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Jacob Becker
Sabine Salla
Ursula Dohmen
Claudia Redbrake
Martin Reim

Explorative study of interleukin levels in the human cornea

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J. Becker (✉) · S. Salla · U. Dohmen
C. Redbrake · M. Reim
Klinikum der RWTH Aachen,
Augenklinik, D-52057 Aachen, Germany
Tel.: 241-8089147,
Fax: 241-8888438

Abstract ● Background: The presence of interleukins has been demonstrated in the cornea and other ocular tissues. Although pathogenic mechanisms are unknown, interleukins seem to be involved in inflammatory disorders of the cornea. The present study was undertaken to analyse concentrations of interleukin-1β (IL-1β) and interleukin-6 (IL-6) in human corneas with various clinical diagnoses. ● Methods: Immediately after keratoplasty 127 explanted human corneas with various corneal diseases were snap frozen and cryosections were prepared for histological examination. Furthermore, the protein content was measured according to the method of Bradford and the concentration of IL-1β and IL-6 were determined using a specific immunosorbent test (ELISA). ● Results: It was found that IL-1β and IL-6 level were clearly higher in corneas with ulcerations and distinct inflammatory signs. Lower levels of both interleukins were found in corneas with a weak expression of inflammatory signs. ● Conclusions: Keratitis, keratoconus with inflammatory signs, and ulcerating processes showed higher interleukin levels than corneas with non-inflammatory disorders like scar formation, corneal dystrophy and keratoconus. The results could show that, depending on the clinical diagnosis, the inflammatory status of the cornea may be evaluated by the interleukin levels determined in the corneal tissue.

Introduction

In recent years it has become clear that inflammation of the eye may be mediated by interleukins. Interleukins are involved in the differentiation and proliferation of lymphocytes and macrophages, but they also influence many other cell types, such as granulocytes, fibroblasts and endothelial cells. Interleukin-1β (IL-1β) is responsible for B-cell activation and proliferation and immunoglobulin (IG) secretion. Interleukin-6 (IL-6) can modulate IL-2 receptor expression, proliferation and differentiation of B- and T-cells and stimulation of IgG secretion [5, 9].

There is considerable experimental evidence for the concept that interleukins play a major role in intraocular inflammations. Interleukins have been detected in ocular tissues obtained from patients with uveitis and have been demonstrated to induce uveitis in animal experiments.

High IL-6 levels in the aqueous humour could be demonstrated in patients with heterochromic cyclitis, a chronic idiopathic form of anterior uveitis [26], and in patients with panuveitis due to toxoplasmosis [14].

Staats and Lausch [24] assayed extracts of HSV-1-infected corneas from mice for the presence of IL-1α and IL-6 and found higher levels after infection. After endotoxin or lipopolysaccharide (LPS)-induced uveitis in rats, higher IL-6 levels could be observed in aqueous humour and serum [9–11]. After endotoxin addition to cultured bovine pigmented ciliary epithelial cells, IL-1 could be detected in the culture supernant [8].

Rosenbaum et al. [18] observed induction of iridocyclitis after injection of IL-1α into the vitreous of rabbits. Intravitreal injection of IL-6 induced uveitis in both rat and rabbits [10, 11]. Bhattacherjee and Henderson [2] compared the effects of anterior chamber injections of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and LPS with injections of IL-1 in a rabbit model. The highest amounts of leucocytes in the aqueous humour were observed in the IL-1-treated eyes followed according to the cell number of LTB_4, LPS and PGE_2. BenEzra et al. [1] reported that IL-1α and IL-1β are potent angiogenic factors and can induce neovascularisation of the cornea in a rabbit model.

Several animal studies and in vitro experiments were performed concerning the involvement of interleukins in corneal disorders of corneal tissue. Niederkorn et al. [16] showed that corneal epithelial cells can secrete IL-1 after trauma and stimulation of phagocytosis. IL-1 and IL-6 are detectable in normal corneal epithelial cells in organ culture [21].

Corneal fibroblasts produced 3–10times more thromboxane if IL-1 was added to the culture medium [22]. IL-1β can induce an increase of collagenase and prostaglandin synthesis by fibroblasts in culture [4]. Similar results could be obtained if tumour necrosis factor (TNF) was added to the culture medium [6, 15]. Human corneal fibroblasts enhance the production of C3 and C5 components if they are cultured with IL-1, IL-2, interferon-α and interferon-1β [19]. As for the inflamed corneal stroma, Saal et al. [20] postulated a possible action of interleukins produced by an infiltration of T-lymphocytes and macrophages in elastase-mediated autodegradation of corneal stroma in patients with rheumatoid arthritis. In an in vitro study, Fabre et al. [7] compared corneal fibroblasts of patients with keratoconus to those of healthy corneas. Fibroblasts from keratoconus contained four times more IL-1 binding sites than normal fibroblasts. In cell culture it could be shown that endothelial cells produce IL-6 if LPS or IL-1α is added to the culture medium [21].

These references suggest that corneal cells and inflammatory cells are capable of modulating inflammatory reactions via interleukin secretion and simultaneously can react with interleukins to produce lytic enzymes and/or inflammatory mediators such as complement components and arachidonic acid metabolites.

IL-1 and IL-6 have proved to be primary regulators of local inflammation in man. In our study we tried to measure the concentrations of these interleukins in human corneal tissue with various diagnoses such as keratoconus, corneal dystrophy, corneal scar, ulcerative process and keratitis.

Methods

Investigation specimens

The tenets of the declaration of Helsinki were followed concerning the examination of human material. We investigated 127 human corneas obtained after keratoplasties. The obtained corneal buttons were snap frozen immediately after explantation. Cryosections 5 µm thick were prepared for histological examination. Haematoxylin and eosin (HE) stain, Giemsa stain and periodic acid–Schiff (PAS) stain were performed and the specimens evaluated for inflammatory signs. Specimens were graded as inflammatory if distinct invasion of the corneal tissue by granulocytes, lymphocytes and macrophages was observed.

Assessment of protein levels and interleukins in the corneal tissue

For optimal disintegration of the tissue the frozen specimens were processed under liquid nitrogen in an oscillating mill. The final homogenate was lysed with ice-cold distilled water. The samples were centrifuged for 20 min at 4° C, 14000 *g* to obtain the water-soluble protein fraction. The supernatant was measured for protein content according to the method of Bradford [3].

Interleukin concentrations were determined in enzyme-linked immunosorbent assays. The procedures for analysis of duplicate samples were identical to those described in the commercial kits from H. Biermann (Bad Nauheim, Germany). A standard curve was used to determine the concentrations of interleukins. The sensitivity for IL-1β ranged from 0.125 to 8 pg/ml and for IL-6 from 0.156 to 10 pg/ml. The interleukin levels were calculated from the protein content.

Statistical evaluation

The median and the correspondingly calculated quantiles (25% and 75%) were preferred for descriptive statistics because of possible outliers. The 25% and 75% quantiles include values which are lower and exactly calculated. Significance testing was performed according to the Mann-Whitney *U* procedure [23].

Results

The explants could be divided into different groups according to clinical diagnosis and inflammatory signs observed in the histological sections, e.g. inflammatory cells (Table 1).

Interleukin-1β

Eight samples were classified "not measurable" because IL-1β values were lower ($n=1$) or higher ($n=7$) than the standard range. If higher values were found the samples were diluted and measured again. The sample was classified not measurable if recurrent dilutions led to unchanged results, and these were not included in statistical analysis. The distribution of the IL-1β levels regarding the different clinical diagnoses is shown in Table 2. A median of 24.22 pg IL-1β/mg protein was found in the

Table 1 Classification of the corneas

1. Corneas with inflammatory disorders (total)	n=46
Keratitis	n=12
Keratoconus[a]	n=11
Ulcerating process	n=23
2. Corneas with non-inflammatory disorders (total)	n=81
Scar formation	n=17
Corneal dystrophy	n=24
Corneal decompensations for various reasons	n=28
Keratoconus	n=12

[a] With inflammatory cellular reaction

group of inflammatory disorders (n=39). In contrast to this, a median of 5.03 pg IL-1β/mg protein could be determined for the non-inflammatory disorders (n=80). The difference in correlation between IL-1β levels was statistically significant (P<0.0001). A higher median for IL-1β in cases of inflammatory disorders is obvious. Furthermore, minimum and maximum values show a wide scatter in some groups. The quantiles may demonstrate the polarisation within the different diagnoses. For example, 25% of the measured samples from keratoconus with cellular infiltration have values below 11.71 pg IL-1β/mg protein, but 75% have values up to

Table 2 Descriptive statistics of IL-1β values (pg/mg protein)

Diagnosis	Minimum	Maximum	Median	25% Quantile	75% Quantile	n	Not measurable
1. Inflammatory disorders (total)	0.48	1750.37	24.22	5.09	164.77	39	7
Keratitis	1.42	1750.37	7.12	3.85	291.02	12	0
Keratoconus[a]	10.68	292.97	32.59	11.71	164.77	11	0
Ulceration	0.48	839.58	42.57	4.50	274.88	16	7
2. Non-inflammatory disorders (total)	0.53	468.86	5.03	2.12	10.69	80	1
Decompensation	0.53	468.86	5.52	2.65	29.8	28	0
Dystrophy	0.76	64.65	5.03	1.73	10.7	24	0
Keratoconus	1.26	7.91	4.16	2.31	5.57	12	0
Scar formation	0.95	48.31	5.48	1.60	19.45	16	1

[a] With inflammatory cellular reaction

Table 3 Correlations of IL-1β values

	Keratoconus	Scar formation	Dystrophy	Decompensation	Ulceration	Keratitis
Keratoconus[a]	P=0.000*	P=0.001*	P=0.000*	P=0.003*	P=0.328	P=0.088
Keratoconus		P=0.243	P=0.230	P=0.070	P=0.009*	P=0.005*
Scar formation			P=0.423	P=0.200	P=0.017*	P=0.097
Dystrophy				P=0.135	P=0.008*	P=0.090
Decompensation					P=0.037*	P=0.230
Ulceration						P=0.321

[a] With inflammatory cellular reaction
* Significant differences, P<0.05

Table 4 Descriptive statistics of IL-6 values (pg/mg protein)

Diagnosis	Minimum	Maximum	Median	25% Quantile	75% Quantile	n	Not measurable
1. Inflammatory disorders (total)	0.07	3327.60	31.32	5.94	228.38	30	16
Keratitis	2.11	228.38	36.90	17.55	76.80	9	3
Keratoconus[a]	0.07	88.85	12.62	5.94	16.02	9	2
Ulceration	3.67	3327.60	264.60	66.40	400.90	12	11
2. Non-inflammatory disorders (total)	0.14	957.11	6.92	1.59	18.49	80	1
Decompensation	0.14	957.11	10.08	3.60	35.97	28	0
Dystrophy	0.82	123.47	10.84	1.84	27.64	24	0
Keratoconus	0.77	9.62	1.23	0.98	2.63	11	1
Scar formation	0.97	74.00	1.89	1.54	9.90	17	0

[a] With inflammatory cellular reaction

Table 5 Correlations of IL-6 values

	Keratoconus	Scar formation	Dystrophy	Decompensation	Ulceration	Keratitis
Keratoconus[a]	$P=0.010$*	$P=0.147$	$P=0.389$	$P=0.389$	$P=0.010$*	$P=0.043$*
Keratoconus		$P=0.032$*	$P=0.002$*	$P=0.000$*	$P=0.000$*	$P=0.000$*
Scar formation			$P=0.097$	$P=0.012$*	$P=0.000$*	$P=0.003$*
Dystrophy				$P=0.330$	$P=0.001$*	$P=0.024$*
Decompensation					$P=0.003$*	$P=0.078$
Ulceration						$P=0.023$*

[a] With inflammatory cellular reaction
* Significant differences, $P<0.05$

164.77 pg IL-1β/mg protein. Statistical analysis of IL-1β values, placed in order to clinical diagnoses, is shown in Table 3.

Significant differences between IL-1β levels of several inflammatory and non-inflammatory disorders could be observed. Ulcerative processes have statistically significantly higher IL-1 values than all non-inflammatory disorders. In contrast to these findings, IL-1β levels of samples with keratitis were similar result to those in keratoconus. The correlation of cellular infiltrated keratoconus, ulceration and keratitis showed no statistical differences.

Interleukin-6

A median of 31.32 pg IL-6/mg protein was found in the group of inflammatory disorders ($n=30$). In contrast, a median of 6.92 pg IL-6/mg protein could be determined for the non-inflammatory disorders ($n=80$). The difference in correlation between IL-6 levels was statistically significant ($P<0.0001$). The distribution of the IL-6 levels regarding the different clinical diagnoses is shown in Table 4. A higher median for IL-6 in cases of inflammatory disorders is obvious. Furthermore, minimum and maximum values show a wide scatter in some groups. The quantiles may demonstrate the polarisation within the different dignoses. For example, 25% of the measured samples with ulcerative processes have values below 66.4 pg/mg protein, but 75% have values up to 400.9 pg/mg protein. Sixteen samples were classified not measurable because IL-6 values were higher than the standard range, and in one sample of non-infiltrated keratoconus no IL-6 concentrations could be measured.

Statistical analysis of IL-6 values, placed in order to cellular infiltration and clinical diagnosis, is shown in Table 5. Significant differences could be observed between IL-6 levels in severe inflammatory disorders such as ulceration and keratitis and levels in non-inflammatory disorders. An exception to these findings was observed in a comparison of interleukin-6 levels in cases of keratitis and decompensation, where no statistical difference was found. Ulcerative processes have statistically significantly higher IL-6 values than all non-inflammatory disorders and also keratitis and infiltrated keratoconus. Furthermore, infiltrated keratoconus showed significantly, lower IL-6 levels than keratitis.

Discussion

Interleukin-1β was detected in 126 of the 127 investigated specimens, 7 of which showed values higher than the standard range for IL-1β. These results were obtained in samples of ulcerative processes. No IL-1β was found in the group without inflammatory cellular reaction in one case of scar formation. The significant difference of IL-1β concentrations between the groups with and without inflammatory cellular infiltration suggests a cell-dependent production of IL-1β. These findings are confirmed by the clinical features. Relatively low levels could be obtained from corneas without inflammatory cellular infiltrations, e.g. decompensation, dystrophy, keratoconus and scar formation of the cornea. Descriptive statistics showed lowered values for the median. Furthermore, this observation is reflected in the calculated quantiles.

The group of corneas suffering from proteolytic alterations and keratitis showed the highest IL-1β concentrations. Nevertheless, a wide range of scattering is obvious within the groups and may due to the intensity of both diseases. Because of this observation, quantiles are once again useful in showing the accumulation of similar findings in one group. For example, IL-1β levels in corneas obtained from patients with keratitis showed a broad spectrum, between 1.42 and 1750.37 pg IL-1β/mg protein. The 75% quantile showed 9 of 12 samples up to 291.02 pg IL-1β/mg protein. Only 3 of the 12 samples showed levels lower than 3.85 pg IL-1β/mg protein. Similar results could be obtained from ulcerative processes. According to the clinical diagnoses, severe inflammatory processes obviously show correlating IL-1β concentrations in the corneal stroma.

Subgroups could be formed and compared statistically according to the clinical picture. Inflammatory disorders like ulceration, keratitis and keratoconus with inflammatory cellular reactions showed concentrations

distinctly different from those in decompensation, dystrophy, scar formation and keratoconus without cellular reaction. These observations were not supported by a statistical comparison of keratitis against scar formation, dystrophy and decompensation. In some cases these findings may be explained by IL-1β secretion by non-inflammatory cells. In vitro investigations demonstrated IL-8 gene expression, IL-6 production and expression of IL-2 receptors [7, 21]. In some cases of decompensation, trauma caused by preliminary surgical interventions seemed to be responsible for higher IL-1β levels.

IL-6 was detected in 110 of the 127 investigated samples. Sixteen specimens from patients suffering from keratitis, keratoconus with inflammatory cellular reaction and ulcerative processes showed IL-6 concentrations higher than the standard range. Within the group with no inflammatory cellular reaction, no IL-6 could be detected in one case. Statistical evaluations of IL-6 levels showed results similar to those obtained in the comparison of IL-1β in samples with and without inflammatory reactions. Therefore, the suggested cell-dependent production of interleukins can be confirmed. Again, the corneas without inflammatory cellular infiltrations showed relatively low levels for IL-6. Absolute data for IL-6 showed concentrations higher than IL-1β levels. The highest values for IL-6 were found in samples of ulcerative processes, with a maximum of 3327.6 pg/mg protein.

To summarise the major data, it is obvious that IL-1β and IL-6 can trigger inflammation in the human cornea. Moreover the hypothesis could be proven that IL-1 is one of the first triggers of inflammatory reactions and is found in lower concentrations than IL-6, which is considered to be an endpoint mediator of inflammation [12]. Both are involved in proteolytic processes and may activate inflammatory cells as well as fibroblasts. Although significant differences were not found in all groups, the results suggest that the inflammatory status of the cornea may be evaluated by the interleukin levels, depending on the clinical diagnosis.

The exact contribution of cytokines to the inflammatory response of the cornea is still unknown, but the experimental results suggest that cytokines play a central role in the response. The injection of interleukins in experimental animal models of inflammation induces reactions similar to those observed for uveitis in patients with elevated interleukin levels in aqueous humour and serum. As already mentioned, results contrary to the common pathway of interleukins, e.g. high IL-6 levels in non-inflamed scars, are possible and suggest a different action of interleukins in some cases of local inflammatory response to the cornea. Rosenbaum and Boney [17] discussed results of endotoxin-induced uveitis and of an active Arthus reaction and presumed other pathways of inflammation, i.e. IL-1β is important but not itself sufficient for the inflammatory process. They suggested that tumour necrosis factor-α, IL-6 and/or IL-8 may subsume the activity of IL-1. This may be confirmed by our findings of higher levels of IL-6 and elevated IL-1β levels in ulcerative processes. Other aspects of the inflammatory response, such as the influence of products of the arachidonic acid pathway, should also be considered [13]. On the other hand, Kulkarni [13] reported the failure of polymorphonuclear leukocyte chemotaxis inhibition with lipoxygenase inhibitors and discussed the fact that prostaglandins and leukotrienes are not the primary mediators of intraocular inflammation induced by endotoxin in rabbits. Verhagen et al. [25] reported that it is essential that the actions and interactions of interleukins with aracidonic acid metabolites, which are also released during inflammtion, be investigated. Furthermore, it is still unknown at which levels of the immune system the interleukins act to modulate the inflammatory reaction. More studies are needed for detailed examination of the role of interleukins in the inflammatory response in corneal inflammation.

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