</i>
<b>FGFb</b>	0.64 ± 0.43	0.2 ± 0.10	0.87 ± 1.14	0.22 ± 0.10	<b>&lt;0,01</b>	<i>0,86</i>	<b>&lt;0,01</b>
<b>VEGF</b>	7.07 ± 3.80	3.39 ± 1.94	5.96 ± 3.86	6.33 ± 2.97	<b>&lt;0,01</b>	<i>0,30</i>	<i>0,55</i>
<b>HGF</b>	16.84 ± 10.01	12.95 ± 7.56	13.82 ± 9.73	11.08 ± 3.26	<i>0,23</i>	<i>0,29</i>	<i>0,09</i>
<b>KGF</b>	0.62 ± 0.30	0.46 ± 0.28	0.46 ± 0.26	0.27 ± 0.15	<b>0,04</b>	<i>0,07</i>	<b>&lt;0,01</b>
<b>IL-6</b>	0.81 ± 0.31	0.95 ± 0.37	0.99 ± 0.36	2.59 ± 1.08	<i>0,44</i>	<i>0,29</i>	<b>&lt;0,01</b>
<b>IL-8</b>	7.44 ± 2.84	6.23 ± 1.80	10.85 ± 3.99	6.63 ± 2.47	<i>0,12</i>	<b>&lt;0,01</b>	<i>0,30</i>

\* p-values indicate the difference between "Keratocytes" versus "Keratocytes + UVA" groups (Wilcoxon-Mann-Whitney test).

† p-values indicate the difference between "Keratocytes" versus "Keratocytes + riboflavin" groups (Wilcoxon-Mann-Whitney test).

‡ p-values indicate the difference between "Keratocytes" versus "Keratocytes + riboflavin + UVA" groups (Wilcoxon-Mann-Whitney test).

Significant p-values are shown in bold.

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